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Quantification of the rice false smut pathogen *Ustilaginoidea virens* from soil in Japan using real-time PCR

Taketo Ashizawa · Mami Takahashi · Jouji Moriwaki · Kazuyuki Hirayae

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Abstract *Ustilaginoidea virens* is the causal agent of false smut disease of rice. In this study, we developed a real-time polymerase chain reaction (PCR) assay to clarify the relationship between false smut occurrence on rice and quantification of *U. virens* from soil in Japan. The method here described is sensitive, detecting less than 50 fg of pathogen DNA, and specific to the nuclear ribosomal DNA for *U. virens* when tested across 27 rice-pathogenic fungi and bacteria, 26 other fungi and bacteria and four plant species. As few as eight chlamydospores of *U. virens* per gram soil were detected when added to sterilized Gley and Ando soils. The real-time PCR assay for the soil samples was at least 100-fold more sensitive than the conventional and nested-PCR assays tested. By quantification of *U. virens* with real-time PCR using DNA extracted from naturally contaminated Gley soils and visual assessment of the disease in agricultural fields, a linear correlation between cycle threshold (C_T) values and the number of false smut balls was revealed. Therefore, this specific quantitative assay could be a useful tool for optimization of disease control strategies, and for studying the ecology of *U. virens*.

Keywords LightCycler · *Villosiclava*

T. Ashizawa (✉) · M. Takahashi
Rice Disease Resistance Research Team, Hokuriku Research Center, National Agricultural Research Center, National Agriculture and Food Organization,
1-2-1 Inada, Joetsu,
Niigata 943-0193, Japan
e-mail: toketa@affrc.go.jp

J. Moriwaki
Toyama Prefectural Agricultural, Forestry and Fisheries, Research Center,
1124-1 Yoshioka,
Toyama 939-8153, Japan

K. Hirayae
National Agricultural Research Center for Kyusyu Okinawa Region,
2421 Suya, Goushi,
Kumamoto 861-1192, Japan

Introduction

False smut, caused by *Ustilaginoidea virens* (Che.) Tak. (teleomorph *Villosiclava virens*) (Tanaka et al. 2008; White et al. 2000), is one of the most serious diseases of rice (Bischoff et al. 2004; Takahashi 1896). The disease is widespread in the major rice-growing areas of Asia (including Japan), Africa, the United States, South America, Italy, Fiji and Papua-New Guinea (Ou 1972). The fungus transforms individual grains of the panicle into greenish spore balls (false smut balls) of a velvety appearance during maturity, the surface of which are covered by powdery dark-green spores (chlamydospores) (Ou 1972). The chlamydospores survive the winter in the soil and become a primary source of infection of the rice plants. The chlamydospores germinate on coleoptile epidermal cells of rice seedlings, and infection hyphae invade intercellular

spaces and reach the meristematic tissues of rice plants (Ikegami 1963). Before heading of rice plants (at the booting stage), the fungus would invade into rice spikelets (Ashizawa and Kataoka 2005; Zhou et al. 2003) and infect rice florets. While the epidemiology of the disease is relatively well studied, the relationship between the pathogen population density in the soil and disease severity in the paddy fields is unknown.

The control of false smut disease depends on hygiene and use of disease-resistant cultivars, although it is not known if such strategies decrease the pathogen population density in soil, and most commercial rice cultivars are highly susceptible (Deng 1989; Yaegashi et al. 1989). Effective fungicides have only recently become available (Tsuda et al. 2006). At present, however, there are no reliable methods for determining whether fungicides should be applied. Quantification of chlamydospores in soils would allow the farmer to make informed decisions for effective control of the disease.

Conventional polymerase chain reaction (PCR) and nested-PCR techniques were able to detect *U. virens* in symptomless tissues of artificially or naturally infected rice plants (Ashizawa and Kataoka 2005; Zhou et al. 2003). However, these methods have not been applied for detection of *U. virens* in soil. Based on the limited knowledge of the ecology of *U. virens* in the soil and rice plants, the objective of this study was to develop a rapid, sensitive and reproducible real-time PCR assay to detect and quantify the population density of *U. virens* in soil. The sensitivity and specificity of real-time PCR assays using a species-specific primer set were compared with visual assessment of the levels of false smut disease in agricultural fields. This method could be used to detect and quantify the risk of false smut disease in farmers fields.

Materials and methods

Isolates, cultures and plants

Forty-seven Japanese isolates and an Italian isolate of *Ustilaginoidea virens* (Table 1, Fig. 1), 27 other rice-pathogenic fungi and bacteria, 26 other fungi and bacteria, and four plant species were used in this study (Table 1).

An isolate (U2003-1) of *U. virens* was obtained from a false smut ball produced on a floret of rice planted in a paddy field at Daisen (Akita), Japan in 2003. A chlamydospore suspension (5×10^3 spores ml^{-1}) was prepared and then streaked on 2% aqueous agar plates incubated at 15°C. After 2–4 weeks incubation, a single hyphal tip was transferred to potato dextrose agar (PDA) and cultured at 28°C. The hyphal tips were transferred to sterilized barley seeds pre-soaked for 12 h in 2% sucrose solution and cultured at 28°C for one month. The isolate U2003-1 grown on the seeds was dried and maintained at 4°C. For a standard curve, the isolate was cultured in yeast peptone sucrose (YPS) broth for 2 weeks at 25°C, washed twice with sterilized water, and harvested mycelia were stored at −80°C prior to DNA extraction. To obtain chlamydospores in this study, conidia were produced by incubation of the dried seeds in 25 ml of 2% potato dextrose broth on a horizontal shaker at 120 rpm for 5 days. Before heading, 2 ml of conidial suspension (5×10^4 conidia ml^{-1}) was injected into the flag leaf sheath of a highly susceptible rice cultivar (*Oryza sativa* cv. Toride1), and false smut balls with numerous chlamydospores that developed after heading were harvested and used for generation of soil standard curves.

