Characterization of nonpathogenic *Cadophora gregata*, a potential biological control agent, concomitantly isolated from soil infested with *Cadophora gregata* f. sp. *adzukicola*, the cause of adzuki bean brown stem rot

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Abstract

We collected 555 isolates of *Cadophora gregata* from adzuki bean field soils in Hokkaido, Japan, from 1997 to 2000. To identify the brown stem rot (BSR) pathogen *C. gregata* f. sp. *adzukicola*, we screened these isolates for pathogenicity to adzuki beans. Of the isolates, all of which originated in Tokachi District, Hokkaido, Japan, 23 were avirulent to adzuki bean, soybean, or mung bean. However, polymerase chain reaction (PCR) with specific primers for *C. gregata* f. sp. *adzukicola* (BSRA1 and BSRA2) detected the specific identifying DNA fragment in these isolates, and cluster analysis with inter-simple sequence repeat markers showed that the isolates were phylogenetically closer to strains that are virulent to adzuki bean. Thus, we concluded that the isolates were nonpathogenic *C. gregata.* A few selected isolates of the nonpathogenic *C. gregata* were effective at

reducing BSR *in vivo* and show potential for development as biological control agents.

Keywords: adzuki bean, biological control, brown stem rot, *Cadophora gregata*, nonpathogenic

1. Introduction

Brown stem rot (BSR), an economically important disease of adzuki bean [*Vigna angularis* (Willd.) Ohwi et Ohashi], is caused by *Cadophora gregata* Harrington & McNew (Harrington and McNew, 2003). Isolates of *C. gregata* from adzuki bean and soybean are host-specific and are recognized as two different formae speciales, *C. gregata* f. sp. *adzukicola* (CGA) and *C. gregata* f. sp. *sojae* (CGS), respectively (Kobayashi et al., 1983, 1991). The pathogens can be differentiated from each other and other fungi based on isozyme banding patterns (Yamamoto et al., 1990), DNA sequences of internal transcribed spacer (ITS) regions of rDNA (Chen et al., 1990, 1999; Harrington et al., 2000), DNA sequences of intergenic spacer (IGS) regions of rDNA (Chen et al., 2000), microsatellite markers (Chen et al., 2002), and amplified fragment length

polymorphisms (AFLPs) or inter-simple sequence repeats (ISSRs; Meng and Chen, 2001). Once soilborne CGA is inside adzuki bean plants by invading root tissues of young plants, it spreads into the vascular and pith tissue via mycelia and conidia production (Narita et al., 1971). This fungus infects vascular tissue and causes pith and vascular tissue discoloration of the stem and petiole, in conjunction with foliar chlorosis or necrosis. Consequently, the pathogen can cause susceptible plants to wilt and reduce adzuki bean yields. Although phytotoxic gregatins produced by CGA previously were thought to be associated with these symptoms (Kobayashi and Ui, 1977), Tanaka et al. (2007) found that the toxins were unlikely to be essential for pathogenicity.

Extensive studies have been performed on phenotypical variations (races) of CGA isolates (Kondo et al., 1998, 2002), adzuki bean resistance to BSR (Adachi et al., 1988; Chiba, 1982, 1985; Chiba et al., 1987; Fujita et al., 1995, 2002, 2007), and the close

ecological association of *C. gregata* with nematodes (Djiwanti, 1999; Sugawara et al., 1997a; Yamada et al., 2005a, 2005b). To date, disease-resistant cultivars and crop rotation are the most practical means of controlling BSR. Indeed, Fujita et al. (2007) and Kondo et al. (2009) found one cultivated adzuki bean variety and one wild adzuki bean accession that tolerated all of the CGA races, making them useful for breeding BSR-resistant adzuki bean cultivars. Yamada et al. (2005b) showed that using wild oats [*Avena strigosa* Schreb.] as green manure decreased the degree of BSR damage by suppressing nematodes. However, as shown by the quick appearance of a strain virulent to recently developed resistant breeding lines (Kondo et al., 2005), BSR outbreaks in new races may be inevitable, due to the co-evolutionary relationship between crop varieties and their pathogens.

The distribution of CGA races has been examined using isolates collected from

adzuki bean field soils in Hokkaido (Kondo et al., 2002). Pathogenicity tests revealed that most isolates were virulent to the susceptible cultivar Erimo-shozu, although several avirulent isolates were discovered. Therefore, we examined the pathogenicity of additional isolates to investigate the distribution of nonpathogenic *C. gregata* (NPC). Some nonpathogenic strains of plant pathogenic species may have the potential to protect the plant against infections caused by the virulent strains (Sneh, 1998). Moreover, understanding NPC may enable us to optimize ecological conditions to enhance its suppressive ability in crop rotations. Our objectives were to identify NPC strains, their characteristics, and their potential as biological control agents of adzuki bean BSR.

2. Materials and methods

2.1. Sources and cultivation of isolates

As described in a previous study (Kondo et al., 2002), *C. gregata* was isolated from 44 adzuki bean field soil samples collected from five districts in Hokkaido from 1997 to 2000 using a modified selective medium soil dilution method (Table 1). Single-spored isolates were stored in green-pea agar (GPA; 200 g frozen green peas boiled 15 min, filtered through four layers of cheesecloth, and solidified using 20 g agar/liter) at 4˚C. Sporulation ability and cultural morphology were determined using V8-juice agar (200 ml V8 juice and 2 g $CaCO_3$ centrifuged at 5,000 rpm for 15 min, with the supernatant diluted to 1 L with distilled water and solidified using 20 g agar/liter) and potato dextrose agar (PDA; Difco, Lawrence, Kansas, USA), respectively. For long-term storage, agar disks containing spores of each isolate grown on GPA were placed in

individual cryovials containing 20% sterile glycerol and maintained at -80˚C. In Table 2 four nonpathogenic isolates that were originally obtained as CGA and CGS from diseased adzuki bean and soybean in Japan, respectively, were included and also used in this study: a virulence-deficient mutant A'31-2 (Kobayashi et al., 1981); isolates A60K68 and A60To, which were originally identified as virulent (Yamamoto, 1994); and isolate A57T22, which was uncertain for virulence.

