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FUNGAL DISEASES

First report of leaf sheath rot of Welsh onion caused by nine taxa of *Rhizoctonia* spp. and characteristics of the pathogens

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Abstract From 2007 to 2013, a disease of Welsh onion, causing leaf sheath rot and concomitant death of outer leaves was found in 20 fields in Hokkaido, Japan. We obtained 20 *Rhizoctonia* isolates from diseased tissues and identified them based on the number of nuclei, hyphal fusion reactions, and molecular techniques using specific PCR primers and sequence of the rDNA-ITS region. The 20 isolates consisted of 16 multinucleate and four binucleate isolates. Of the multinucleate isolates, five were found to be so far unknown and designated here as *Rhizoctonia solani* AG-4 hybrid subgroup between HG-I and HG-II. Others were identified as AG-1 IB (three isolates), AG-2-2 IIIB (two isolates), AG-4 HG-I (two isolates), AG-1 IC (one isolate), AG-2-1 (one isolate), AG-4 HG-II (one isolate) and AG-5 (one isolate). All four binucleate isolates were binucleate *Rhizoctonia* AG-U. Original symptoms were reproduced on all plants inoculated with these isolates. Thus, we revealed that as many as nine taxa of *Rhizoctonia* spp. were associated with the disease. This is the first report of leaf sheath rot of Welsh onion caused by *Rhizoctonia* spp.

Keywords *Allium fistulosum* · Hokkaido · Hybrid · Leaf sheath · Subgroup · *Rhizoctonia*

The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases as accessions LC090068–090073.

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Introduction

Welsh onion (*Allium fistulosum* L.) is native to Southwest China and commonly cultivated in East Asian countries such as Japan, China and Korea (Ford-Lloyd and Armstrong 1993; Kumazawa and Katsumata 1965). In the 2014 growing season, approximately 22,900 ha were planted with Welsh onion in Japan. In Hokkaido, the northernmost island of Japan, 2-month-old seedlings of Welsh onion are raised in a greenhouse, transplanted into open fields from late April to mid June, then harvested 4 months later (from August to October). Leaf sheaths of the plants are covered three times with soil by hilling with an interval of 1 month to promote etiolated growth. Some farmers also cultivate the plants in greenhouses from autumn to the following spring. Welsh onion is usually cultivated continuously without rotation with other crops in Hokkaido. Leaf sheath rot and outer leaf death of Welsh onion were first found to occur in fields and a greenhouse in Hokkaido from 2007 to 2013, and *Rhizoctonia* spp. were consistently isolated from diseased tissues.

Rhizoctonia solani is a species complex separated by hyphal anastomosis interactions. Currently, the *R. solani* complex is divided into 13 anastomosis groups (AGs): AGs 1 through 13 (Carling et al. 2002). Several AGs are further divided into subgroups on the basis of pathogenicity, biochemical and genetic makers. Binucleate *Rhizoctonia* is also classified based on hyphal anastomosis interactions. To date, 18 AGs A through W, except for AG-J, M, T, N and V, are recognized (Sharon et al. 2008; Yang et al. 2015).

Leaf sheath rot of Welsh onion caused by *Rhizoctonia* spp. has not been reported so far, therefore, we aimed to confirm their pathogenicity, to identify and to characterize the isolates.

Materials and methods

Disease occurrence and fungal isolation

The disease was surveyed in 20 Welsh onion fields, including one greenhouse in major production areas in Hokkaido from 2007 to 2013 (Table 1). Rotted basal parts of leaves (Fig. 1b) were sampled from each field. A total of 40–60 leaf pieces (ca. 5 × 5 mm) were cut from ca. 10 leaves collected randomly from each field. Leaf pieces were washed in running water, blotted on sterilized filter paper, and then placed on 9-cm-diameter petri dishes containing potato dextrose agar (PDA) amended with 20 mg/L streptomycin sulfate. After incubation at 25 °C for 2 days, 24–30 isolates typical of *Rhizoctonia* spp. were selected for each field, transferred to water agar (WA) plates, and grown for 2 days at 25 °C for hyphal tip isolation. Hyphal tip isolates were placed on PDA plates (6 isolates per plate) and incubated at 25 °C. Ten days after incubation, one isolate was selected from isolates with similar cultural morphology to maintain as barley grain culture (Naito et al. 1993) at − 20 °C until use.

Identification of isolates

The isolates were examined for the number of nuclei per cell and cultural morphology as described previously (Misawa and Kuninaga 2010, 2013). Hyphal anastomosis tests were conducted according to Rinehart et al. (2007) with a modification. All the isolates were paired with tester isolates belonging to *R. solani* AGs 1 – 5 (Misawa et al. 2015) or with the binucleate *Rhizoctonia* AGs A through U, excluding AGs J, M, T and N (Misawa and Toda 2013). Isolates were grown on PDA for 3 days at 25 °C. Mycelial plugs (5 mm diameter) were taken from colony margins of each Welsh onion isolates and AG tester isolates and paired ca. 2 cm apart on glass slides (76 × 26 mm) coated with WA, placed on a paper towel moistened with distilled water in a plastic box (24 × 17 × 8.5 cm) and incubated at 25 °C. From 2 to 3 days after incubation, the overlapping portion of mycelia was observed microscopically, and fusion frequency was determined as follows: low, <30%; moderate, 30–50%; high, >50% (Sneh et al. 1991), and the interactions were scored as C0–C3 reactions based on the scale developed by Carling (1996).