DNA extraction

For fungi, 100 mg of one- to two-week-old mycelia scraped from a culture on PDA, and 100 mg of frozen mycelia of the isolate U2003-1, were placed in a 2.0 ml microcentrifuge tube containing 500 mg of 0.6 mm zirconia/silica beads and a 6 mm stainless steel bead. Similarly, individual colonies of bacteria on YPS agar were harvested. In addition, 100 mg of fresh plant leaves were harvested. The isolates or tissues frozen under liquid nitrogen were ground to a powder using a Shake Master (BioMedical Science, Tokyo, Japan) at 1100 rpm for 2 min. DNA of pure cultures was extracted using the DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA) following the manufacturer's protocol.

The DNA extraction and additional purification procedure from soils was described in a previous report (Kageyama et al. 2003). Briefly, soil (500 mg) was added to a sterilized 2.0 ml microcentrifuge tube containing 500 mg of 0.6 mm zirconia/silica beads. The mixture was suspended in 625 µl extraction buffer (100 mM Tris-HCl, pH 9.0; 40 mM EDTA; 2% sodium dodecyl sulfate; 0.8% skimmed milk), and RNase A (10 mg ml^{-1}), then vigorously vortexed for 1 min. Next, 375 µl benzyl chloride was added to the tube, vigorously vortexed again for 2 min, then

Table 1 Specificity of primer/probe set for detection of *Ustilaginoidea virens* DNA

Species	MAFF number[a]	Source of isolation	Origin[b]	PCR[c]	Real-time PCR[c]
Fungal pathogens					
Ustilaginoidea virens	U2003-1	*Oryza sativa*	Akita (1)	+	+
	U2003-2	*Oryza sativa*	Miyagi (2)	+	+
	U2003-3	*Oryza sativa*	Fukushima (3)	+	+
	U2003-4	*Oryza sativa*	Aomori (4)	+	+
	U2003-5	*Oryza sativa*	Iwate (5)	+	+
	U2003-6	*Oryza sativa*	Miyagi (6)	+	+
	U2006-1	*Oryza sativa*	Tochigi (7)	+	+
	U2006-2	*Oryza sativa*	Toyama (8)	+	+
	U2006-5	*Oryza sativa*	Ishikawa (9)	+	+
	U2006-7	*Oryza sativa*	Ishikawa (10)	+	+
	U2006-8	*Oryza sativa*	Fukui (11)	+	+
	U2006-9	*Oryza sativa*	Fukui (12)	+	+
	U2006-10	*Oryza sativa*	Toyama (13)	+	+
	U2006-11	*Oryza sativa*	Niigata (14)	+	+
	U2007-3	*Oryza sativa*	Niigata (15)	+	+
	U2007-7	*Oryza sativa*	Gifu (16)	+	+
	U2007-15	*Oryza sativa*	Nagano (17)	+	+
	U2007-16	*Oryza sativa*	Nagano (18)	+	+
	U2007-21	*Oryza sativa*	Niigata (19)	+	+
	U2007-28	*Oryza sativa*	Niigata (20)	+	+
	U2009-2	*Oryza sativa*	Shiga (21)	+	+
	U2009-3	*Oryza sativa*	Mie (22)	+	+
	U2009-4	*Oryza sativa*	Mie (23)	+	+
	U2009-5	*Oryza sativa*	Aichi (24)	+	+
	U2009-7	*Oryza sativa*	Nara (25)	+	+
	U2009-8	*Oryza sativa*	Chiba (26)	+	+
	U2009-9	*Oryza sativa*	Shizuoka (27)	+	+
	U2009-10	*Oryza sativa*	Aichi (28)	+	+
	U2009-13	*Oryza sativa*	Shizuoka (29)	+	+
	U2009-14	*Oryza sativa*	Chiba (30)	+	+
	U2009-16	*Oryza sativa*	Shimane (31)	+	+
	U2009-17	*Oryza sativa*	Shimane (32)	+	+
	U2009-19	*Oryza sativa*	Hyogo (33)	+	+
	U2009-20	*Oryza sativa*	Hiroshima (34)	+	+
	U2009-24	*Oryza sativa*	Hiroshima (35)	+	+
	U2009-27	*Oryza sativa*	Tottori (36)	+	+
	U2009-28	*Oryza sativa*	Kyoto (37)	+	+
	U2009-29	*Oryza sativa*	Kyoto (38)	+	+
	U2009-31	*Oryza sativa*	Hyogo (39)	+	+
	U2009-36	*Oryza sativa*	Ehime (40)	+	+
	U2009-37	*Oryza sativa*	Ehime (41)	+	+
	U2009-45	*Oryza sativa*	Kumamoto (42)	+	+
	U2009-46	*Oryza sativa*	Kumamoto (43)	+	+

Table 1 (continued)