2.2. Cultural conditions and screening of nonpathogenic strains

The inoculum was grown in V8-juice broth at 25˚C on a reciprocal shaker at 120-oscillations/min. After 3 weeks of incubation, mycelia and spores were collected by filtration through Whatman No. 1 filter paper and washed by suspending them in distilled

water, followed by centrifugation. The fungal pellets were homogenized in distilled water with a homogenizer (10,000 rpm for 3 min; AN-5, Shin-nihonseiki, Tokyo, Japan) and the concentration of mycelial fragments and spores was determined using a hemocytometer; mycelial fragments of all sizes were included in counts. Blended cultures were then diluted with distilled water to a concentration of 10^7 propagules per milliliter. The adzuki bean varieties used to determine fungal races were cvs. Erimo-shozu (susceptible to all races), Kita-no-otome (resistant to races 1 and 3, but susceptible to race 2) and Acc259 (resistant to races 1 and 2, but susceptible to race 3). Seedlings were grown in plastic containers (15 × 20 × 5 cm) with sterilized vermiculite for about 10 to 14 days in greenhouse, then the roots were washed gently with running tap water. The roots of 10 seedlings of each cultivar were dipped into each inoculum suspension (50 ml) for 12 hr, then transplanted into a soil (Pot –ace, Katakura Chikkarin

K.K. Tokyo, Japan)/ vermiculite mixture (1:1, v/v) in 12-cm diameter plastic pots. The response to the pathogen was evaluated after eight weeks growth in greenhouse. Nighttime low and daytime high temperatures during these tests were 15/32°C. Plants received supplemental lighting from metal halide sodium lamps (400W) to maintain a 14-h photoperiod. The inoculation experiments were completely randomized, with two replications (pots) per isolate per cultivar and five plants per pot. Pathogenicity tests were repeated twice with these cultivars, and nonpathogenic isolates were determined. Soybean [*Glycine max* (L.) Merrill. cv. Sapporo-midori] and mung bean [*Vigna radiata* (L.) R. Wilczek, susceptible to both CGS and CGA] were also used to determine formae speciales of *C. gregata*. The roots of 10 seedlings of each crop grown as described above were dipped into a suspension (50 ml) of each inoculum (Table 3) for 12 h. The seedlings were then transplanted into the sterilized mixed soil in 18-cm-diameter plastic pots.

Isolates T96-1 (race 1 CGA), T96-5 (race 2 CGA), and S58KS (CGS) also were used as controls. For BSR assessment, the number of diseased plants in the greenhouse with foliar symptoms (stunted and necrotic) or vascular discoloration was counted eight weeks after inoculation.

2.3. Production of gregatins

Isolates were grown in adzuki bean stem medium (5 g dry stem pieces boiled 15 min, filtered through four layers of cheesecloth, with the filtrate diluted to 1 L using distilled water) containing 5% glucose (Kobayashi and Ui, 1975) on a reciprocal shaker at 120-oscillations/min for 4 weeks at 25˚C. The culture filtrate (200 ml) was adjusted to pH 7.0, extracted once with an equal volume of ethyl acetate, and washed with distilled water.

The ethyl acetate solution was evaporated, yielding an oily residue. This extract was dissolved in acetone (200 μl), and 3 μl was spotted onto a thin-layer chromatography (TLC) silica gel plate (Silica gel 60 F_{254}; Merck, Darmstadt, Germany). The solvent system was chloroform: methanol (98:2, v/v). Gregatins were visualized by UV irradiation (254 nm). For positive and negative controls, extracts from a culture of wild-type strain T96-5 and non-inoculated medium were used, respectively.

2.4. DNA extraction from mycelia

For DNA extraction, agar plugs were removed from the growing margin of 1-week-old cultures and transferred to V8-juice broth. After a 14-day incubation period at 25˚C, mycelial mats were harvested by filtering the broth through Whatman No.1 filter

paper in a Buchner funnel then cleaned by rinsing each mat several times with distilled water. After removing the excess water, all mycelial mats were frozen at -80˚C until they were ground to a fine powder in liquid nitrogen using a sterilized mortar and pestle. The DNA extraction was conducted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The DNA concentration was determined by UV/Vis spectrophotometer (DU640; Beckman Coulter Inc., Fullerton, CA, USA) and the purity of the DNA samples was examined by electrophoresis in 0.8% agarose (Wako Pure Chemical Industries, Osaka, Japan) gel (TBE buffer).

2.5. Design and specificity of primers specific for C. gregata

Two polymerase chain reaction (PCR) primers, BSRA1

(5’-GCTTGCTCCGTGGTGGGCTA-3’) and BSRA2 (5’-GATTTGGGGGTTGCTGGAAG-3’), were designed for CGA based on the ITS sequence (GenBank accession no. U66731; Chen et al., 1996; Sakuma et al., 1999). The PCR primers, BSR1 (5’-GCTTGCTCCGTGGCGGGCTG-3’) and BSR2 (5’-AATTTGGGTGTTGCTGGCATG-3’) (Chen et al., 1996), also were used to confirm CGS. To check DNA quality, PCR was also performed on the DNAs, using the primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’), which amplify ITS rDNA through ITS 1, the 5.8S gene, and ITS 2 (White et al., 1990). PCR amplifications were generally performed in 25 μl reactions, with 0.2 mM dNTP mixture, 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$), 0.75 units *Taq* polymerase (Takara Inc., Shiga, Japan), 0.16 μM of each specific primer, and 4 pg template DNA, under the following

temperature regimes: initial denaturation at 94˚C for 2 min, followed by 30 denaturation cycles at 94˚C for 1 min; annealing at 53˚C (for ITS rDNA), and 60˚C or 62˚C for 1 min; and extension at 72˚C for 1 min, with a final extension at 72˚C for 5 min using a DNA thermal cycler (TaKaRa Inc., Shiga, Japan). A negative control without the DNA template was included in each PCR set to monitor possible contamination. To visualize the DNA fragments, 3 μl PCR products was loaded in a gel containing 1.5% agarose, along with φ*Hae*Ⅲ. After running 30 min at 100 V, gels were stained with ethidium bromide and photographed under ultraviolet light.

2.6. Detection of C. gregata in adzuki bean plants

The colonization of plant roots by *C. gregata* was determined when the plants

were assessed eight weeks after inoculation. Root samples (≥ 10 pieces) from each leguminous crop, which comprised four plants hosting each isolate, were washed gently with running tap water for 3 h. DNA was extracted from the samples using the DNeasy Plant Mini Kit. PCR, using the primers described above, was then performed to detect *C. gregata* inoculates.

2.7. Genetic analysis of C. gregata using ISSR

Isolates used in this study are listed in Table 2. DNA extractions were performed as described above. Microsatellite primers used in this study and the annealing temperature of each primer are shown in Table 4**.** The PCR reaction mixture (25 μl) consisted of 25 ng template DNA, 0.625 units *Taq* polymerase, 10 × PCR buffer (10 mM

Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$), 0.2 mM dNTP mixture, and 0.5μ M primer. The PCR thermal cycler conditions were 94˚C for 1 min, followed by 35 cycles at 94˚C for 1 min, each annealing temperature for 1 min (Table 3), and 72˚C for 2 min. A final elongation was performed at 72˚C for 10 min to ensure a double-stranded amplification product. The temperature was then reduced and maintained at 4˚C. All amplifications were repeated at least twice. Negative controls were run with all amplifications to confirm that the reactions were free of contamination. PCR products were electrophoresed separately in 1.5% agarose gel (TBE buffer) at 100 V for 40–60 min, along with λ*Hind*Ⅲ. The gels were stained with ethidium bromide and visualized under UV light. A digital image of the gel was analyzed using Kodak Digital Science, Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA).

