J Gen Plant Pathol (2005) 71:414–417
DOI 10.1007/s10327-005-0232-1



FUNGAL DISEASES

*Short communication*

**Norio Kondo · Ayumi Notsu · Shohei Fujita
Hisanori Shimada · Shigeo Naito**

# DNA sequence analysis of ribosomal ITS region 1 of *Phytophthora vignae* f. sp. *adzukicola*, the pathogen that causes stem rot of adzuki bean

Received: January 17, 2005 / Accepted: June 13, 2005

**Abstract** Sequences of the internal transcribed spacer (ITS) region 1 were used to examine the phylogenetic relationships among races of 19 isolates of *Phytophthora vignae* f. sp. *adzukicola* and between this forma specialis and three isolates of the closely related *P. vignae* f. sp. *vignae.* The ITS 1 sequences were highly conserved (> 98.7% similarity) among representatives of both formae speciales groups. The results of this study indicate that *P. vignae* is a monophyletic group.

**Key words** *Phytophthora vignae* f. sp. *adzukicola* · *Phytophthora vignae* f. sp. *vignae* · ITS sequence · Adzuki bean · Cowpea

Phytophthora stem rot of adzuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi) is a common problem in northern Japan (Kitazawa et al. 1978, 1979; Makino et al. 1997). The disease is caused by *Phytophthora vignae* Purss f. sp. *adzukicola*, which is distinct from the pathogen that causes *Phytophthora* stem and root rot on cowpea [*V. unguiculata* (L.) Warp], *P. vignae* f. sp. *vignae* (Purss 1957; Tsuchiya et al. 1986). Four races of the pathogen are known to exist, with race 3 being the most common, followed by races 1 and 4 (Kondo et al. 2004; Notsu et al. 2003).

The phylogenetic relationships among *Phytophthora* species have been investigated by analyzing the internal transcribed spacer (ITS) region 1 of the ribosomal DNA unit (Förster et al. 2000). Using this technique, *P. vignae* from cowpea and *P. sojae* from soybean (*Glycine max*) were found to be closely related. This close relationship has also been confirmed by restriction fragment length polymorphism (RFLP) analyses (Whisson et al. 1993) and ITS sequence analyses (Crawford et al. 1996a,b). In this study we investigated the phylogenetic relationships by comparing sequences of the ITS region 1 between two formae speciales of *P. vignae* and among races of the *P. vignae* f. sp. *adzukicola*.

The isolates of *P. vignae* f. sp. *adzukicola* investigated here were collected and identified during a previous study (Kondo et al. 2004). The list of the isolates is provided in Table 1, including *P. vignae* f. sp. *vignae* (ATCC 46735) from the American Type Culture Collection. To obtain DNA for polymerase chain reaction (PCR) analyses, mycelial mats grown in pea broth were harvested by filtration after 14 days, as described by Kondo et al. (2004). The mycelial mats were ground to a fine powder using a sterilized mortar and pestle with liquid nitrogen. DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Hildew, Germany) following the manufacturer's instructions. The PCR was used to amplify the ITS rDNA (through ITS 1, the 5.8S gene, and ITS 2) of each isolate using the primers AB28 (5′-GCGGATCCATATGCTTAAGTTCAGCG GGT-3′) and TW81 (5′-GCGGATCCGTTTCCGTAGG TGAACCTGC-3′) (Howlett et al. 1992). PCR reactions (50 µl volume) contained 0.2 µM of each primer, 5 ng of extracted DNA, 4 µl dNTP mixture (Takara, Shiga, Japan), and 2.5 units of *Taq* DNA polymerase (Takara) in 1× PCR buffer (Takara). An initial denaturation step was carried out at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 45 s, and a final extension step of 70°C for 10 min. PCR products were separated on 1% agarose gels following standard protocols for gel electrophoresis to result in the amplification of an about 900-bp rDNA fragment from all of the isolates examined in this study. The PCR products were purified directly using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Tokyo, Japan). All purified DNA samples were stored at –20°C.