Species	MAFF number[a]	Source of isolation	Origin[b]	PCR[c]	Real-time PCR[c]
	236576	*Oryza sativa*	Ibaraki (44)	+	+
	240420	*Oryza sativa*	Ishikawa (45)	+	+
	240421	*Oryza sativa*	Shiga (46)	+	+
	305626	*Oryza sativa*	Akita (47)	+	+
	NBRC5923	–	Italy	+	+
Alternaria alternata	235991	*Oryza sativa*	Ibaraki	–	–
	235998	*Oryza sativa*	Ibaraki	–	–
Alternaria padwickii	235994	*Oryza sativa*	Ibaraki	–	–
Ceratobasidium setariae	237403	*Oryza sativa*	Fukuoka	–	–
	237410	*Oryza sativa*	Niigata	–	–
Cochliobolus miyabeanus	305065	*Oryza sativa*	Okayama	–	–
	305066	*Oryza sativa*	Hokkaido	–	–
	510752	*Oryza sativa*	Chiba	–	–
	511109	*Oryza sativa*	Akita	–	–
Curvularia inaequalis	235530	*Oryza sativa*	Ibaraki	–	–
	237999	Soil	Ibaraki	–	–
Curvularia senegalensis	235538	*Oryza sativa*	Okinawa	–	–
Curvularia inaequalis	237999	Soil	Ibaraki	–	–
Drechslera gigantea	305583	*Oryza sativa*	Miyagi	–	–
Epicoccum nigrum	235995	*Oryza sativa*	Ibaraki	–	–
Gibberella fujikuroi	235949	*Oryza sativa*	Ibaraki	–	–
	235950	*Oryza sativa*	Ibaraki	–	–
	235953	*Oryza sativa*	Fukushima	–	–
	235964	*Oryza sativa*	Fukushima	–	–
Nigrospora oryzae	111239	*Oryza sativa*	Ibaraki	–	–
Pyricularia grisea	101511	*Oryza sativa*	Aichi	–	–
Pythium graminicola	238432	*Oryza sativa*	Saitama	–	–
	238433	*Oryza sativa*	Saitama	–	–
Pythium inflatum	305863	Soil	Fukuoka	–	–
Pythium irregulare	305572	Soil	Kanagawa	–	–
Pythium spinosum	425458	Paddy soil	Hokkaido	–	–
Pythium sylvaticum	237779	Soil	Ibaraki	–	–
Rhizoctonia zeae	237360	*Oryza sativa*	Fukuoka	–	–
	237367	Paddy soil	Wakayama	–	–
Sarocladium oryzae	305006	*Oryza sativa*	Kanagawa	–	–
Sclerotium fumigatum	237402	*Oryza sativa*	Saga	–	–
Sclerotium hydrophilum	237356	*Oryza sativa*	Fukuoka	–	–
	237359	*Oryza sativa*	Fukui	–	–
Tilletia barclayana	305958	*Oryza sativa*	Ibaraki	–	–
	305959	*Oryza sativa*	Ibaraki	–	–
	306156	*Oryza sativa*	Miyazaki	–	–
	306445	*Oryza sativa*	Hyogo	–	–

Table 1 (continued)

Species	MAFF number[a]	Source of isolation	Origin[b]	PCR[c]	Real-time PCR[c]
Bacterial pathogens					
Burkholderia glumae	106541	*Oryza sativa*	Kanagawa	–	–
	106551	*Oryza sativa*	Saitama	–	–
Burkholderia plantarii	106640	Rice nursery soil	Ibaraki	–	–
	106672	*Oryza sativa*	Ishikawa	–	–
Pantoea ananatis	106630	*Oryza sativa*	Ibaraki	–	–
	730149	*Oryza sativa*	Iwate	–	–
Pseudomonas fuscovaginae	106631	*Oryza sativa*	Hokkaido	–	–
	301177	*Oryza sativa*	Hokkaido	–	–
Xanthomonas oryzae pv. *oryzae*	210548	*Oryza sativa*	Kagoshima	–	–
	210559	*Oryza sativa*	Niigata	–	–
Other fungi					
Athelia rolfsii	102020	*Glycine max*	Gunma	–	–
Botrytis cinerea	235990	*Allium cepa*	Hokkaido	–	–
Cladosporium herbarum	235117	*Triticum aestivum*	Ibaraki	–	–
Claviceps panicoidearum	511351	*Miscanthus sinensis*	Tochigi	–	–
Claviceps paspali	306124	*Paspalum* sp.	Tokyo	–	–
Claviceps purpurea	237656	*Hordeum vulgare*	Toyama	–	–
	237657	*Poa annua*	Toyama	–	–
	510877	*Festuca arundinacea*	Tochigi	–	–
Claviceps sorghicola	306571	*Sorghum bicolor*	Tochigi	–	–
	306572	*Sorghum bicolor*	Tochigi	–	–
	306573	*Sorghum bicolor*	Tochigi	–	–
Claviceps sp.	306574	*Sorghum bicolor*	Tochigi	–	–
Curvularia intermedia	235531	*Triticum aestivum*	Ibaraki	–	–
Epichloe sp.	236054	*Agropyron ciliare*	Ibaraki	–	–
Epichloe typhina	241255	*Phleum pratense*	Hokkaido	–	–
	306230	*Brachypodium sylvaticum*	Nagano	–	–
	306601	*Agropyron ciliare*	Ibaraki	–	–
	306602	*Agropyron ciliare*	Ibaraki	–	–
	511345	*Phleum pratense*	Hokkaido	–	–
Epheris japonica	306623	*Paspalum urvillei*	Okinawa	–	–
Fusarium oxysporum	236429	*Triticum aestivum*	Hokkaido	–	–
Gaeumannomyces graminis	305163	*Triticum aestivum*	Tokyo	–	–
Gibberella avenacea	101041	*Triticum aestivum*	Hokkaido	–	–
Hypocrea lutea	NBRC9061	–	–	–	–
Hypocrea rosellus	NBRC6911	–	–	–	–
Hypocrea schweinitzii	NBRC9063	–	–	–	–
Phytophthora megasperma f. sp. *glycinea*	235802	*Glycine max*	Hokkaido	–	–
Rhizoctonia oryzae	235850	*Agrostis palustris*	Kagawa	–	–
	235849	*Agrostis palustris*	Kagawa	–	–
Rhizopus arrhizus	238040	*Vigna radiata*	Tokyo	–	–

Table 1 (continued)

Species	MAFF number[a]	Source of isolation	Origin[b]	PCR[c]	Real-time PCR[c]
Thanatephorus cucumeris	237258	*Glycine max*	Miyagi	−	−
	237259	*Triticum aestivum*	Iwate	−	−
Trichoderma viride	236543	Decayed tree	Nagano	−	−
Verticillium dahliae	103002	*Solanum melongena*	Nagano	−	−
Other bacteria					
Pseudomonas fluorescens	520043	*Triticum aestivum*	Hokkaido	−	−
Streptomyces sp.	109053	Soil	Hokkaido	−	−
Plant species					
Glycine max	−	−	−	−	−
Hordeum vulgare	−	−	−	−	−
Oryza sativa	−	−	−	−	−
Triticum aestivum	−	−	−	−	−

[a] Samples from the Ministry of Agriculture, Forestry and Fisheries (MAFF) Gene Bank; Isolates from U2003-1 to U2009-46 collected in Japan; NBRC5923, 6911, 9061, and 9063 from National Institute of Technology and Evaluation, Biological Resource Center (NBRC).