PCR amplifications using AG- or subgroup-specific primers were used to detect *R. solani* AG-1 IA, IB and IC (Kuninaga 2003), AG-2-1 (Carling et al. 2002), AG-2-2 IIIB, IV and LP (Carling et al. 2002), AG-4 HG-I, HG-II

Table 1 Summary of occurrence of Welsh onion leaf sheath rot in 20 fields investigated in this study from 2007 to 2013

Year	Field	Date of study	City	Cultivated area (m^2)	Diseased plants (%)	No. dead leaves[b]	Cultivar
2007	1	23-Aug	Hokuto	1,400	50	3–5	Shirahane-ipponbuto
2008	2	7-Aug	Hokuto	8,000	80	3–5	Hikarino-tsurugi
2009	3[a]	21-Dec	Yakumo	330	5	2–3	Fuyuougi-3go
2011	4	5-Sep	Hokuto	650	30	3–5	Shirahane-ipponbuto
2012	5	8-Aug	Hokuto	8,000	80	1–2	Syuga
	6	21-Aug		40	100	2–4	Kan-u ipponbuto
2013	7	14-Aug	Hokuto	1,450	5	1–2	Kitano-takumi
	8	14-Aug		400	30	1–3	Kitano-takumi
	9	14-Aug		1,400	100	3–5	Kitano-takumi
	10	14-Aug		2,700	100	2–4	Kitano-takumi
	11	14-Aug		2,300	80	1–3	Kan-u ipponbuto
	12	19-Aug		250	80	2–3	Motokura
	13	16-Aug	Yakumo	2,700	60	1–2	Natsuougi-4go
	14	16-Aug		3,500	80	3–4	Natsuougi-4go
	15	6-Sep	Naganuma	2,700	100	1–2	White tiger
	16	6-Sep	Nanporo	36,000	15	1–2	Syuga
	17	6-Sep		8,000	60	1–2	Natsuougi-4go
	18	6-Sep		27,000	90	1–2	White tiger
	19	11-Sep	Date	3,500	100	2–4	White sword
	20	11-Sep		10,000	100	3–5	White sword

[a]Plants were grown in a greenhouse. In the other 19 fields, plants were grown in open fields

