Evolution of an *Eleusine*-Specific Subgroup of *Pyricularia oryzae* Through a Gain of an Avirulence Gene

Soichiro Asuke,¹ Masaki Tanaka,¹ Gang-Su Hyon,¹ Yoshihiro Inoue,² Trinh Thi Phuong Vy,¹ Daisuke Niwamoto,¹ Hitoshi Nakayashiki,¹ and Yukio Tosa^{1,†}

¹ Graduate School of Agricultural Science, Kobe University, Nada, Kobe 657-8501, Japan

² Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Accepted 10 September 2019.

Eleusine isolates (members of the *Eleusine* pathotype) of *Pyricularia oryzae* are divided into two subgroups, EC-I and EC-II, differentiated by molecular markers. A multilocus phylogenetic analysis revealed that these subgroups are very close to *Eragrostis* isolates. EC-II and *Eragrostis* isolates were exclusively virulent on finger millet and weeping lovegrass, respectively, while EC-I isolates were virulent on both. The avirulence of EC-II on weeping lovegrass was conditioned by an avirulence gene, *PWL1*. All EC-II isolates shared a peculiar structure (P structure) that was considered to be produced by an insertion (or translocation) of a DNA fragment carrying *PWL1*. On the other hand, all EC-I and *Eragrostis* isolates were noncarriers of *PWL1* and shared a gene structure that should have predated the insertion of the *PWL1*-containing fragment. These results, together with phylogenetic analyses using whole-genome sequences, suggest that the *Eleusine*-specific subgroup (EC-II) evolved through a loss of pathogenicity on weeping lovegrass caused by a gain of *PWL1*.

Keywords: blast, *Magnaporthe oryzae*, pathotype, *Pyricularia oryzae*

Pyricularia oryzae (Zhang et al. 2016) (syn. *Magnaporthe oryzae*) is a causal agent of blast disease of staple crops, e.g., rice, wheat, and finger millet (Kato et al. 2000). Roughly speaking, isolates from each crop are pathogenic on plants in the same genus as the original host crop. For example, isolates from finger millet (*Eleusine coracana*) are pathogenic on other plant species within the genus *Eleusine*, such as *Eleusine indica* and *Eleusine africana* in addition to *Eleusine coracana*. Kato et al. (2000) designated those host genus-specific groups as pathotypes. Several pathotypes have been recognized in *P. oryzae*, e.g., the *Eleusine* pathotype pathogenic on finger millet, *Oryza* pathotype pathogenic on rice, *Setaria* pathotype

pathogenic on foxtail millet, *Triticum* pathotype pathogenic on wheat (Cruz and Valent 2017), and *Lolium* pathotype pathogenic on ryegrass (Tosa et al. 2004).

The question is how these pathotypes evolved from ancestral strains of *P. oryzae*. The recent emergence of the *Triticum* pathotype (wheat blast fungus) in 1985 (Urashima et al. 1993) made it possible to analyze mechanisms of pathotype evolution, because *Triticum* isolates (isolates from *Triticum* spp.) collected just after its emergence have been preserved in laboratories and are available together with records of wheat cultivars that have been cultivated in 1980s. Inoue et al. (2017) suggested that the *Triticum* isolates evolved from a *Lolium* isolate or its relatives through a host jump that was mediated by a wide cultivation of wheat cultivars lacking the resistance gene *Rwt3*, followed by functional losses of its corresponding avirulence gene *PWT3*.

Eleusine isolates (isolates from *Eleusine* spp.) are highly fertile with *P. oryzae* isolates from various hosts. This characteristic contributed to the finding of its teleomorph (Barr 1977; Kato et al. 1976; Ueyama and Tsuda 1975; Yaegashi and Nishihara 1976), which led to the establishment of a system for genetic analyses of the blast fungus (Taga et al. 1978). Yaegashi (1978) crossed *Eleusine* isolates with *Eragrostis* isolates (isolates from weeping lovegrass, *Eragrostis curvula*), inoculated finger millet and weeping lovegrass with their F₁ progeny, and suggested that the specific pathogenicity on each plant was conditioned by a single gene. Valent et al. (1986) crossed another *Eleusine* isolate with an *Eragrostis* isolate, inoculated goosegrass (*Eleusine indica*) and weeping lovegrass with their progeny, and recognized, again, that a single gene difference between the parental isolates accounts for the pathogenicity difference on each host. The gene conditioning the pathogenicity toward weeping lovegrass was named *PWL1* (Valent and Chumley 1994). In a series of crosses involving *Oryza* isolates, they identified *PWL2*, another gene conditioning the pathogenicity toward weeping lovegrass (Valent and Chumley 1994). Subsequently, Sweigard et al. (1995) isolated the *PWL2* gene through positional cloning. Kang et al. (1995) isolated the *PWL1* gene by using its homology to *PWL2*. These successes of molecular cloning revealed that *PWL1* and *PWL2* condition avirulence of *Eleusine* isolates and *Oryza* isolates, respectively, toward weeping lovegrass (Kang et al. 1995; Sweigard et al. 1995).