[b] Number in parenthesis indicates site from which *U. virens* was isolated in Japan (refer to Fig. 1).

[c] +, positive; −, negative

incubated at 50°C for 1 h. Following incubation, 375 μl of 3.0 M NaOAc was added to the suspension, gently vortexed, and incubated on ice for 15 min. The suspension was centrifuged for 10 min at 18,000× *g*, and the upper layer was transferred to a clean tube. This step was repeated twice. DNA was precipitated by addition of an equal volume of isopropanol and centrifugation for 20 min at 18,000 × *g*. The DNA

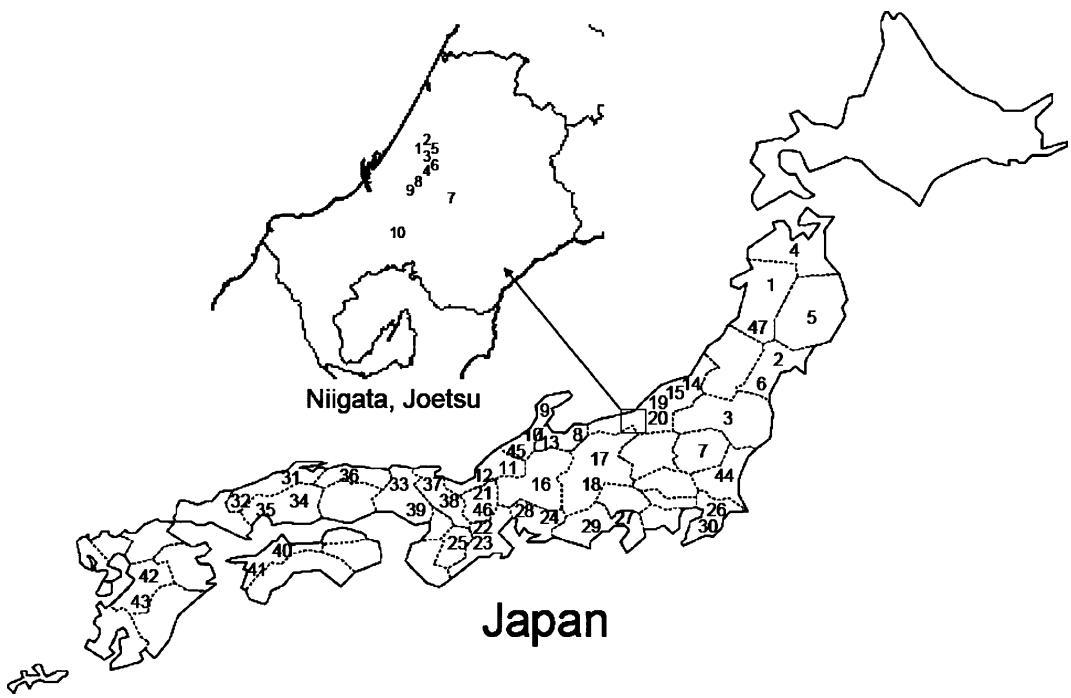

Fig. 1 Sites (prefectures) in Japan from which *Ustilaginoidea virens* was isolated, and sampling sites of Gley soil in agricultural paddy fields in Joetsu, Niigata

was washed with 70% ethanol and dried under vacuum. The DNA was dissolved in 200 μl Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0).

The extracted DNA was purified using the Geneclean Spin Kit following the manufacturer's recommendations (Qbiogene, Carlsbad, CA). Ultimately, 15–20 μl of DNA solution was obtained from an initial volume of 200 μl, and was stored at −20°C. The solution was diluted 1:5 with autoclaved Milli Q water prior to conventional PCR, nested-PCR and real-time PCR assays.

Real-time PCR primer/probe design and specificity

The 2852-bp and 2834-bp ribosomal DNA (rDNA) sequences (GenBank accession nos. AB105954 and AB116645, which share the same 2,834-bp sequences) were analyzed using Primer3 and Probe Finder software at the Universal ProbeLibrary Assay Design Center website (Roche Diagnostics, Mannheim, Germany) to identify species-specific nucleotide sequences for the primers and fluorochrome-labeled probe used in this study. The following primers were synthesized (Greiner Bio-One, Tokyo, Japan): forward primer uvr1497F 5′-CCGCTGCCTAAGATAAAGTCC-3′; reverse primer uvr1586R 5′-AGGCTCCCGGTTGTTTTTAC-3′ (Fig. 2). The Universal ProbeLibrary Probe #57 (5′-GGCCCCAG-3′) was selected (Fig. 2). The probe labeled the 5′ terminus with fluorescein (FAM) and near the 3′ terminus with a dark quencher dye. The primer/probe set uvr1497F-Probe #57-uvr1586R amplified a product 109 bp in length.