[b]Number of dead leaves per plant

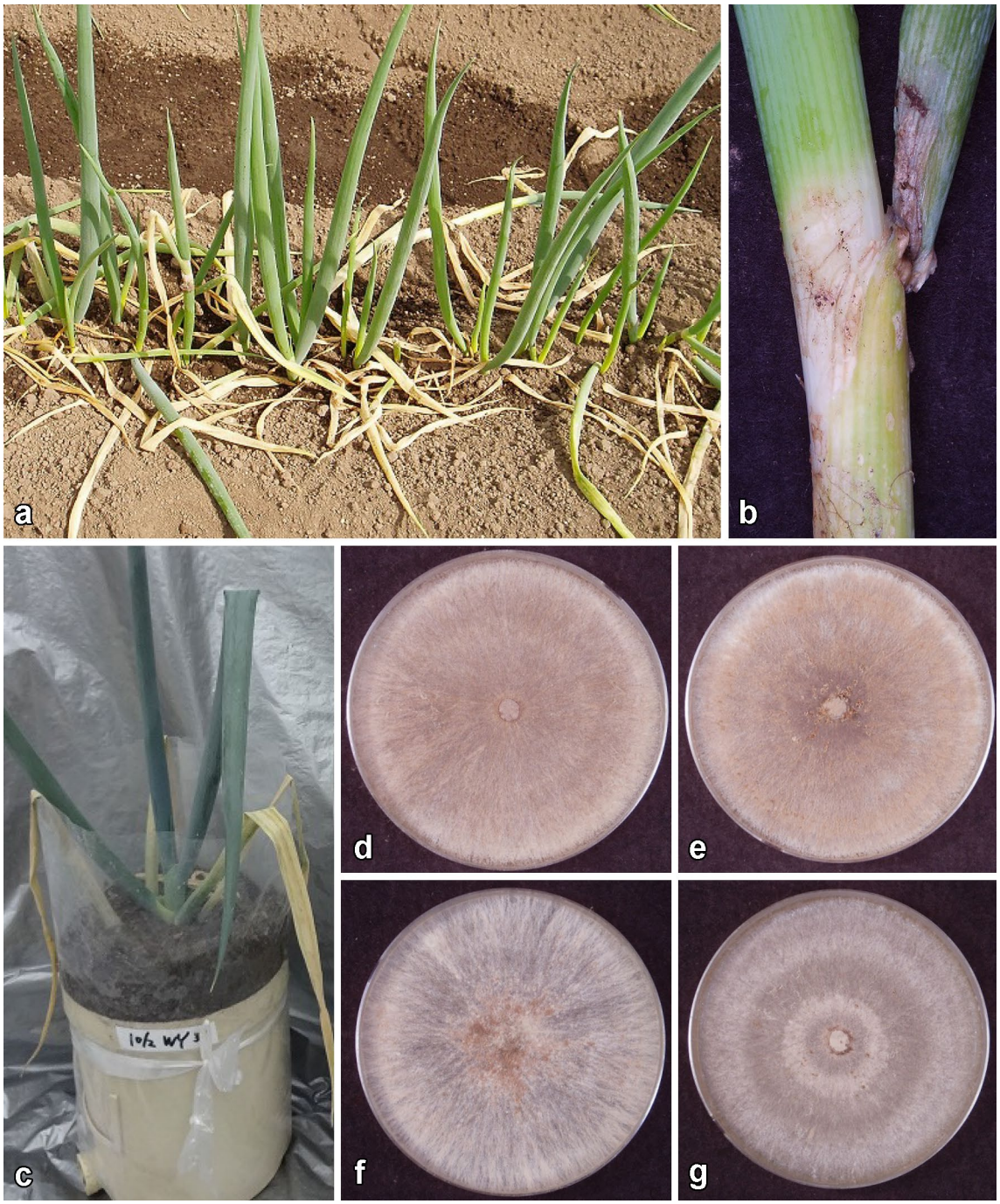

Fig. 1 Leaf sheath rot and outer leaf death of Welsh onion caused by *Rhizoctonia* spp. **a** Outer leaf death on Welsh onion plants infected with *Rhizoctonia solani* AG-4 HG-I in Hokuto, Hokkaido in 2007. **b** Rot symptoms of leaf sheath and basal part of outer leaf near the soil line on diseased plant. **c** Outer leaf death of Welsh onion plant 7 days after inoculation with *R. solani* AG-4 hybrid subgroup between HG-I and HG-II isolate WY31. **d–g** Cultural morphology of four isolates after 22 days on PDA at 25 °C. **d** Isolate WH31 (AG-4 hybrid subgroup between HG-I and HG-II). **e** Isolate WH41 (AG-4 hybrid subgroup between HG-I and HG-II). **f** Isolate WLS11 (AG-4 HG-I). **g** Isolate WNn11 (AG-4 HG-II)

and HG-III (Kuninaga 2003) and AG-5 (Arakawa and Inagaki 2014) (Table 2). These primers were designed for the internal transcribed spacer region on ribosomal DNA (rDNA-ITS). Their sequences, annealing temperatures, and product size are shown in Table 2. Fungal DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA) from 10-day-old mycelia grown at 25 °C on PDA plates. PCR amplification was

Table 2 AG- or subgroup-specific primers for detection of *Rhizoctonia solani* used in this study

AG, subgroup	Primer	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	References
AG-1 IA	AG-1 IA-F	CCTTAATTTGGCAGGAGGG	58	540	Kuninaga (2003)
	AG-1 IA-R	GACTATTAGAAGCGGTTCA			
AG-1 IB	AG-1 IB-F	TGTAGCTGGCCTTTTAAC	58	580	Kuninaga (2003)
	AG-1 IB-R	GGACTATTAGAAGCGGTTCG			
AG-1 IC	AG-1 IC-F	GAGTTGTTGCTGGCCTCTGG	58	550	Kuninaga (2003)
	AG-1 IC-R	CCAAGTCAATGGACTATTG			
AG-2-1	P21	CAAAGGCAATRGGTTATTGGAC	60	480	Carling et al. (2002)
		CCTGATTTGAGATCAGATCATAAAG			
AG-2-2 IIIB	P22-IIIB	AGGCAGAGRCATGGATGGGAG	62	500	Carling et al. (2002)
		ACCTTGGCCAMCCTTTTTATC			
AG-2-2 IV	P22-IV	AGGCAGAGACATGGATGGGAA	62	500	Carling et al. (2002)
		CTTGCCACCCMTTTTTTAC			
AG-2-2 LP	P22-LP	AGGCAGAGAAACATGGATGGGC	62	400	Carling et al. (2002)
		CCTCCAATACCAAAGTGAAACCAAATC			
AG-4 HG-I	AG-4 HG-I-F	GGACCTACTCTCYTTGG	55	420	Kuninaga (2003)
	AG-4 HG-I-II-R	ACAGGGTGTCCTCAGCGA			
AG-4 HG-II	AG-4 HG-II-F	GGACCTTCTACTCCCCCT	55	420	Kuninaga (2003)
	AG-4 HG-I-II-R	ACAGGGTGTCCTCAGCGA			
AG-4 HG-III	AG-4 HG-III-F	GTTGTAGCTGGCATTTCC	58	560	Kuninaga (2003)
	AG-4 HG-III-R	CCACCCCTCCCAAACTCT			
AG-5	AG-5 F	GGTTGTAGCTGGCTCATGAA	55	350	Arakawa and Inagaki (2014)
	ThanaCera-R	TGATACTCAAACAGGCATGC			

performed in 25 µL mixture containing 0.2 µL template DNA, 200 µM each of primer pairs, and 12.5 µL HotStarTaq Master Mix Kit (Qiagen) in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Cycling conditions consisted of 95 °C for 15 min; followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at the temperature specific for each primer pair for 1 min, and extension at 72 °C for 1 min; then a final extension for 5 min at 72 °C. PCR products were separated on 2% agarose gels, stained with ethidium bromide, and viewed using a UV transilluminator.