The PCR reaction fragments were treated as putative distinct loci, and presence

or absence was scored as 1 or 0, respectively. Only intense fragments of the isolates were scored, if reproducible in independent reactions. A binary data matrix was constructed from the scores of all of the isolates. Distance and similarity matrix computations for binary data were performed using the Windist program in PHYLIP (http://evolution.genetics.washington.edu/phylip.html). Cluster analysis, using the unweighted pair-group method with arithmetic averages (UPGMA), was performed using the NEIGHBOR program to produce a dendrogram. For each branch of the phenogram, a supported bootstrap value was generated with 1,000 bootstrapped samples, using the Winboot program (Yap et al., 1996).

2.8. Screening trials for biocontrol effectiveness

Seedlings of cv. Erimo-shozu were co-inoculated with each nonpathogenic isolate and T96-1 (CGA); suspensions (10^7 mycelial fragments and spores/ ml) were mixed at 9:1 (v/v) to yield 50 ml inoculum. Adzuki bean seedlings, spores, and mycelial fragments were prepared as described above. For each nonpathogenic isolate, 16 seedlings were immersed in the suspension for 24 h and then planted, four per pot, in 12-cm-diameter vinyl pots filled with sterilized soil mixture, consisting of 1:1 (v/v) vermiculite/Pot-ace. This procedure was conducted twice for all combinations. Nighttime low and daytime high temperatures during these tests were 15°/ 32°C. Plants received supplemental lighting from metal halide sodium lamps (400W) to maintain a 14-h photoperiod. In all tests, controls were inoculated with only the BSR pathogen (10^6 mycelial fragments and spores/ ml). The suppression of BSR was assessed based on the occurrence of vascular browning 45 days after inoculation. Average disease severity for

each isolate was calculated from the proportion of nodes with browning to the total number of nodes in each replicate (pot), using the following formula: [Σ(number of nodes discolored/ number of total nodes) × 100/total number of plants].

2.9. Statistical analysis

All data expressed as percentages were arcsine-transformed ($\text{sine}^{-1}\sqrt{x}$) prior to analysis. The means were compared using Dunnett's test, with reference to a positive control.

3. Results

3.1. Identification of NPC

Of the 555 isolates, which included 483 virulent isolates (Kondo et al., 2002), 23 avirulent isolates were obtained only from Tokachi district (Table 1). Although 13 nonpathogenic isolates did not produce conidia on V8 agar (Table 3), nine others (isolate Hir6-4 was not tested) were morphologically identical to *C. gregata*: hyaline and septate hyphae on GPA; hyaline, ovoid or ellipsoidal conidia forming a false head on phialides with short collarettes; and slow growth on PDA, with colonies reddish-brown or salmon pink to red. However, five of 43 isolates that were isolated from 1976 to 1996 and maintained in our laboratory were avirulent; with one exception (mutant isolate A'31-2), they originated from Tockachi district or the experimental field of Hokkaido University.

When subjected to PCR, using the primers BSRA1 and BSRA2 designed

specifically for CGA, all of the NPC isolates tested at an annealing temperature of 62˚C (Fig. 1A) yielded the expected 483-bp fragment, but the primers BSR1 and BSR2, which were specific for CGS, did not produce the fragment (Fig. 1B). Occasionally, at 60˚C annealing temperature, PCR fragments were slightly amplified, even when primers BSR1 and BSR2 were used with CGA isolates (data not shown). This indicates that NPC isolates are much closer to CGA than to CGS. The PCR products of all isolates resulting from ITS1 and ITS4, which amplify ITS rDNA, were about the same size, except that of isolate Taka-2, which was smaller (data not shown).

3.2. Host specificity and gregatins productivity

All nonpathogenic isolates, including four virulence-deficient isolates, were

not pathogenic to either soybean or mung bean, indicating that they were not CGS (Table 3). Although cononization of treated isolates inside roots is not determined clearly by PCR because a trace of each fungus on the root surface might be included in the sample, infected isolates, except isolate Taka-2, were identified and seemed to be colonized in the inoculated roots due to gentle surface sterilization. The production of gregatins varied, depending on the isolate; 10 isolates produced gregatins as profusely as did wild-type CGA isolates, but TLC detected no gregatin in the 16 other isolates. Thus, gregatin production does not necessarily determine CGA pathogenicity.

3.3. Cluster analysis with ISSR

Each of the eight primers tested amplified 10 to 18 fragments (Table 4), ranging

in size from about 400 to 2500 bps (data not shown). The frequency of polymorphic fragments was greater among CGA isolates (79.6%) than among isolates of CGS (68.0%) and NPC (64.1%), indicating higher genetic variation in the CGA population.

Cluster analysis based on 103 ISSR markers separated isolates into four groups (G1–G4): CGA isolates from commercial fields (G1); those from Tokachi Agricultural Experiment Station (G2); NPC isolates, except isolate Taka-2 (G3); and CGS isolates (G4) supported by high bootstrap values (82–100%; Fig. 2). The UPGMA tree created with ISSR revealed that G1 was resolved from the clade consisting of G2 and G3, and G1 and G4 were clearly distinguished.

For all of the groups, the ISSR profiles were closely associated with geographical origins, and all of the CGA races were scattered throughout the tree. Isolates from the same field tended to cluster in subgroups within CGA. Only isolate Taka-2 was

distinct from other NPC or CGA isolates.

3.4. Effect of NPC on BSR incidence

Effective isolates were found within 26 NPC isolates, including four virulence-deficient mutants. Co-inoculation with NPC isolates Oto1-5-1 or To-konT3c-2 and CGA isolate T96-1 significantly ($P < 0.05$) reduced BSR incidence in both trials, compared with inoculation solely with the CGA isolate (Fig. 3). Isolate Oto1-5-1 was particularly effective at reducing the disease, with reductions of 84% and 87% in the first and second trials, respectively.