Before a potential primer/probe set was used, its putative amplicon sequence (109 bp long) was compared with available sequences in GenBank and EMBL using the BLAST sequence alignment search tool (available online from the National Center for Biotechnology Information, Bethesda, MD), as a first step in eliminating non-specific primer/probe candidate sets. In the second step, the primer/probe set was screened for specificity using the 48 *U. virens* isolates and other fungal and bacterial pathogens of rice, other fungi and bacteria and plants (Table 1) by amplification using real-time PCR following the protocol below. For each isolate, the DNA extract concentration was 1.0 ng μl^{-1}, and 5 μl of DNA extract was included in each reaction. For the primer set, the appropriate positive control was included at 5 ng per reaction, and PCR-grade water was used as the negative control. In addition, conventional PCR was also carried out in a GeneAmp PCR system 9700

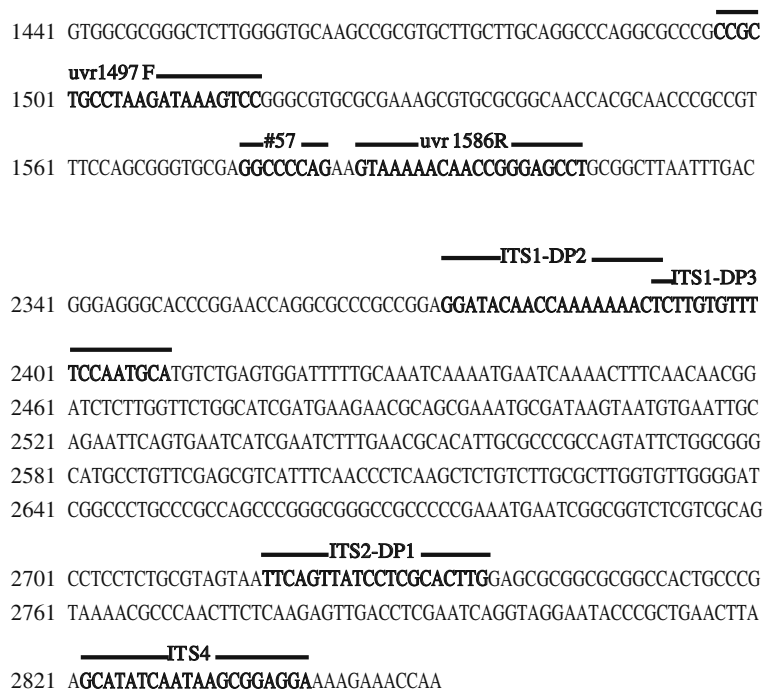

Fig. 2 Sequence, locations of primers and fluorogenic probes within the ribosomal DNA region of *Ustilaginoidea virens* (Genbank accession numbers AB105954 and AB116645, which share the same sequences). Uvr1497F, uvr1586R and #57, forward (F) and reverse (R) primers and universal ProbeLibrary probe for real-time PCR; ITS1-DP2 and ITS4, F and R primers for PCR; ITS1-DP3 and ITS2-DP1, F and R primers for nested-PCR

(Applied Biosystems, Foster City, CA) with 0.5 U HotStar *Taq* polymerase (QIAGEN), 1 × HotStar buffer, 250 μM each dNTP, 0.5 μM each primer (uvr1497F and uvr1586R), and 2 ng template DNA in a final volume of 20 μl. The PCR conditions were 95°C for 15 min, followed by 30 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were separated on ethidium bromide-stained 1.0% (wt vol^{-1}) agarose gel run in TBE buffer and exposed to ultraviolet light to visualize the DNA fragment of 109 bp.

Standard curves

A standard curve based on threshold cycles (C_T) for a 10-fold dilution series, with the addition of a 0.5 dilution, of pure DNA from *U. virens* (in ng μl^{-1}; 1×10^{0}, 0.5×10^{0}, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6}) were constructed in triplicate real-time reactions. The C_T values were automatically calculated with LightCycler version 1.2 (Roche Diagnostics, Tokyo, Japan) to indicate the cycle at which increased fluorescence signals exceeded the background during the exponential growth phase of the PCR amplification process. A standard curve was obtained by plotting the C_T value versus the logarithm of the concentration of each dilution of *U. virens* DNA.

To relate C_T to number of *U. virens* chlamydospores per gram of soil, a separate standard curve was generated. For the soil standard curves, representative and widely distributed Gley and Ando soils collected from paddy fields at Joetsu (Niigata), Japan were also used to determine whether the DNA extraction method was effective for the pathogen occurring in different soil types. The soils were air-dried for 2 weeks at room temperature, debris were removed, and then the soils were mixed thoroughly and autoclaved. A 500 mg sample of each soil type was placed in a 2.0-ml microcentrifuge tube containing 500 mg of 0.6 mm zirconia/silica beads and was mixed with 10 μl of a *U. virens* chlamydospore suspension corresponding to either 8, 80, 800, or 8000 chlamydospores ml^{-1} in autoclaved Milli Q water. The triplicate samples were stored at −20°C prior to DNA extraction. The samples were extracted as described earlier for DNA extraction from soil. A soil standard curve was obtained by plotting the C_T value versus the logarithm of the concentration of each 10-fold dilution series of chlamydospores.

Real-time PCR conditions and analysis

DNA extracted from pure cultures of rice-pathogenic fungi and bacteria, other fungi and bacteria, plant tissues, and from the inoculated paddy soils and naturally contaminated agricultural soil samples, was amplified with the primer/probe set uvr1497F-Probe #57-uvr1586R. For each 1/5 diluted DNA sample, triplicate reactions were performed in 20 μl volumes consisting of 5 μl DNA template, 10 μl 2 × LightCycler 480 Probe Master (Roche), 1000 nM each forward and reverse primer, and 200 nM probe. The primer concentration was first optimized, and then probe optimization was performed using the primer concentration. Amplification and detection of fluorescence were conducted using a LightCycler 480 system (Roche). The thermal-cycling conditions consisted of a single cycle at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s, 59°C for 25 s, and 50°C for 15 s. All reactions were performed in triplicate, and included parallel reactions of three separate dilution series of pure standard DNA in all PCR amplification runs.

Comparison among real-time PCR, conventional PCR and nested-PCR assays for detection of *U. virens* in soil

The conventional PCR and nested-PCR were carried out as described earlier, and the forward and reverse primers ITS1-DP2 (Ashizawa and Kataoka 2005) and ITS4 (White et al. 1990) for conventional PCR, and ITS1-DP3 and ITS2-DP1 (Ashizawa and Kataoka 2005) for nested-PCR, were used (Fig. 1). In addition, these primer specificities were tested using all species listed in Table 1. The template DNA (2 μl) for the conventional PCR was used as described earlier for DNA extraction from soil, and 1 μl of the first-round PCR amplicon was used for the nested-PCR amplification. The real-time PCR assay as described above, and conventional PCR and nested-PCR assays, were compared for their sensitivity to detect *U. virens* in soil (Table 2).