The rDNA-ITS regions of the binucleate isolates were amplified using primers ITS4 and ITS5 (White et al. 1990) since specific primers were only available for AGs A–F and K (Arakawa and Inagaki 2014). DNA was extracted as described above. PCR reactions consisted of 95 °C for 15 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; then final extension for 10 min at 72 °C. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and rDNA-ITS region of the isolates were directly sequenced using the same primers for amplification. DNA was sequenced by Hokkaido System Science Co. Ltd (Sapporo, Japan).

A part of the rDNA-ITS region of *R. solani* AG-4 hybrid subgroup between HG-I and HG-II (AG-4 HG-I + II h.s.) isolate WLY21 was amplified using the subgroup-specific primer pairs for AG-4 HG-I or HG-II (described above) to compare its sequence with the sequences of *R. solani* AG-4 isolates deposited in the DDBJ/EMBL/GenBank databases. The PCR products were directly sequenced using the same primers for amplification.

Mycelial growth temperature relations and cultural morphology

Radial mycelial growth rate of the isolates on PDA was determined at different temperatures, i.e., 5, 10, 15, 20, 25, 30, 35 and 40 °C. The radius of each colony was measured at 24 h intervals until 72 h after incubation. Each treatment was made with three replicates. For description of culture morphology, a 6-mm-diameter agar disk from the edge of a PDA colony was placed in the center of a PDA plate and incubated at 25 °C for 22 days in the dark. The morphology of each culture was then evaluated in terms of color, zonation and formation of sclerotia.

Genetic similarity among field isolates of binucleate *Rhizoctonia*

To clarify genetic relationship among the field isolates of binucleate *Rhizoctonia* belonging to the same AG, we tested hyphal fusion between each isolate and the other isolates as described above.

Pathogenicity

Six-month-old Welsh onion plants (cv. Motokura) grown in 1/5000-a (15.9 cm diameter × 19.3 cm depth) plastic pots filled with commercial potting soil (Pottace, Katakura Chikkarin Co., Tokyo, Japan), which had been autoclaved at 121 °C for 60 min, were used for pathogenicity tests. Pots each with single plants were girdled around the mouth with a plastic film (30 × 60 cm), secured with thick plastic strings, and received soil-inoculum mixture up to 10 cm thick on the soil surface (Fig. 1c). The soil-inoculum mixture was prepared as follows: 6-mm-diameter mycelia disks were placed on sterilized wheat bran and incubated at 25 °C for 2 weeks; and 5 g of wheat bran culture were mixed with 1.5 L of sterilized soil and used as the soil-inoculum mixture. Inoculated plants were grown in a greenhouse with average temperatures of 19.4–25.0 °C. Sterilized wheat bran served as the control. Virulence on Welsh onion was determined 7 days after inoculation. Each inoculation test consisted of two replicate pots.

Results

Disease occurrence

Leaf sheath rot and outer leaf death of Welsh onion (Fig. 1a) were found on 10 cultivars in 20 fields varying in acreage from 40 to 36,000 m^2 including one greenhouse in five cities i.e., Hokuto, Yakumo, Naganuma, Nanporo and Date, from early August to mid September, except Field 3 (Table 1). Plants cultivated in Field 3 (greenhouse) showed symptoms in December (Table 1). The disease occurred from 2 to 3 months after transplanting. Leaf sheaths of diseased plants turned brown and became soft 0–10 cm below the soil line. Outer leaves submerged in the soil were also diseased (Fig. 1b). Outer leaves ultimately died when they were girdled by lesions. Inner leaves, which were not in contact with soil, remained healthy (Fig. 1a). The incidence of plants with symptoms ranged between 5 and 100% (Table 1). All plants were diseased to various extents in six fields, i.e., Fields 6, 9, 10, 15, 19 and 20. Nine other fields, i.e., Fields 1, 2, 5, 11, 12, 13, 14, 17 and 18, were severely damaged with 50–90% diseased plants. One to five leaves were diseased per plant (Table 1).