In this article we focus on evolution of the *Eleusine* pathotype, which maintains ancestral characteristics (e.g., very high fertility). Dobinson et al. (1993) suggested that, on the basis of the presence or absence of a long terminal repeat (LTR) retrotransposon, *grasshopper*, *Eleusine* isolates could be divided into at least two genetically distinct subgroups. Tanaka et al. (2009) examined the population structure of *Eleusine* isolates

[†]Corresponding author: Y. Tosa; tosayuki@kobe-u.ac.jp

This is a resubmission of a paper published by Tanaka et al. in 2010 (Mol. Plant-Microbe Interact. 23:771-783) and retracted on June 3, 2015, reporting on further experiments performed to check whether arguments made were scientifically correct.

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary text, three supplementary figures, and one supplementary table are published online.

The author(s) declare no conflict of interest.

collected from various countries, using nucleotide sequences of ribosomal (r)DNA and DNA fingerprint profiles with transposable elements (MGR586, MGR583, and *grasshopper*). Again, *Eleusine* isolates were divided into two subgroups. One subgroup, named Ec-I, was composed of isolates from Japan and Uganda and did not contain *grasshopper*. The other, named Ec-II, was composed of isolates from Japan, China, Nepal, and India and contained *grasshopper*. In the present article, we will designate Ec-I and Ec-II as EC-I and EC-II (using an upper case C), respectively, to differentiate these subgroup names from isolate codes. Intriguingly, a preliminary infection assay showed that EC-II isolates were avirulent on weeping lovegrass while EC-I isolates were virulent, which led us to assume that *PWL1* may be a key for revealing the course of evolution of the two subgroups. In this article we report pathways of their differentiation inferred from rearrangements around *PWL1*.

RESULTS

Multilocus phylogenetic analysis.

To clarify phylogenetic positions of EC-I and EC-II in the *Pyricularia* population, six EC-I isolates and 34 EC-II isolates (Supplementary Table S1) were subjected to multilocus phylogenetic analysis. A portion was amplified from the rRNA gene (rDNA ITS1-5.8S-ITS2 region), the actin gene (*ACT*), the β -tubulin gene (*TUB*), and the calmodulin gene (*CAL*). The size of amplified fragments of rDNA, *ACT*, *TUB*, and *CAL* were 453, 294, 494, and 482 bp, respectively. Nucleotide sequences of these fragments were combined and were aligned with those of 73 isolates from various hosts (Supplementary Table S1) deposited into GenBank by Hirata et al. (2007). Hirata et al. (2007) found 21 (rd1-rd21), 15 (ac1-ac15), 16 (be1-be16), and 16 (cal-ca16) alleles at the rDNA, *ACT*, *TUB*, and *CAL* loci, respectively, and defined 29 genotypes based on the combination of these alleles at the four loci. The isolates used in the present study were classified into 17 genotypes among the 29 genotypes. Maximum parsimony (MP) trees of the 17 genotypes were constructed using INA-B-92-45 (*Bambusicularia brunnea*, genotype Ss) and HYZiM101-1-1-1 (*Proxipyrularia zingiberis*, genotype Zm1) (Klaubauf et al. 2014), which are morphologically distinguishable from *P. oryzae* and *P. grisea*, as outgroup taxa. The isolates were plotted on the MP tree according to their genotypes.