Evaluation of disease

At maturity of the commercial rice cv. Koshihikari, the severity of false smut disease (Brooks et al. 2009; Osada 1995) was evaluated in each sampled agricultural paddy field (Fig. 1, Table 3) during August 2008. A total of 10 fields (10 hills per sampling point; a total of 100 hills per field) were observed and the

Table 2 Polymerase chain reaction (PCR) and nested-PCR detection of *U. virens* DNA extracted from soil

	Soil	Number of chlamydospores[a]			
		8	80	800	8000
PCR	Gley	–[b]	–	–	+
	Ando	–	–	–	–
Nested-PCR	Gley	–	–	+	+
	Ando	–	–	–	+

[a] Sterilized soil samples were inoculated with chlamydospores per gram dry weight of soil.

[b] +, detected;–, not detected

number of false smut balls (greenish balls) in florets was counted. The disease severity was determined by calculating the average number of smut balls per hill from the 10 sampling points per field.

Agricultural soil samples

Soil samples (approximately 100 g per sample) were collected during June 2008 from paddy fields containing machine-planted rice seedlings in Joetsu, Niigata (Fig. 1, Table 3), because the chlamydospores germinate and infect rice seedlings during the life cycle of *U. virens* (Ikegami 1963). All soil samples were air-dried for 2 weeks at room temperature, debris were removed and the soils were mixed thoroughly. DNA was extracted from 10 samples per field as described earlier for DNA extraction from soil.

Data analysis

The relationships between C_T values and log-transformed fungal DNA concentration, number of chlamydospores in the artificially inoculated Gley and Ando soils, and disease severity (number of false smut balls per hill) in the agricultural paddy fields were analyzed by correlation analyses using SAS version 9.2 (SAS Institute, Inc., Cary, NC).

Results

Primer/probe design for real-time PCR

The primer specificity test was conducted using conventional PCR and the primer/probe specificity test was conducted using real-time PCR. The primer/probe set was shown to be specific for detection of all the 48 isolates of *U. virens* that formed no dimers and no non-specific amplification was obtained (Table 1).

Table 3 Cycle threshold (C_T) values in real-time polymerase chain reaction assays of *U. virens* DNA extracted from agricultural Gley soil samples and severity of false smut disease of rice

Sample	Origin	Number of smut balls per hill[a]	C_T[b]
1	Kakizaki, Joetsu	4.23±3.35	31.49±0.66
2	Kakizaki, Joetsu	3.08±2.7	31.67±0.29
3	Kakizaki, Joetsu	1.52±1.29	33.36±0.22
4	Kakizaki, Joetsu	0.92±0.8	33.03±0.27
5	Kakizaki, Joetsu	0.63±0.58	34.25±0.64
6	Kakizaki, Joetsu	0.57±0.6	34.54±0.48
7	Yoshikawa, Joetsu	0.41±0.2	33.98±0.5
8	Kakizaki, Joetsu	0.04±0.07	35.15±0.5
9	Kakizaki, Joetsu	0.0	38.46±0.58
10	Inada, Joetsu	0.0	39.18±1.04 (7/10 detected)[c]

[a] Values are means±standard deviation from 10 sampling points per field (10 hills per sampling point, a total of 100 hills per field).

[b] Values are the mean±standard deviation for the 10 sampling points per field (three replicates per sample, a total of 30 replicates per field).

[c] Seven out of 10 sampling points were used for calculation of the mean C_T value. No amplification was observed in soil samples from the other three sampling points

Standard curves for real-time PCR

A standard curve was obtained with reproducible amplification, and the $\log_{10}$ *U. virens* DNA concentration and C_T were highly correlated (R^2=0.9977) for the primer pair (Fig. 3). The DNA was quantifiable at concentrations from 50 fg to 5 ng, whereas 5 fg DNA was not amplified.

Quantification of *U. virens* in soil using real-time PCR

To determine whether soil type affected the precision of *U. virens* DNA quantification by real-time PCR, the values of amplified DNA extracted from soil with those obtained from serially diluted pure chlamydospore suspensions were compared. The quantitative

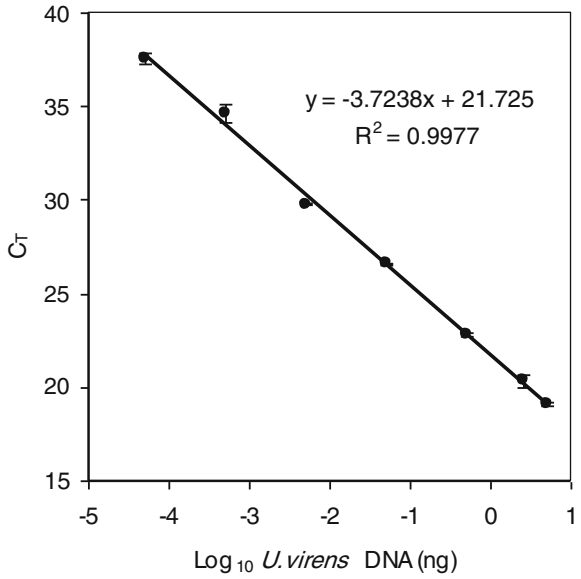

Fig. 3 Standard curve for *U. virens* DNA in a real-time polymerase chain reaction probe assay. Cycle threshold (C_T) values are plotted against the DNA concentration diluted with sterilized Milli Q water. Each point represents the mean of three replicates. Error bars represent the standard deviation

real-time PCR assay was sensitive enough to detect the lowest numbers of *U. virens* chlamydospores (eight chlamydospores per gram) added to soil samples. The assay did not constantly detect *U. virens* chlamydospores lower than the number per gram (data not shown). A linear relationship (Fig. 4) was obtained between chlamydospore concentration and the amount of DNA quantified from the soil (Gley soil: R^2=0.993; Ando soil: R^2=0.999). It also demonstrated that there was no significant difference in DNA extraction efficiency between the two soil types.