Identification of isolates

Rhizoctonia-like fungi were frequently recovered from diseased tissues in all 20 fields (Table 3). All isolates collected from the same fields were similar to each other in culture morphology; therefore, one isolate each representing 20 fields was examined in detail. Hyphae lacked clamp connections, and young hyphae branched near the distal septum and were constricted near the base of branching. These features indicated that the isolates belonged to the genus *Rhizoctonia* (Ogoshi 1987). Sixteen isolates were multinucleate (3–18 nuclei), and hyphal diameter ranged from 5.2 to 11.3 µm (mean 7.3–8.6 µm) (Table 3). They were identified as *R. solani*, according to the descriptions of Ogoshi (1987). Four other isolates (isolates WLS21, WH71, WY11 and WNg11) had two nuclei per cell, and hyphal diameter ranged from 3.7 to 6.8 µm (mean 4.7–5.5 µm) (Table 3). These four isolates were identified as binucleate *Rhizoctonia* according to the descriptions of Ogoshi (1987).

Thirteen isolates of *R. solani*, i.e., WLS1, WLS11, WLY21, WH11, WH31, WH41, WH51, WY31, WNn11, WNn21, WNn31, WD11 and WD21, anastomosed with the tester isolate of AG-1 or AG-4 or AG-5 each with a high fusion frequency of more than 50% with their respective testers (Table 3). One isolate (isolate WLS91) and two isolates (isolates WLS81 and WH21) showed the C2 reaction, e.g., cell wall fused, with the AG-2-1 and AG-2-2 tester isolates, respectively, with high fusion frequencies of more than 50%, and the C1 reaction with tester isolates AG-2-2 and AG-2-1, respectively, with frequencies less than 30%, respectively (Table 3). All isolates failed to anastomose with other tester isolates.

Based on the results of the hyphal fusion tests (Table 3) and PCR with the specific primers (Fig. 2), three isolates (isolates WH51, WNn21 and WD11), one isolate (isolate WNn31), one isolate (isolate WLS91), two isolates (isolates WLS81 and WH21), two isolates (isolates WLS1 and WLS11), one isolate (isolate WNn11) and one isolate (isolate WD21) were identified as *R. solani* AG-1 IB, AG-1 IC, AG-2-1, AG-2-2 IIIB, AG-4 HG-I, AG-4 HG-II and AG-5, respectively (Table 3). DNA of five isolates (isolates WLY21, WH11, WH31, WH41 and WY31) that anastomosed with the AG-4 tester isolate, reacted with the primer sets both for AG-4 HG-I and HG-II, producing PCR products of the expected size (Fig. 2). Here, we refer to them as *R. solani* AG-4 HG-I + II h.s. Sequence of isolate WLY21 analyzed by AG-4 HG-I specific primers (deposited in GenBank as LC090072) was closely related to sequences from AG-4 HG-I isolates with identities of 99.7–100% (AB000018, KF907732–907733, KC405624–405628, FJ480862–480865, DQ102446, AY154307). Isolate WLY21 was then analyzed using AG-4 HG-II specific primers (deposited in GenBank as LC090073) to obtain

Table 3 Origin, morphology and identification results based on anastomosis reaction, PCR with AG- or subgroup-specific primers and rDNA-ITS sequences of each isolate of *Rhizoctonia* spp. from Welsh onion

Year	City	Field	Isolate	MAFF[a]	Nucleus[b]	Hyphae (μm)[c]	AG[d]	Subgroup
2007	Hokuto	1	WLS1	241950	3–8 (5.7)	6.6–9.5 (7.7)	AG-4	HG-I[e]
2008	Hokuto	2	WLS81	242301	3–10 (5.8)	6.4–9.7 (8.2)	AG-2-2	IIIB[e]
2009	Yakumo	3	WLS91	242302	4–12 (7.1)	6.5–10.2 (8.3)	AG-2-1	AG-2-1[e]
2011	Hokuto	4	WLS11	244465	3–8 (4.9)	7.0–10.9 (8.6)	AG-4	HG-I[e]
2012	Hokuto	5	WLS21	244466	2	3.7–6.0 (4.7)	AG-U	AG-U[f]
		6	WLY21	244467	3–8 (4.2)	5.8–9.4 (7.3)	AG-4	HG-I + II[e]
2013	Hokuto	7	WH11	244468	3–7 (5.2)	5.2–11.3 (7.8)	AG-4	HG-I + II[e]
		8	WH21	244469	5–18 (9.1)	6.1–9.6 (8.1)	AG-2-2	IIIB[e]
		9	WH31	244470	3–9 (5.7)	5.8–9.8 (7.6)	AG-4	HG-I + II[e]
		10	WH41	244471	4–7 (4.7)	5.4–10.1 (7.6)	AG-4	HG-I + II[e]
		11	WH51	244472	5–15 (9.1)	5.6–9.7 (8.0)	AG-1	IB[e]
		12	WH71	244473	2	4.2–6.7 (5.5)	AG-U	AG-U[f]
	Yakumo	13	WY11	244474	2	4.2–6.8 (5.0)	AG-U	AG-U[f]
		14	WY31	244475	3–7 (4.2)	5.8–9.6 (7.4)	AG-4	HG-I + II[e]
	Naganuma	15	WNg11	244476	2	4.3–6.8 (5.4)	AG-U	AG-U[f]
	Nanporo	16	WNn11	244477	3–8 (5.2)	5.8–9.8 (7.4)	AG-4	HG-II[e]
		17	WNn21	244478	4–12 (6.5)	5.3–8.7 (7.4)	AG-1	IB[e]
		18	WNn31	244479	3–15 (8.2)	6.7–9.6 (7.9)	AG-1	IC[e]
	Date	19	WD11	244480	3–13 (7.0)	6.3–10.8 (8.1)	AG-1	IB[e]
		20	WD21	244481	3–11 (6.5)	6.3–8.5 (7.7)	AG-5	AG-5[e]