4. Discussion

The 23 isolates obtained from adzuki bean field soils in Tokachi district were morphologically similar to CGA but were determined to be avirulent to adzuki bean (cv. Erimo-shozu). In addition, PCR, using the CGA-specific primers BSRA1 and BSRA2, detected a specific DNA fragment in these isolates (Fig. 1A). Thus, we concluded that nonpathogenic or saprophytic *C. gregata* exists concomitantly in soil infested with the adzuki bean BSR pathogen. Molecular markers for easy discrimination of CGA and CGS have been developed: a variable DNA region in the ITS (Chen et al., 1996) or a variable DNA region in the IGS of nuclear rDNA (Chen et al., 2000). However, genotype C, designated from a variable DNA region in the IGS, does not always coincide with CGA isolates (Ito et al., 2008). Accordingly, we used the marker from the variable DNA region in the ITS to identify CGA. Currently, we are genotyping CGA and CGS in Japan using

the specific primers BSRIGS1 and BSRIGS2 (Chen et al., 2000).

Although it is not clear why NPC isolate populations are restricted only to Tokachi district, the UPGMA tree with ISSR revealed that NPC isolates—with the exception of isolate Taka-2, which is distinct from other NPC or CGA isolates—are phylogenetically closer to CGA isolates from Tokachi Agricultural Experiment Station (TAES) in Memuro than to isolates from other adzuki bean fields (Fig. 2). Thus, when the infested fields of TAES were created for breeding about 40 years ago, diseased residues of adzuki bean collected from a common location could have been incorporated. Although the CGA-specific fragment was also detected in isolate Taka-2, the size of the amplified fragment of the ITS region was smaller than that in other tested NPC isolates, as well as that of CGA. Of course, this isolate is not pathogenic to soybean (it is not a CGS pathogen). Thus, it is unique, and its close relationship to CGS in the UPGMA tree

could occur by chance. Isolate A57T22, which was also collected from Shimizu in Tockachi district in 1983 and preserved in our laboratory, is avirulent and included in NPC group G3 (Fig. 2), indicating that the isolate is probably not a virulence-deficient mutant but is nonpathogenic by nature. The tree also showed that adzuki bean isolates (G1–G3) and soybean isolates (G4) of *C. gregata* are clearly distinguished. Generally, our results were consistent with previous findings (Meng et al., 2001; Yamamoto, 1994; Yamamoto et al., 1990), in that CGA and CGS are genetically distinct from each other. For all of the groups, ISSR profiles are closely associated with geographical origins, although isolates from the same district are generally not tightly clustered in the AFLP analysis (Kondo et al., 2002).

We determined a varied range of NPC isolates after screening for the reduction of BSR symptoms on adzuki bean stems *in vivo*. One potential isolate, Oto1-5-1, shows

potential to control the disease (Fig. 3), due to its disease suppression ability and lack of gregatin productivity (Table 3). Previously, the pre-inoculation of CGS isolates was reported to induce resistance to BSR in adzuki bean (Sugawara et al., 1997b). Although split-root experiments in the same report demonstrated that some CGS isolates induced systemic resistance to adzuki bean BSR, no systemic resistance with isolate Oto1-5-1 was determined in preliminary tests using a split-root method (unpublished data). Hence, the reduction of BSR symptoms in this study could be attributed to parasitic competition for infection sites on the root.

In naturally occurring suppressive soils, antagonistic *Fusarium* spp. population levels typically must be 10–100 times greater than that of the pathogen to be effective (Alabouvette, 1986; Alabouvette et al., 1993; Paulitz et al., 1987). Although no suppressive soil has been found in adzuki bean BSR, indigenous NPC isolates may

induce disease suppression if appropriate crops or varieties of adzuki bean are cultivated, and application methods of effective NPC isolates, such as Oto1-5-1, are improved. Further research is required to develop this antagonist as a biocontrol agent, as well as to investigate the conditions under which it is effective and ways to improve its consistency and level of effectiveness.

Acknowledgement

This work was supported by the Japan Beans and Peas Foundation.

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Fig. 1. Agarose gels showing PCR amplification of nonpathogenic isolates of *Cadophora gregata* and virulence-deficient mutants of *C. gregata* f. sp. *adzukicola* with specific primers BSRA1 and BSRA2 for *C. gregata* f. sp. *adzukicola* (**A**) and with specific primers BSR1 and BSR2 for *C. gregata* f. sp. *sojae* (**B**). Lane M: size marker φ*Hae*Ⅲ; lanes 1 through 3: isolate A'31-2, A60K68 and A60To of virulence-deficient *C. gregata* f. sp. *adzukicola* mutated during culturing or preserving; lanes 4 through 26: isolates A57T22 (suspected to be nonpathogenic by nature), To-konT3c-2, Kyo2-1, Kyo2-2, Kyo2-3N, Kyo2-5N, Kyo3-1N, Kyo3-2N, Kyo4-2, Kyo4-3, Skt2-3, Skt5-1, Hir4-1, Hir2-3, Sot8-1, A8-7-5, Oto1-3-3N, Oto1-3-4, Oto1-5-1, Tyo6-1, Taka-2, Mem6-4 and Seo7-1 of nonpathogenic *C. gregata*; lane 27: isolate T96-1 of *C. gregata* f. sp. *adzukicola*; lane 28: isolate S58KS of *C. gregata* f. sp. *sojae*; and lane N: negative control without template DNA.

Fig. 2. Dendrogram depicting the genetic variation and relationships among *Cadophora gregata* f. sp. *adzukicola*, *C. gregata* f. sp. *sojae* and nonpathogenic *C. gregata* isolates with the unweighted pair-group method with arithmetic averages (UPGMA). Lower bar is a genetic distance scale. Bootstrap values of major clusters (1000 replicates) are indicated (larger than 80). G1: *C. gregata* f. sp. *adzukicola* isolates from commercial fields; G2: *Cadophora gregata* f. sp. *adzukicola* isolates from Tokachi Agricultural Experiment Station; G3: nonpathogenic *C. gregata* isolates; and G4: *C. gregata* f. sp. *sojae* isolates.

Fig. 3. Screening of nonpathogenic *Cadophora gregata* isolates for biocontrol of brown stem rot of adzuki bean (*C. gregata* f. sp. *adzukicola*). Disease severity is the average percent of nodes with browning to the total number of the nodes in each replicate. Vertical bars represent the standard errors of means (n = 4). (*) Significantly different from each control [inoculation of *C. gregata* f. sp. *adzukicola* alone (isolate T96-1)] according to Dunnett test ($P \leq 0.05$).

Table 1. Distribution of virulent and avirulent isolates of *Cadophora gregata* on adzuki bean collected from brown stem rot (BSR) infested fields during 1997 to 2000 in Hokkaido

District	No. of fields surveyed	No. of isolates	
		Virulent	Avirulent
Shiribeshi	5	45(45)[2]	0
Iburi	4	52(52)	0
Kamikawa	8	43(40)	0
Ishikari	1[1]	36	0
Tokachi	26	379(346)	23
Total	44	555(483)	23

[1] The experiment field of Hokkaido University, Sapporo

[2] Number in parenthesis shows the number of isolates described in the result (Table 1) of Kondo et al. (2002).