N. Kondo (✉) · A. Notsu · S. Naito
Laboratory of Plant Pathology, Graduate School of Agriculture, Hokkaido University, Kita 9 Nishi 9, Sapporo 060-8589, Japan
Tel. +81-11-706-3829; Fax +81-11-706-4938
e-mail: norikon@res.agr.hokudai.ac.jp

S. Fujita · H. Shimada
Tokachi Agricultural Experiment Station, Memuro, Japan

The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession nos. AB120062–AB120080 and AB120122

**Table 1.** Isolates of *Phytophthora vignae* compared in this study and DDBJ accession numbers of their rDNA ITS 1 region sequence data

| *Phytophthora vignae* f. sp. | Race | Isolate | Location | DDBJ accession no. |
|---|---|---|---|---|
| *adzukicola* | 1 | Pv-tbt2 | Tobetsu | AB120062 |
| | | Pv-spr | Sapporo | AB120063 |
| | | Pc-ngn2 | Naganuma | AB120064 |
| | | Pv-hnb4 | Honbetsu | AB120065 |
| | 3 | Pv-tbt1 | Tobetsu | AB120066 |
| | | Pv-sbt4 | Shibetsu | AB120067 |
| | | Pv-bei1 | Biei | AB120068 |
| | | Pv-bei2 | Biei | AB120069 |
| | | Pv-bei3 | Biei | AB120070 |
| | | Pv-ash | Asahikawa | AB120071 |
| | | Pv-kgk2 | Kyogoku | AB120072 |
| | | Pv-atm1 | Atsuma | AB120073 |
| | 4 | Pv-sns1 | Shinshinotsu | AB120074 |
| | | Pv-wsm | Wassamu | AB120075 |
| | | Pv-asb1 | Ashibetsu | AB120076 |
| | | Pv-o1 | Oiwake | AB120077 |
| | | Pv-o2 | Oiwake | AB120078 |
| | | Pv-o3 | Oiwake | AB120079 |
| | Nonpathogenic | Pv-kcn2 | Kucchan | AB120080 |
| *vignae* | – | ATCC46735 | Australia | AB120122 |

Both strands of the amplified ITS region 1 from each purified DNA sample were sequenced following the protocol with the Thermo Sequence Cy5 Dye Terminator Cycle Sequencing kit (Amersham Biosciences) with 2.5 pmol of each primer: S1 (5′-CCGTAGGTGAACCTGCGGAGG-3′) and S4 (5′-CCCGGAAGTGCAATATGCG-3′) (Howlett et al. 1992). Sequencing was carried out using an ALFexpress II auto sequencer (Amersham Biosciences). Sequences of ITS region 1 ranged in size from 230 to 233 bp and were deposited in the DDBJ (accession nos. AB120062–120080 and AB120122) (Table 1). The sequences were aligned together with those of *P. sojae* from *G. max* [AF242799 (Wisconsin, USA) (Förster et al. 2000)], *P. vignae* from *V. unguiculata* [AF242830 (Australia) (Förster et al. 2000); L41388 (Australia) (Crawford et al. 1996a)] and *P. cinnamomi* from *Persea americana* [AF242795 (California, USA) (Förster et al. 2000)] as an outgroup. Sequence similarities were evaluated using the program CLUSTAL X version 1.8 (Thompson et al. 1997). The ITS 1 sequences of the *P. vignae* f. sp. *adzukicola* were found to be highly conserved (99.3%–100% similarities) regardless of race or geographical origin. These sequences also had almost identical similarities (98.7%–100%) to *P. vignae* f. sp. *vignae*. High similarities (95.7%) to *P. sojae* were also found.

Following introduction of gaps for alignment purposes, there were 239 bp total, of which 194 were constant, 34 were variable and parsimony uninformative, and 11 were parsimony informative. Phylogenetic analyses, carried out using the maximum parsimony method with a heuristic tree search (PAUP* 4.0 beta version) (Swofford 2002) showed that all *P. vignae* isolates were grouped into a single clade with 100% bootstrap support (Fig. 1). All isolates of *P. vignae* also clustered together in neighbor-joining analysis (bootstrap value 100%, data not shown). It was reported that there are no morphological or physiological differences between the adzuki bean pathogen and the cowpea pathogen (Kitazawa et al. 1978, 1979; Tsuchiya et al. 1986). As a result, the pathogens have been subdivided for taxonomic purposes into two formae speciales: *P. vignae* f. spp. *adzukicola* and *vignae* (Tsuchiya et al. 1986). Liew et al. (1991) demonstrated a close similarity between adzuki bean and cowpea isolates using RFLP analysis. It was speculated that these two geographically isolated groups of *P. vignae* were derived from a single source, rather than each being an indigenous pathogen of their respective hosts (cowpea and adzuki bean). The phylogenetic analysis reported in the present study supports the hypothesis that *P. vignae* is a monophyletic species with a distinct host specificity (Fig. 1).