[a]MAFF accession numbers in NARO Genebank for all 20 isolates used in this study

[b]Number of nuclei per cell; minimum–maximum (mean)

[c]Width of hyphae; minimum–maximum (mean)

[d]Anastomosis group was determined by hyphal fusion reactions with tester isolates of *Rhizoctonia solani* AGs 1–5 and binucleate *Rhizoctonia* AGs A–U, except for AG-J, M, T and N

[e]Identification based on the PCR with AG- or subgroup-specific primers

[f]Identification based on rDNA-ITS sequence. Sequences of isolates WLS21, WH71, WY11 and WNg11 were deposited in the DDBJ/EMBL/GenBank databases with accession numbers LC090069, LC090068, LC090070 and LC090071, respectively

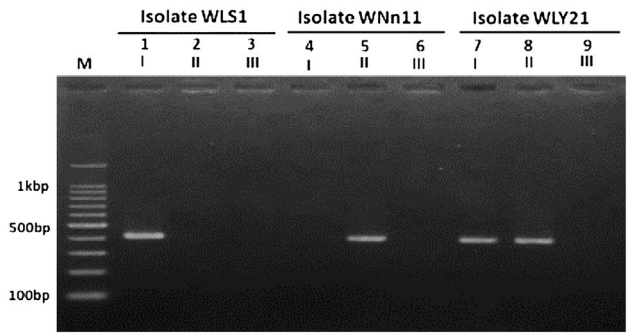

Fig. 2 Agarose gel electrophoresis of PCR products amplified from DNA of *Rhizoctonia solani* isolates from Welsh onion. Amplifications were done using primer pairs designed for specific detection of *R. solani* AG-4 HG-I (*lanes 1, 4* and *7*), HG-II (*lanes 2, 5* and *8*) and HG-III (*lanes 3, 6* and *9*). Lane M 100-bp ladder. Genomic DNA from isolate WLS1 (*lanes 1–3*), isolate WNn11 (*lanes 4–6*) and isolate WLY21 (*lanes 7–9*)

sequence. The sequence was identical with those of AG-4 HG-II isolates (AB000008, JX843818, FM867593–867594, AF354373–354074, HQ629858–629861). These results indicate that isolate WLY21 has the rDNA-ITS regions of both AG-4 HG-I and AG-4 HG-II.

All four binucleate *Rhizoctonia* isolates (isolates WLS21, WH71, WY11 and WNg11) had the C2 reaction with the AG-U tester, with a high fusion frequency and also with the AG-P tester, but with a low fusion frequency. They failed to react to other tester isolates. Sequences of the rDNA-ITS regions of the four binucleate isolates (deposited in GenBank as LC090068–090071) were closely related to sequences from AG-U isolates with identities of 99.9–100% (HQ269825) or 99.3–99.8% (HQ269809–269811, HQ269816, HQ269820, AB196664–196666, AB731590). These results agreed with the results from hyphal fusion tests, and these four isolates were identified as binucleate *Rhizoctonia* AG-U (Table 3). Twenty isolates obtained in this study were deposited in Genebank, National Agriculture and Food Research Organization (NARO) with the accession numbers as shown in Table 3.

Mycelial growth temperature relations and cultural morphology

Most isolates, including binucleate *Rhizoctonia* did not grow at 5 or 40 °C on PDA (Table 4). Growth was optimal at 25–30 °C in most isolates, and isolates of *R. solani* AGs 1 and 4 tended to grow faster (Table 4). The results agreed with Watanabe and Matsuda (1966), Ogoshi (1976), and Hyakumachi and Sumino (1984) for *R. solani* and with Priyatmojo et al. (2001) and Hyakumachi et al. (2005) for binucleate *Rhizoctonia*.