Table 2. Isolates of *Cadophora gregata* used in this study

Forma specialis	Isolate No.	Location			Race	Year
adzukicola	Oto1-2-3	Otofuke	Tokachi	Hokkaido	2	1997
	Oto1-2-1	Otofuke	Tokachi	Hokkaido	3	1997
	Mem3-4	Memuro	Tokachi	Hokkaido	1	1997
	Mem1-3	Memuro	Tokachi	Hokkaido	1	1997
	Mur-3	Memuro	Tokachi	Hokkaido	2	1998
	Msek	Memuro	Tokachi	Hokkaido	2	2000
	Mem1-2	Memuro	Tokachi	Hokkaido	2	1997
	T96-1	Memuro	Tokachi	Hokkaido	1	1996
	Skt2-4	Shihoro	Tokachi	Hokkaido	2	1997
	Smo1-3	Shihoro	Tokachi	Hokkaido	2	1997
	Smo3-3	Shihoro	Tokachi	Hokkaido	1	1997
	Skt1-3	Shihoro	Tokachi	Hokkaido	1	1997
	Kyo5-2	Churui	Tokachi	Hokkaido	2	1997
	Seo1-2	Sarabetsu	Tokachi	Hokkaido	1	1997
	A54-25	Makubetsu	Tokachi	Hokkaido	1	1979
	A56-25	Makubetsu	Tokachi	Hokkaido	1	1982
	A57T14	Makubetsu	Tokachi	Hokkaido	1	1983
	Towa3-3	Taiki	Tokachi	Hokkaido	1	1997
	NT96-V	Obihiro	Tokachi	Hokkaido	1	1996
	T96	Obihiro	Tokachi	Hokkaido	1	1996
	A10	TAES[1]	Tokachi	Hokkaido	1	1976
	A13	TAES	Tokachi	Hokkaido	1	1970
	KT1	TAES	Tokachi	Hokkaido	1	1990
	KT2	TAES	Tokachi	Hokkaido	1	1990
	HY-2	TAES	Tokachi	Hokkaido	1	1990
	TAES93	TAES	Tokachi	Hokkaido	1	1993
	TTF95-Ha	TAES	Tokachi	Hokkaido	2	1995
	To-konT3c-5	TAES	Tokachi	Hokkaido	2	1997
	To-konT7b-2	TAES	Tokachi	Hokkaido	2	1997

To-konT7a-2	TAES	Tokachi	Hokkaido	1	1997
T96-5	TAES	Tokachi	Hokkaido	2	1996
Pg-A8-2	TAES	Tokachi	Hokkaido	2	1996
MAR92-1	Maruseppu	Abashiri	Hokkaido	1	1992
SET92	Maruseppu	Abashiri	Hokkaido	1	1992
Tak-1	Kucchan	Shiribeshi	Hokkaido	2	1999
Tak-2	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-1	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-2	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-3	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-4	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-5	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-6	Kucchan	Shiribeshi	Hokkaido	2	1999
Yah-7	Kucchan	Shiribeshi	Hokkaido	1	1999
Yah-8	Kucchan	Shiribeshi	Hokkaido	1	1999
Gak-2	Kyowa	Shiribeshi	Hokkaido	3	1999
Gak-3	Kyowa	Shiribeshi	Hokkaido	2	1999
Gak-4	Kyowa	Shiribeshi	Hokkaido	2	1999
Oiw-2	Oiwake	Iburi	Hokkaido	2	1999
Oiw-3	Oiwake	Iburi	Hokkaido	2	1999
Oiw-4	Oiwake	Iburi	Hokkaido	1	1999
Oiw-5	Oiwake	Iburi	Hokkaido	2	1999
Oiw-6	Oiwake	Iburi	Hokkaido	1	1999
Oiw-8	Oiwake	Iburi	Hokkaido	1	1999
Oiw-9	Oiwake	Iburi	Hokkaido	2	1999
Oiw-10	Oiwake	Iburi	Hokkaido	2	1999
Oiw-11	Oiwake	Iburi	Hokkaido	1	1999
Oiw-12	Oiwake	Iburi	Hokkaido	2	1999
Oiw-13	Oiwake	Iburi	Hokkaido	2	1999
Tom-4	Atsuma	Iburi	Hokkaido	2	1999
Tom-7	Atsuma	Iburi	Hokkaido	2	1999
Toy-1	Atsuma	Iburi	Hokkaido	1	1999

Toy-2	Atsuma	Iburi	Hokkaido	2	1999
Toy-3	Atsuma	Iburi	Hokkaido	1	1999
Kan-1	Atsuma	Iburi	Hokkaido	1	1999
Kan-2	Atsuma	Iburi	Hokkaido	1	1999
A54W	Wassamu	Kamikawa	Hokkaido	1	1979
A59W	Wassamu	Kamikawa	Hokkaido	1	1984
A60W1	Wassamu	Kamikawa	Hokkaido	1	1985
A54-2	Aibetsu	Kamikawa	Hokkaido	1	1979
A59K	Kenbuchi	Kamikawa	Hokkaido	1	1984
A60K2	Kenbuchi	Kamikawa	Hokkaido	1	1985
KEN90-3	Kenbuchi	Kamikawa	Hokkaido	1	1990
A60N1	Asahikawa	Kamikawa	Hokkaido	1	1985
Shu-1	Asahikawa	Kamikawa	Hokkaido	1	1998
Shu-2	Asahikawa	Kamikawa	Hokkaido	2	1998
Shu-3	Asahikawa	Kamikawa	Hokkaido	1	1998
Shk-1	Asahikawa	Kamikawa	Hokkaido	2	1999
Shk-2	Asahikawa	Kamikawa	Hokkaido	2	1999
Shk-3	Asahikawa	Kamikawa	Hokkaido	2	1999
Shk-4	Asahikawa	Kamikawa	Hokkaido	2	1999
Shk-5	Asahikawa	Kamikawa	Hokkaido	1	1999
A62B3-1	Higashikawa	Kamikawa	Hokkaido	1	1988
Kos-13	Biei	Kamikawa	Hokkaido	2	1999
Kos-17	Biei	Kamikawa	Hokkaido	2	1999
Kos-18	Biei	Kamikawa	Hokkaido	2	1999
A62S-2	Shibetsu	Kamikawa	Hokkaido	1	1988
SHI90-3	Shibetsu	Kamikawa	Hokkaido	1	1990
Shi-2	Shibetsu	Kamikawa	Hokkaido	1	1999
Shi-3	Shibetsu	Kamikawa	Hokkaido	1	1999
Shi-4	Shibetsu	Kamikawa	Hokkaido	1	1999
Ksh-1	Kami-shibetsu	Kamikawa	Hokkaido	1	1999
Ksh-4	Kami-shibetsu	Kamikawa	Hokkaido	1	1999
Fur-2	Furano	Kamikawa	Hokkaido	2	1999