Comparison among real-time PCR, conventional PCR and nested-PCR assays for detection of *U. virens* in soil

Overall, the real-time PCR assay described above was at least 100-fold more sensitive than the conventional and nested-PCR assays (Table 2). While the real-time PCR assay showed no difference in sensitivity between the two soil types, both the conventional and nested-PCR assays for detection from the Gley soil samples was approximately 10-fold greater than those from the Ando soil samples. In addition, conventional and nested-PCR primer/probe sets detected all 48 isolates of *U. virens*, forming no dimers and showing no non-specific amplification (data not shown).

Analysis of agricultural samples and disease severity

Real-time PCR assays were positive for nine of the 10 fields (a total of 90 soil samples), but in one of the fields three of the 10 soil samples were negative (Table 3). The average C_T value ranged from 31.49 to 38.46 for nine fields, and was 39.18 for the single field in which DNA was amplified in seven of the 10 soil samples.. The average number of false smut balls per hill ranged from 0.04 to 4.23 for eight of the 10 fields. No smut balls were observed on rice panicles in two fields, whose C_T values were 38.46 and 39.18, respectively. The relationship between C_T (x) values and observed number of false smut balls per hill (y) in the eight fields, excluding the two fields in which no false smut balls were observed, was expressed by the equation y=−1.0418x+36.255 (R^2=0.872).

Discussion

In this study, we demonstrated the utility of a real-time PCR assay developed for quantification of *U. virens* in soil. The specificity of the primers and probe was confirmed by testing them against 47 Japanese isolates and an Italian isolate of *U. virens*, 27 rice-pathogenic

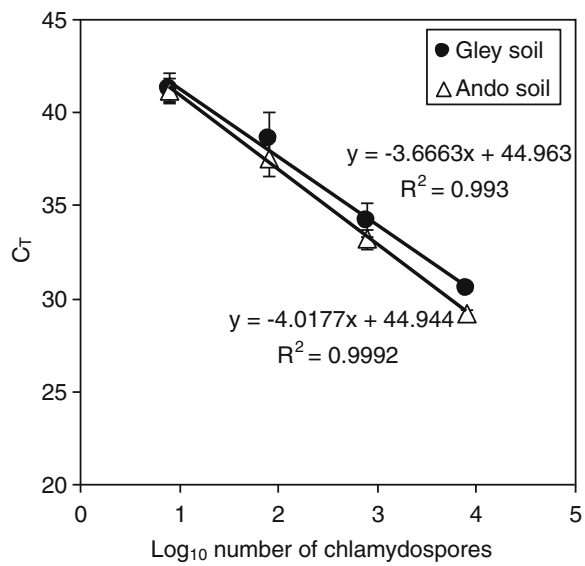

Fig. 4 Standard curve for *U. virens* DNA extracted from soil. Sterilized Gley and Ando soil samples were inoculated with either 8, 80, 800 or 8000 chlamydospores per gram dry weight of soil. Each point represents the mean of three replicates. Error bars represent the standard deviation

fungi and bacteria, 26 other fungi and bacteria, and four plant species using conventional and real-time PCR.

The standard curve was generated using a range of DNA concentrations from 5 ng to 50 fg. In all DNA concentrations, the real-time PCR amplification was very reproducible with an average standard deviation of 0.177 (within 0.5) for the concentration estimates.

The real-time PCR was able to detect and quantify *U. virens* both in artificially and naturally contaminated soil samples, and the soil DNA extractions were highly reproducible. DNA of *U. virens* was detected at concentrations <8 chlamydospores per gram sterilized soil but was not detected in the sterilized uninoculated control. The detection and quantification of chlamydospores both in the Gley and Ando soil types was generally consistent, while average standard deviations of 0.82 and 0.54, respectively, for these concentration estimates were higher than that for the standard curve. In contrast, the conventional PCR and nested-PCR detection of *U. virens* in artificially contaminated soil samples was apparently less sensitive than the real-time PCR assay, and soil type also affected the sensitivity. Although not tested in this study, inhibitors, lower amplification efficiency and other factors may have affected the PCR amplification (Volossiouk et al. 1995). These data indicate that the conventional and nested-PCR may seriously limit its applicability as a detection method for soil DNA extracts.

Phylogenetic analyses have demonstrated that Ustilaginoideae species are not allied with the genus *Claviceps* (Bischoff et al. 2004; Tanaka and Tanaka 2008; White et al. 2000). A new genus, *Villosiclava*, was proposed for the teleomorph of *U. virens* by Tanaka et al. (2008), indicating that the morphological and biological characteristics of the species are distinct from those of *Claviceps*. In addition, the rDNA sequence for *U. virens* deposited in GenBank (accession nos. AB105954 and AB116645) possessed a unique region not present in any of the other organisms tested, including *Claviceps*, and allowed for designing the species-specific primers for the real-time PCR. Similarly, the PCR and nested-PCR primers (Ashizawa and Kataoka 2005; Zhou et al. 2003) were specific for the 47 Japanese isolates and an Italian isolate. On the basis of the rDNA sequences of *U. virens* deposited in GenBank and EMBL, the specificity may be consistent among other Chinese and Japanese isolates (Bischoff et al 2004; Tanaka and Tanaka 2008; Zhou et al 2003). However, there are some variations of the rDNA sequences in some other Chinese and Indian isolates deposited in GenBank or EMBL (unpublished data). Therefore, if such sequence variations in the rDNA exist within the *U. virens* population, further analysis of the PCR and nested-PCR primer specificities may be required.