Morphology of 22-day-old PDA cultures of AG-4 HG-I + II h.s. isolates were compared with the related subgroups; all eight AG-4 isolates had slight aerial mycelia, and all five AG-4 HG-I + II h.s. isolates showed radial mycelia growth as was the case with AG-4 HG-I isolate WLS11 (Fig. 1d–f). While cultures of all five AG-4 HG-I + II h.s. isolates and AG-4 HG-I isolate WLS1 were creamy to pale brown and mealy (Fig. 1d–e), those of isolates WLS11 (AG-4 HG-I) and WNn11 (AG-4 HG-II) were creamy and mealy (Fig. 1f–g). Isolate WNn11 was characterized by a concentric mycelia growth pattern (Fig. 1g). A few medium-brown sclerotia were produced, which ranged from 0.2 to 0.5 mm across for isolates WH11 and WNn11 (Fig. 1g), 0.5–2 mm across for isolate WH41 (Fig. 1e) and 1–1.5 mm across (isolate WY31). Thus, mycelial growth temperature relations and cultural morphology of AG-4 HG-I + II h.s. isolates were similar to those of AG-4 isolates reported previously (Ogoshi 1976; Stevens Johnk and Jones 2001). Cultural morphology of isolates belonging to other AGs agreed with that in previous papers (Hyakumachi and Sumino 1984; Hyakumachi et al. 2005; Ogoshi 1976; Priyatmojo et al. 2001; Watanabe and Matsuda 1966) (data not shown).

Genetic similarity among field isolates of binucleate *Rhizoctonia*

The four binucleate *Rhizoctonia* AG-U isolates from Welsh onion exhibited the C2 reaction with each other, indicating that they were not clonal.

Table 4 Mycelial growth of isolates of 20 *Rhizoctonia* spp. from Welsh onion on potato dextrose agar at different temperatures for 24 h (mm)

	Rhizoctonia solani									
AG	AG-1				AG-2-1	AG-2-2		AG-4		
Subgroup	IB			IC		IIIB		HG-I		HG-II
Isolate (°C)	WH51	WNn21	WD11	WNn31	WLS91	WLS81	WH21	WLS1	WLS11	WNn11
5	0.2	0	0.2	0.3	2	0	0	0	0	0
10	5.2	5.4	7	4.1	3.5	1.6	3.6	1.4	0.2	4.4
15	9.8	8.7	11.6	11.8	8.4	5.3	6.8	8.4	6.2	9.3
20	20.4	14.5	22.4	22.4	10.8	9.4	13.9	18.8	13.4	14.5
25	20.4	17	27.7	31.3	14.2	15	18.2	23.4	20.7	17.4
30	17.1	16.6	20.9	26	6.9	19.8	20.1	21.2	17.3	15.5
35	1.3	1.1	2.6	3.8	0	8.1	6.9	5.3	1.1	0
40	0	0	0	0	0	0	0	0	0	0

	Rhizoctonia solani						Binucleate *Rhizoctonia*			
AG	AG-4					AG-5	AG-U			
Subgroup	Hybrid subgroup between HG-I and HG-II									
Isolate (°C)	WLY21	WH11	WH31	WH41	WY31	WD21	WLS21	WH71	WY11	WNg11
5	0	0	0	0	0	0.1	0	0	0	0
10	1	3.9	4.8	4.8	4	3.6	0.7	4	3.7	3.9
15	7	7.7	8.4	9	8.5	6.3	8	8.6	6.9	8.3
20	13.4	15.3	17	16.7	14.8	12.9	13	16.5	14.5	14.2
25	18.1	18.4	21.3	19.9	19.6	18.9	18.5	18.2	18.6	18
30	19.9	4.9	16	14.3	18	16.2	13.7	16.3	16.5	16.8
35	0.2	0.7	5.9	0.5	0.9	0.4	0.2	2.3	0.3	1.5
40	0	0	0	0	0	0	0	0	0	0

Each value is the mean of three replicates

Pathogenicity

All 20 isolates consistently incited leaf sheath rot and outer leaf death on Welsh onion plants (Fig. 1c). The symptoms were similar to those of naturally diseased plants, and leaves attached to the infested soil died. The isolates were reisolated from inoculated, diseased plants. There was no significant difference in virulence among the 20 isolates. Control plants remained healthy, and the pathogen was not isolated.

Discussion

This study demonstrated that as many as nine groups of *Rhizoctonia* spp. (*R. solani* AG-1 IB, AG-1 IC, AG-2-1, AG-2-2 IIIB, AG-4 HG-I, AG-4 HG-II, AG-4 HG-I + II h.s., and AG-5, in addition to binucleate *Rhizoctonia* AG-U) were associated with the leaf sheath disease of mature Welsh onion plants. Young plants are more susceptible to the pathogen (Baker 1970), and several AGs and subgroups of *Rhizoctonia* spp. have been reported in Japan as damping-off pathogens of single crops, e.g., seven groups from sugarbeet seedlings, i.e., *R. solani* AG-1, AG-2-1, AG-2-2, AG-3, AG-4, AG-5 and unidentified binucleate *Rhizoctonia* (Naito et al. 1975) and four groups from broccoli seedlings, i.e., *R. solani* AG-1 IC, AG-2-1, AG-2-2 IIIB and AG-4 HG-I (Kubota and Abiko 1997; Kubota et al. 2009; Misawa et al. 2013; Yamauchi et al. 2009). In the case of mature plants, only root rot of Japanese radish was reported as the disease being caused by *R. solani* (five groups: AG-1, AG-2-1, AG-2-2, AG-4 and AG-5; Homma and Ishii 1984). The diversity of the Welsh onion leaf sheath rot pathogens suggests that mature Welsh onion plants are, at least in some plant parts, as susceptible to *Rhizoctonia* spp. as seedlings of other crops.