	Fur-3	Furano	Kamikawa	Hokkaido	1	1999
	BIE1	Biei	Kamikawa	Hokkaido	3	1990
	A57B4	Biei	Kamikawa	Hokkaido	1	1983
	Rub-5	Biei	Kamikawa	Hokkaido	1	1999
	NAY90-6	Nayoro	Kamikawa	Hokkaido	1	1990
	A58H	HU[2]	Ishikari	Hokkaido	1	1983
	A59-13	HU	Ishikari	Hokkaido	1	1984
	A61Ta	HU	Ishikari	Hokkaido	1	1986
	A63Ta-1	HU	Ishikari	Hokkaido	1	1988
	FRS91	HU	Ishikari	Hokkaido	1	1991
	HU94A	HU	Ishikari	Hokkaido	1	1994
	AH95	HU	Ishikari	Hokkaido	1	1995
	NG76	unknown			1	1976
sojae	BSRKI91-9	TAES	Tokachi	Hokkaido	-	1991
	BSRKI91-1	TAES	Tokachi	Hokkaido	-	1991
	BSRKI98-1	TAES	Tokachi	Hokkaido	-	1991
	BSR98-2	TAES	Tokachi	Hokkaido	-	1991
	S8002	Memuro	Tokachi	Hokkaido	-	1980
	A58KS	HU	Ishikari	Hokkaido	-	1983
	S62Ha6	HU	Ishikari	Hokkaido	-	1987
	S62Ha	HU	Ishikari	Hokkaido	-	1987
	S62K17	HU	Ishikari	Hokkaido	-	1987
	S60Sa	HU	Ishikari	Hokkaido	-	1985
	Nag92	Naganuma	Sorachi	Hokkaido	-	1992
	CHI-4	Naganuma	Sorachi	Hokkaido	-	1992
	AK89-9	Nishisenboku		Akita	-	1989
	R3	Kariwano		Akita	-	1987
nonpathogenic	A'31-2	Kunneppu	Abashiri	Hokkaido	-	1978
	A59Ta	HU	Ishikari	Hokkaido	-	1984
	A60K68	HU	Ishikari	Hokkaido	-	1985

A60To	HU	Ishikari	Hokkaido	-	1985
A57T22	Shimizu	Tokachi	Hokkaido	-	1983
To-konT3c-2	TAES	Tokachi	Hokkaido	-	1997
A8-7-5	TAES	Tokachi	Hokkaido	-	1997
Kyo2-1	Churui	Tokachi	Hokkaido	-	1997
Kyo2-2	Churui	Tokachi	Hokkaido	-	1997
Kyo2-3N	Churui	Tokachi	Hokkaido	-	1997
Kyo2-5N	Churui	Tokachi	Hokkaido	-	1997
Kyo3-1N	Churui	Tokachi	Hokkaido	-	1997
Kyo3-2N	Churui	Tokachi	Hokkaido	-	1997
Kyo4-2	Churui	Tokachi	Hokkaido	-	1997
Kyo4-3	Churui	Tokachi	Hokkaido	-	1997
Skt2-3	Shihoro	Tokachi	Hokkaido	-	1997
Skt5-1	Shihoro	Tokachi	Hokkaido	-	1997
Sot8-1	Shihoro	Tokachi	Hokkaido	-	1997
Hir2-3	Hiroo	Tokachi	Hokkaido	-	1997
Hir4-1	Hiroo	Tokachi	Hokkaido	-	1997
Hir6-4	Hiroo	Tokachi	Hokkaido	-	1997
Oto1-3-3N	Otofuke	Tokachi	Hokkaido	-	1997
Oto1-3-4	Otofuke	Tokachi	Hokkaido	-	1997
Oto1-5-1	Otofuke	Tokachi	Hokkaido	-	1997
Tyo6-1	Taiki	Tokachi	Hokkaido	-	1997
Taka-2	Memuro	Tokachi	Hokkaido	-	1997
Mem6-4	Memuro	Tokachi	Hokkaido	-	1997
Seo7-1	Sarabetsu	Tokachi	Hokkaido	-	1997

[1] Tokachi Agriculture Experiment Station, Memuro, Tokachi

[2] Experiment field in Hokkaido University, Sapporo, Ishikari

Table 3. Sporulation ability, gregatins productivity of nonpathogenic *Cadophora gregata* isolates, their pathogenicity and detection from artificially inoculated roots of adzuki bean, mung bean and soybean using polymerase chain reaction (PCR)

Formae specialis	Isolate	Sporulation on V8 juice agar	Gregatins production	Pathogenicity/ PCR[1]		
				Adzuki bean (Erimo-shozu)	Mung bean (un-known)	Soybean (Sapporo-midori)
adzukicola	T96-1	+	+	9/+	8/+	0/+
	T96-5	+	+	10/+	6/+	0/+
sojae	S58KS	+	+	0/+	5/+	7/+
nonpathogenic	A'31-2	+	+	0/+	0/+	0/+
	A57T22	+	-	0/+	0/+	0/+
	A60K68	+	+	0/+	0/+	0/+
	A60To	+	-	0/+	0/+	0/+
	To-konT3c-2	+	+	0/+	0/+	0/+
	Kyo2-1	-	-	0/+	0/+	0/+
	Kyo2-2	-	-	0/+	0/+	0/+
	Kyo2-3N	-	+	0/+	0/+	0/+
	Kyo2-5N	-	-	0/+	0/+	0/+
	Kyo3-1N	-	-	0/+	0/+	0/+
	Kyo3-2N	-	+	0/+	0/+	0/+
	Kyo4-2	-	-	0/+	0/+	0/+
	Kyo4-3	-	-	0/+	0/+	0/+
	Skt2-3	+	+	0/+	0/+	0/+
	Skt5-1	-	-	0/+	0/+	0/+
	Hir4-1	+	+	0/+	0/+	0/+
	Hir2-3	+	-	0/+	0/+	0/+
	Sot8-1	+	+	0/+	0/+	0/+