The cowpea pathogen has been reported from India (Nirwan and Upadhyaya 1972), Australia (Purss 1957), the Guangzhow area of China (Hwang and Qi 1984), Taiwan (Kao and Leu 1982) and Sri Lanka (Fernando and Linderman 1993; Sivakadadcham and Fernando 1991), whereas the adzuki bean pathogen has been found only in Hokkaido, Japan (Kitazawa et al. 1978) and Korea (Han et al. 1982). However, it was also reported that the cowpea and adzuki bean pathogens were slightly pathogenic to adzuki bean and cowpea, respectively, depending on the inoculation conditions (Kitazawa et al. 1979; Tsuchiya et al. 1986). Although the cowpea pathogen has not been found in Japan, we are not able to eliminate the possibility that it is also present in Japan. Symptoms or signs of the cowpea disease may have been overlooked; low yield could easily have been attributed to environmental factors rather than to root disease. In Hokkaido, the production area of adzuki bean reached more than 50 000 ha during 1900 to 1935 and is currently maintained at the level of 30 000 ha, whereas

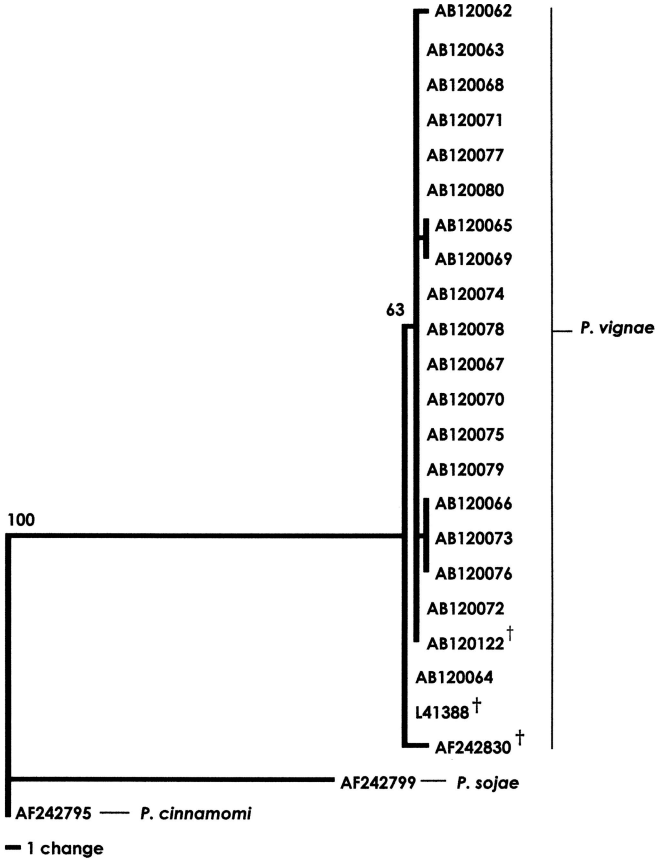


**Fig. 1.** Phylogenetic relationships among *Phytophthora vignae* isolates, including two formae speciales, *adzukicola* and *vignae*, using the internal transcribed spacer (ITS) 1 sequence data based on maximum parsimony. Sequences available in DDBJ, including *P. vignae* (AF242830, L41388), *P. sojae* (AF242799), and *P. cinnamomi* (AF242795) (served as outgroup), were also used. Bootstrap values (1000 replicates) are given above the nodes that support the observed topography (only values above 60% are shown). Of 239 characters, 194 were constant, 34 were variable and parsimony uninformative, and 11 were parsimony informative. Tree length 52, consistency index (CI) 0.981, homoplasy index (HI) 0.021, retention index (RI) 0.933, rescaled consistency index (RC) 0.916. †ITS 1 sequence data from *P. vignae* f. sp. *vignae*

cowpea has been a minor (domestic) crop. This background may have resulted in the dominance of the adzuki bean pathogen.

We did not investigate the evolution of pathogenesis in *P. vignae* in this study. However, it is interesting to note that a universally avirulent (nonpathogenic) isolate of *P. vignae* has been identified as well as in *P. sojae*, indicating that cross-fertilization could occur under natural conditions (Bhat et al. 1992). To obtain a better understanding of the ecology and evolution of these pathogens, genetic crosses (Bhat and Schmitthenner 1993; Förster et al. 1994; Makino et al. 1997) between the two formae speciales of *P. vignae* or among species (Brasier et al. 1999; May et al. 2003) would be useful.