Ustilaginoideae species also include *Ustilaginoidea dichronemae*, a pathogen of upland grass weeds (*Dichronema* and *Paspalum* spp.) collected in Brazil, Costa Rica, Puerto Rico, and Venezuela, and a bamboo (*Chusquea* spp.) pathogen *Neomunkia sydowii* collected in Costa Rica (Bischoff et al. 2004). To date, the two pathogens have not been reported in other rice-growing regions, including Japan, and no cultures presently exist anywhere in the world. Further study of the primer specificity will be needed if these two pathogens are found to be more widely distributed. However, the distribution and epidemiology apparently differ between the two pathogens and *U. virens*.

For samples taken from Gley soil in paddy fields (with a well-mixed soil layer) planted with rice plants at Niigata (Fig. 1, Table 3), a statistically significant correlation was found between the number of false smut balls observed and C_T values detected by the real-time PCR (R^2=0.872, n=8). The detection limit of *U. virens* chlamydospores was in the same order of magnitude as that of *Colletotrichum coccodes* spores (Cullen et al. 2002) and *Helminthosporium solani* spores (Cullen et al. 2001), and seemed to be relatively high compared with those of other soilborne pathogens (see review by Okubara et al. 2005). Although not tested in this study, the real-time PCR assay will be a useful tool for determining the presence of *U. virens* in naturally contaminated Ando soil samples; the soil standard curve for the Ando and Gley soils did not differ statistically. No false smut balls were observed in two fields with high C_T values, which suggests that a low number of chlamydospores would be affected for infection of Koshihikari rice. To our knowledge, this is the first report investigating the relationship between false smut occurrence and DNA quantification using real-time PCR. In addition, almost the same severity was observed in randomly selected four fields (sample 3, 1.35±0.37; sample 7, 0.37±0.31; sample 9, 0; sample 10, 0) in 2009 indicates that the C_T values may be useful to estimate disease risk.

The method for quantification of *Ustilaginoidea virens* in soil outlined in this study is very sensitive

and reproducible compared with the conventional and nested-PCR. The technique, combined with false smut occurrence and meteorological data analyses, will facilitate the risk management of false smut disease. It also will be useful for the development of disease management strategies using fungicides, quantitative host resistance, soil conditioners and crop rotations.

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References

Ashizawa, T., & Kataoka, Y. (2005). Detection of *Ustilaginoidea virens* in rice panicles before and after heading in the field using nested-PCR technique with species-specific primers. *Japanese Journal of Phytopathology, 71*, 16–19.

Bischoff, J. F., Sullivan, R. F., Kjer, K. M., & White, J. F., Jr. (2004). Phylogenetic placement of the anamorphic tribe Ustilaginoideae (Hypocreales, Ascomycota). *Mycologia, 96*, 1088–1094.

Brooks, S. A., Anders, M. M., & Yeater, K. M. (2009). Effect of cultural management practices on the severity of false smut and kernel smut of rice. *Plant Disease, 93*, 1202–1208. doi:10.1094/PDIS-93-11-1202.

Cullen, D. W., Lees, A. K., Toth, I. K., & Duncan, J. M. (2001). Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. *European Journal of Plant Pathology, 107*, 387–398.

Cullen, D. W., Lees, A. K., Toth, I. K., & Duncan, J. M. (2002). Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. *Plant Pathology, 51*, 281–292.

Deng, G. S. (1989). Present status of research on false smut in China. *Plant Protection Beijing, 15*, 39–40.

Ikegami, H. (1963). Invasion of chlamydospores and hyphae of the false smut fungus into rice plants. *Research Bulletin of the Faculty of Agriculture, Gifu University, 18*, 54–60.

Kageyama, K., Komatsu, T., & Suga, H. (2003). Refined PCR protocol for detection of plant pathogens in soil. *Journal of General Plant Pathology, 69*, 153–160. doi:10.1007/s10327-002-0037-4.

Okubara, P. A., Schroeder, K. L., & Paulitz, T. C. (2005). Real-time polymerase chain reaction: applications to studies on soilborne pathogens. *Canadian Journal of Plant Pathology, 27*, 300–313.

Osada, S. (1995). Effect of false smut occurrence on yield and quality of rice. *Annual Report of the Society of Plant Protection of North Japan, 46*, 30–32.

Ou, S. H. (1972). *Rice diseases*. Kew: Commonwealth Mycological Institute.

Takahashi, Y. (1896). On *Ustilago virens* Cooke and a New Species of *Tilletia* parasitic on rice plant. *Botanical Magagine, Tokyo, 10*, 16–20.

Tanaka, E., & Tanaka, C. (2008). Phylogenetic study of clavicipitaceous fungi using acetaldehyde dehydrogenase gene sequences. *Mycoscience, 49*, 115–125. doi:10.1007/s10267-007-0401-5.

Tanaka, T., Ashizawa, T., Sonoda, R., & Tanaka, C. (2008). *Villosiclava virens* gen. nov., com. nov., teleomorph of *Ustilaginoidea virens*, the causal agent of rice false smut. *Mycotaxon, 106*, 491–501.

Tsuda, M., Sasahara, M., Ohara, T., & Kato, S. (2006). Optimal application timing of simeconazole granules for control of rice kernel smut and false smut. *Journal of General Plant Pathology, 72*, 301–304. doi:10.1007/s10327-006-0288-6.

Volossiouk, T., Robb, E. J., & Nazar, R. N. (1995). Direct DNA extraction for PCR-mediated assays of soil organisms. *Applied and Environmental Microbiology, 61*, 3972–3976.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). California: San Diego.

White, J. F., Jr., Sullivan, R., Moy, M., Patel, R., & Duncan, R. (1990). An overview of some current problems in the classification of plant-parasitic Clavicipitaceae. *Studies in Mycology, 45*, 95–105.

Yaegashi, H., Fujita, Y., & Sonoda, R. (1989). Severe outbreak of false smut of rice in 1988. *Plant Protection Tokyo, 43*, 311–314.

Zhou, Y.-L., Izumitsu, K., Sonoda, R., Nakazaki, T., Tanaka, E., Tsuda, M., et al. (2003). PCR-based specific detection of *Ustilaginoidea virens* and *Ephelis japonica*. *Journal of Phytopathology, 151*, 513–518.