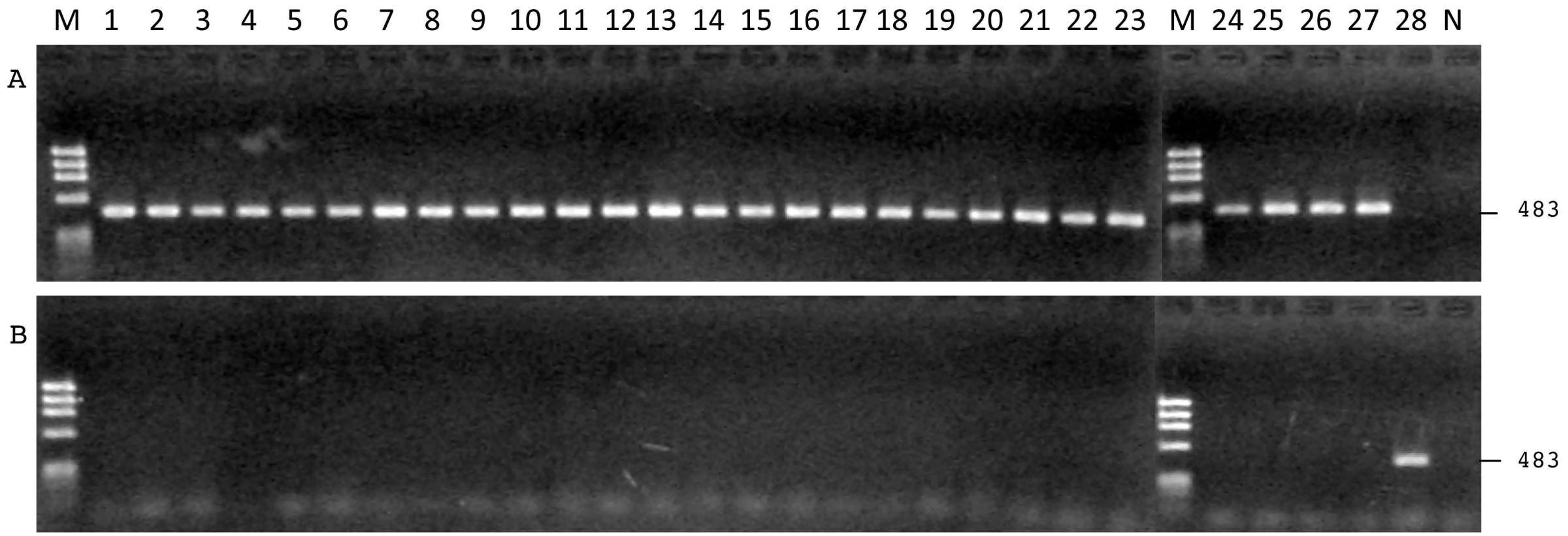
A8-7-5	-	-	0/+	0/+	0/+
Oto1-3-3N	-	-	0/+	0/+	0/+
Oto1-3-4	+	-	0/+	0/+	0/+
Oto1-5-1	+	-	0/+	0/+	0/+
Tyo6-1	+	+	0/+	0/+	0/+
Taka-2	-	-	0/-	0/-	0/-
Mem6-4	-	+	0/+	0/+	0/+
Seo7-1	+	-	0/+	0/+	0/+

[1] Number of plants diseased out of a total 10 tested/ Presence (+) or absence (-) of a specific DNA fragment (483-bp) detected from inoculated roots using PCR with each specific primer set

Table 4. Inter simple sequence repeat (ISSR) primers used in this study and DNA fragments of *Cadophora gregata* isolates

ISSR primers	Anealing Temperature	Total number of fragments	Number of polymorphic fragments		
			CGA[1]	CGS	NP
$(AAG)_7$	51	11	8	9	11
$(AGC)_7$	52	10	10	6	8
$(ACC)_7$	51	10	9	5	8
$(GTG)_7$	50	11	9	6	4
$(GAGA)_4$	48	16	8	12	4
$(TGTC)_4$	48	11	10	7	5
$(TCC)_5$	50	18	14	12	16
$(ACG)_5$	50	16	14	13	10
	Total	103	82	70	66