**Acknowledgment** This work was supported by a grant from the Japan Bean and Pea Foundation.

## References

Bhat RG, Schmitthenner AF (1993) Genetic crosses between physiologic races of *Phytophthora sojae*. Exp Mycol 17:122–129

Bhat RG, Olah AF, Schmitthenner AF (1992) Characterization of universally avirulent strains of *Phytophthora sojae*. Can J Bot 70:1175–1185

Brasier CM, Cooke DEL, Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proc Natl Acad Sci USA 96:5878–5883

Crawford AR, Bassam BJ, Drench A, Maclean DJ, Irwin JAG (1996a) Evolutionary relationships among *Phytophthora* species deduced from rDNA sequence analysis. Mycol Res 100:437–443

Crawford AR, Bassam BJ, Drench A, Maclean DJ, Irwin JAG (1996b) Correction: evolutionary relationships among *Phytophthora* species deduced from rDNA sequence analysis. Mycol Res 100:1218

Fernando WGD, Linderman RG (1993) Occurrence, distribution, and pathogenicity of the cowpea root and stem rot pathogen, *Phytophthora vignae* in soils of Sri Lanka. Plant Dis 77:1158–1164

Förster H, Tyler BM, Coffey MD (1994) *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. Mol Plant Microbe Interact 7:780–791

Förster H, Cummings MP, Coffey MD (2000) Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS I DNA sequence analysis with emphasis on Waterhouse groups V and VI. Mycol Res 104:1055–1061

Han EH, Lee CH, Sin CS, Lee EK (1982) An investigation on the stem rot of red bean caused by *Phytophthora vignae* Purss. Res Per Off Rural Dev Soil Fert Crop Prot Mycol Farm Prod Util 24:69–71

Howlett BJ, Brownlee AG, Guest DI, Adcock GJ, McFadden GI (1992) The 5S ribosomal-RNA gene is linked to large and small subunit ribosomal-RNA genes in the oomycetes, *Phytophthora vignae*, *P. cinnamomi*, *P. megasperma* f. sp. *glycinea* and *Saprolegnia ferax*. Curr Genet 22:455–461

Hwang JK, Qi PK (1984) A study on *Phytophthora* of yardlong bean. Acta Phytopathol Sin 14:193–199 (in Chinese)

Kao CW, Leu LS (1982) Phytophthora stem rot of cowpea caused by *Phytophthora vignae* Purss in Taiwan. Plant Prot Bull 24:189–191

Kitazawa K, Tsuchiya S, Kodama F, Kamjaipai W, Ogoshi A, Yanagita K (1978) Phytophthora stem rot of adzuki bean (*Phaseolus angularis*) caused by *Phytophthora vignae* Purss. Ann Phytopathol Soc Jpn 44:528–531 (in Japanese with English summary)

Kitazawa K, Suzui T, Yanagita K (1979) Pathogenicity of *Phytophthora vignae* Purss to adzuki bean and cowpea. Ann Phytopathol Soc Jpn 45:406–408

Kondo N, Notsu A, Naito S, Fujita S, Shimada H (2004) Distribution of *Phytophthora vignae* f. sp. *adzukicola* races in adzuki bean fields in Hokkaido, Japan. Plant Dis 88:875–877

Liew ECY, MacLean DJ, Manners JM, Dawson D, Irwin JAG (1991) Use of restriction fragment length polymorphisms to study genetic relationships between Australian and Japanese isolates of *Phytophthora vignae*. Aust J Bot 39:335–346

Makino H, Fujita S, Murata K, Kondo N, Ogoshi A (1997) Characteristics of *Phytophthora vignae* isolates collected in Hokkaido. Ann Phytopathol Soc Jpn 63:530 (abstract in Japanese)

May KJ, Drenth A, Irwin JAG (2003) Interspecific hybrids between the homothallic *Phytophthora sojae* and *Phytophthora vignae*. Aust Plant Pathol 32:353–359

Nirwan RS, Upadhyaya J (1972) Phytophthora blight of cowpea new to India. Indian Phytopathol 25:162–163

Notsu A, Kondo N, Fujita S, Murata K, Naito S (2003) New race of *Phytophthora vignae* f. sp. *adzukicola*, the causal agent of Phytophthora stem rot of the adzuki bean. J Gen Plant Pathol 69:39–41

Purss GS (1957) Stem rot: a disease of cowpeas caused by an undescribed species of *Phytophthora*. Queensland J Agric Sci 14:125–154

Sivakadadcham B, Fernando WGD (1991) An investigation on the stem rot of red bean caused by *Phytophthora vignae* Purss. Res Per Off Rural Dev Soil Fert Crop Prot Mycol Farm Prod Util 24:69–71

Swofford DL (2002) PAUP*: Phylogenetic analysis using parsimony (and other methods) 4.0 beta. Sinauer Associates, Sunderland, MA, USA

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tsuchiya S, Yanagawa M, Ogoshi A (1986) Formae speciales differentiation of *Phytophthora vignae* isolates from cowpea and adzuki bean. Ann Phytopathol Soc Jpn 52:577–584

Whisson SC, Howlett BJ, Liew ECY, MacLean DJ, Manners JM, Irwin JAG (1993) An assessment of genetic relationships between members of the *Phytophthora megasperma* complex and *Phytophthora vignae* using molecular markers. Aust Sys Bot 6:295–308