Since the rDNA-ITS region of *R. solani* AG-4 HG-I + II h.s. isolates could not be analyzed by direct sequencing due to their genetic heterogeneity, alternative methods were cloning and PCR amplification using the subgroup-specific primers. Using the latter method, we successfully distinguished AG-4 HG-I + II h.s. from the known three subgroups (HG-I, HG-II and HG-III). To our knowledge, there is no report on genetic heterogeneity among *R. solani* AG-4 subgroups. AG-4 HG-I + II h.s. is likely to incite diseases on other crops in the world. Since this fungus was almost indistinguishable from isolates of AG-4 HG-I and AG-4 HG-II on the basis of cultural morphology, the possibility of their coexistence in single fields cannot be ruled out. Further research on AG-4 HG-I + II h.s. is needed to clarify the geographic distribution and host range of this group. PCR using AG-4 HG-I + II specific primers would facilitate identification of this group.

Field isolates are usually heterokaryons in AG-4 (Anderson et al. 1972), and the new subgroup AG-4 HG-I + II h.s. is one of the examples. Heterokaryosis and genetic heterogeneity normally occur through sexual recombination of genotypes; basidiospores of *Thanatephorus cucumeris*, the teleomorph of *R. solani*, germinate to develop primary mycelia, and hyphal fusion between primary mycelia results in heterogenic, secondary mycelia. Cubeta et al. (1993) paired many homokaryotic single-basidiospore isolates from different field AG-4 isolates to confirm heterokaryosis at the colony junction. We assume that intersubgroup mating of primary mycelia between AG-4 HGs-I and -II to establish HG-I + II h.s.

Alternatively, Kuninaga et al. (2004) examined heterokaryosis in isolates of the *R. solani* AG-1 hybrid subgroup between IA and IE (AG-1 IA + IE h.s.), inciting common bean web blight. They obtained homokaryotic isolates of the pathogen using a protoplast method developed by Phillips (1993) to prepare homokaryons from heterokaryotic field isolates without basidiospore formation and identified genotypes; protoplast progenies were identified as AG-1 IA or AG-1 IE. These subgroups were then paired to generate AG-1 IA + IE h.s. Their results represent the basis of our assumption, although we have neither observed basidiospores of *T. cucumeris* nor found hybrid subgroups in AG-1 in Welsh onion fields.

In this study, we obtained four binucleate *Rhizoctonia* AG-U isolates from three main Welsh onion production cities (Hokuto, Yakumo and Naganuma) in 2012–2013. Hyphal fusion tests using these isolates demonstrated that they were not clonal, implying that AG-U was originally present in the soil of various regions of Hokkaido. AG-U was first isolated from roses with root and stem rot in Gifu, Japan, in 1999–2001 (Hyakumachi et al. 2005; Priyatmojo et al. 2001), and later recognized on other crops (Misawa and Toda 2013; Rinehart et al. 2007). Therefore, AG-U should be monitored on other hosts, and critical assessment of disease damage is necessary.

This study, along with our subsequent routine survey from 2006 to 2014 revealed the epidemiology of the disease except greenhouse cultivation (Field 3) as follows: (1) the disease occurs from early July (in 2006; data not shown) to mid September in Hokkaido with average daily temperatures, ranging from 19 to 23 °C; (2) plants become diseased 5 to 10 days after hilling, but disease does not develop further; (3) outer leaves in contact with soil are killed entirely, whereas inner leaves with no soil contact, remain healthy. (4) Because new inner leaves develop every week, healthy leaves gradually increase in number until next hilling; and (5) if the next hilling is done when the daily average temperature is more than 19 °C, the disease occurs again.

The maximum temperature for pathogenesis could not be clarified from field surveys. The maximum average

daily temperature of 23 °C occurs in Hokkaido in mid August. Since optimal growth temperatures of the pathogens other than AG-2-1 were at 25–30 °C, it may be inferred that the disease occurs between 25 and 30 °C.

Although the damage varied among fields in terms of diseased plant ratio (Table 1), inoculation experiments failed to reveal any significant difference in virulence among the isolates. Since hilling is an important factor to promote disease occurrence, differences in field disease severity seems to correlate with cultural practices such as the height and period of hilling.

The only *Rhizoctonia* disease so far known to occur on Welsh onion in Japan has been damping off of seedlings up to 2 months old caused by *Rhizoctonia* spp. (Yamamoto and Uehara 1972). The pathogens, however, were not identified to the AG. The leaf sheath rot we report here occurs later than damping off, between 2 and 3 months after transplanting (4 to 5 months after sowing), and the symptoms are different. These features distinguish leaf sheath rot from damping off and represent a basis for recognizing leaf sheath rot as a new disease. We refer to this new disease as "leaf sheath rot of Welsh onion (Rhizoctonia youshou-fuhai-byo in Japanese)".

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