[1]*C. gregata* f. sp. *adzukicola*, CGA; *C. gregata* f. sp. *sojae*, CGS; nonpathogenic *C. gregata*, NP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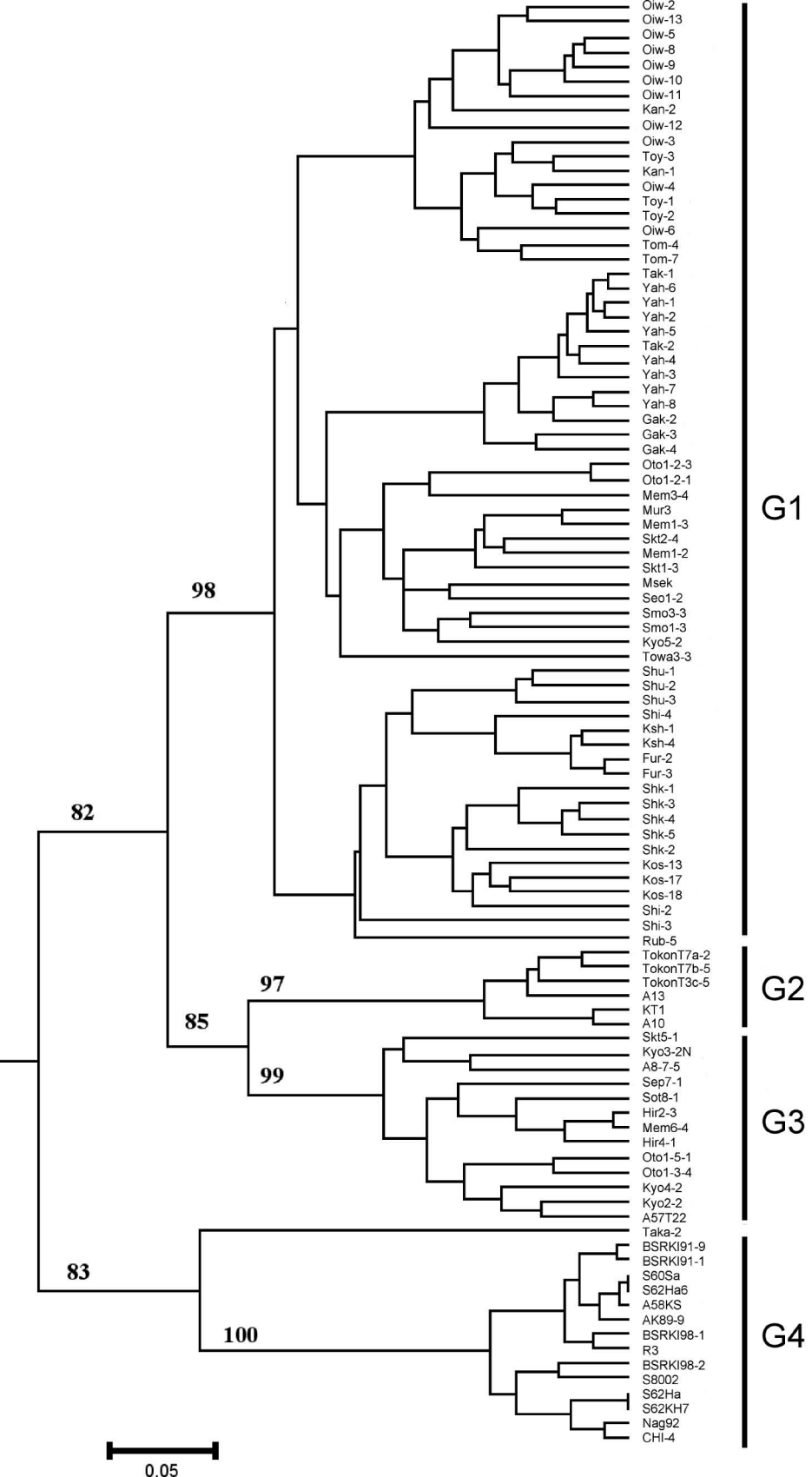
A
B
483
483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Oiw-12
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Kan-1
Oiw-4
Toy-1
Toy-2
Oiw-6
Tom-4
Tom-7
Tak-1
Yah-6
Yah-1
Yah-2
Yah-5
Tak-2
Yah-4
Yah-3
Yah-7
Yah-8
Gak-2
Gak-3
Gak-4
Oto1-2-3
Oto1-2-1
Mem3-4
Mur3
Mem1-3
Skt2-4
Mem1-2
Skt1-3
Msek
Seo1-2
Smo3-3
Smo1-3
Kyo5-2
Towa3-3
Shu-1
Shu-2
Shu-3
Shi-4
Ksh-1
Ksh-4
Fur-2
Fur-3
Shk-1
Shk-3
Shk-4
Shk-5
Shk-2
Kos-13
Kos-17
Kos-18
Shi-2
Shi-3
Rub-5
TokonT7a-2
TokonT7b-5
TokonT3c-5
A13
KT1
A10
Skt5-1
Kyo3-2N
A8-7-5
Sep7-1
Sot8-1
Hir2-3
Mem6-4
Hir4-1
Oto1-5-1
Oto1-3-4
Kyo4-2
Kyo2-2
A57T22
Taka-2
BSRKI91-9
BSRKI91-1
S60Sa
S62Ha6
A58KS
AK89-9
BSRKI98-1
R3
BSRKI98-2
S8002
S62Ha
S62KH7
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CHI-4
G1
G2
G3
G4
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97
85
99
83
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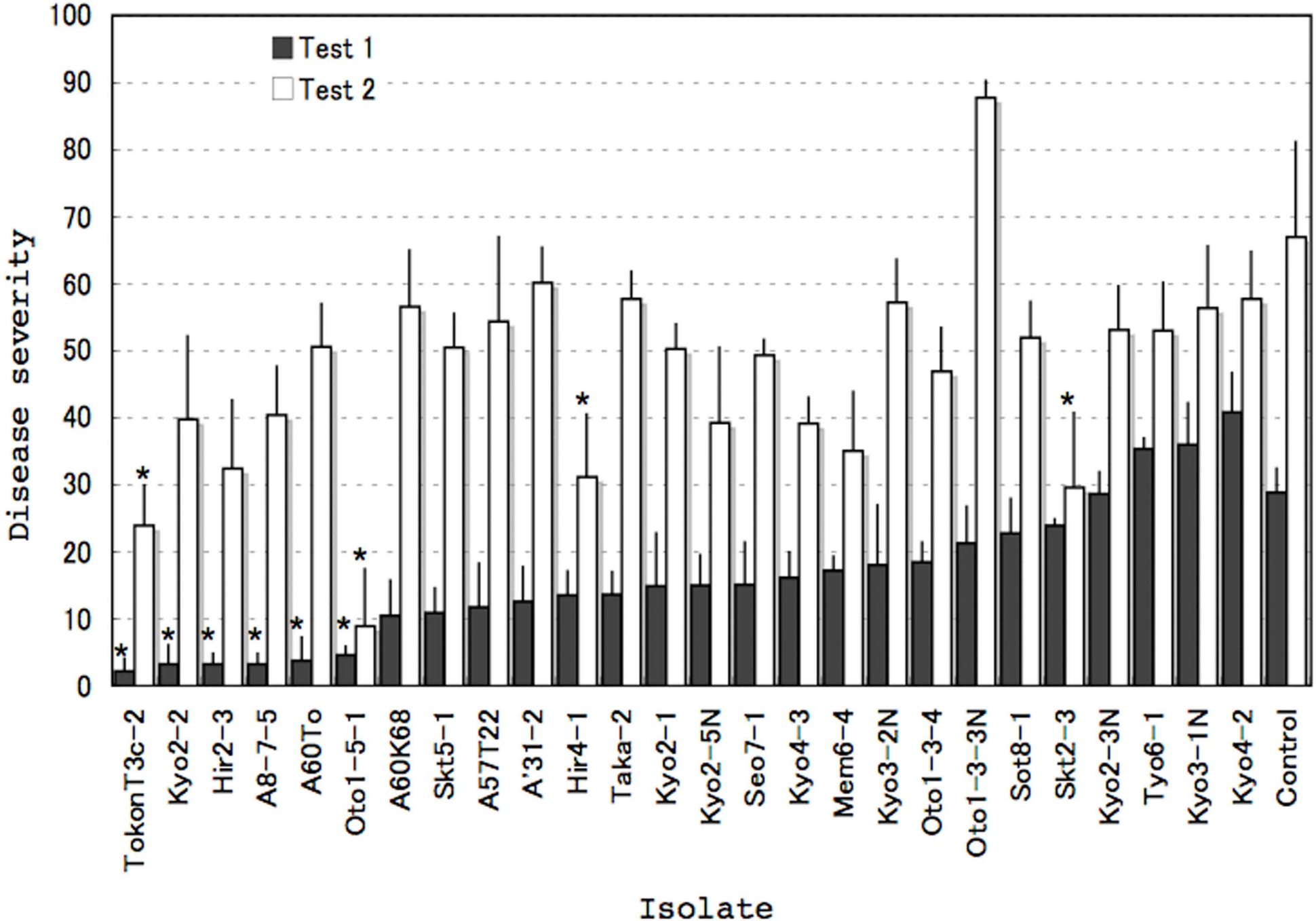
Test 1
Test 2
Disease severity
100
90
80
70
60
50
40
30
20
10
0
TokonT3c-2
Kyo2-2
Hir2-3
A8-7-5
A60To
Oto1-5-1
A60K68
Skt5-1
A57T22
A'31-2
Hir4-1
Taka-2
Kyo2-1
Kyo2-5N
Seo7-1
Kyo4-3
Mem6-4
Kyo3-2N
Oto1-3-4
Oto1-3-3N
Sot8-1
Skt2-3
Kyo2-3N
Tyo6-1
Kyo3-1N
Kyo4-2
Control
Isolate