The 34 EC-II isolates shared an identical combination of alleles and belonged to the TELE1 genotype (Fig. 1). The combined DNA sequences of these EC-II isolates were 100% identical. The six EC-I isolates shared another, identical combination of alleles and belonged to the TELE2 genotype. Interestingly, *Eragrostis* isolates (two from weeping lovegrass, *Eragrostis curvula*, and one from lehmann lovegrass, *Eragrostis lehmanniana*) also belonged to the TELE2 genotype. The combined DNA sequences of these EC-I and *Eragrostis* isolates were 100% identical. The dendrogram suggested that the *Eleusine* isolates are very close to *Eragrostis* isolates. However, relative relationships among EC-I, EC-II, and *Eragrostis* isolates were ambiguous because TELE1 and TELE2 shared the same alleles at the *ACT*, *TUB*, and *CAL* loci and differed only at the rDNA locus (Hirata et al. 2007).

Pathogenicity of *Eleusine* and *Eragrostis* isolates to weeping lovegrass and finger millet.

To evaluate the relationship among the EC-I, EC-II, and *Eragrostis* isolates from the viewpoint of pathogenicity, these isolates were sprayed onto finger millet and weeping lovegrass.

The EC-II isolates and *Eragrostis* isolates showed specific pathogenicity to their original hosts; the EC-II isolates were highly virulent on finger millet and avirulent on weeping lovegrass, while the *Eragrostis* isolates were avirulent on finger millet and highly virulent on weeping lovegrass (Table 1; Fig. 2). On the other hand, the EC-I isolates were highly virulent, not only on finger millet (their original host), but also on weeping lovegrass (Table 1; Fig. 2).

Distribution and function of *PWL1* homologs in *Eleusine* isolates.

The contrasting pathogenicity of the two groups of *Eleusine* isolates to weeping lovegrass led us to a hypothesis that *PWL1*, an avirulence gene against weeping lovegrass derived from *Eleusine* isolates, might be a key gene for elucidating the course of evolution of the *Eleusine* pathotype. To test this hypothesis, we examined molecular structures at the *Pwl1* locus in the *Eleusine* isolates.

A *PWL1* homolog was amplified from Ec-3J (an EC-II isolate) with *PWL1*-2F and *PWL1*-1R (Table 2), primers designed from the *PWL1* sequence in the database (DDBJ, GenBank, and EMBL accession number U36923). The amplified fragment corresponding to 383 bp within the 444-bp open reading frame (ORF) of *PWL1* was inserted into BluescriptII SK(+) and was established as p*PWL1*-ORF. When genomic DNA was digested with *SaII* and was probed with p*PWL1*-ORF, a single band appeared in all EC-II isolates (Supplementary Fig. S1). Its size was almost uniform among isolates, with slight variations. Similar results were obtained with *ClaI* digests (examples can be found in Figure 3A). These results suggest that all EC-II isolates carry a single copy of *PWL1*. On the other hand, no signals were detected in the EC-I isolates (Fig. 3A; Supplementary Fig. S1), indicating that they lack *PWL1* homologs. The *Eragrostis* isolates also lacked *PWL1* homologs (Fig. 3A; Supplementary Fig. S1). Taken together, the presence or absence of *PWL1* homologs was perfectly concordant with the avirulence or virulence on weeping lovegrass (Table 1).

To reveal molecular structures of the *PWL1* homologs in the EC-II isolates, a 1.1-kb region containing the promoter and 444-bp ORF of *PWL1* was amplified from the EC-II isolates with primers *PWL1*-pro-1F and *PWL1*-1R (Table 2; Fig. 4) and was cloned into pBluescriptII SK(+). Sequence analysis of the clones revealed that all EC-II isolates had a homolog with a 100% identical sequence. When the ORF sequence (444 bp) of these clones (DDBJ, GenBank, and EMBL accession number AB480169) was compared with that reported by Kang et al. (1995) (DDBJ, GenBank, and EMBL accession number U36923), 5-bp substitutions were detected. These substitutions caused changes of three putative amino acids.

The 1.1-kb fragment did not contain the 3' untranslated region. To check whether the *PWL1* homolog was functional, a larger fragment (4.2 kb), including the 3' untranslated region, was amplified from Ec-3J (an EC-II isolate) with primers *Fba-PWL1*-2F and *PWL1*-3R (Table 2; Fig. 4), was cloned into pBluescriptII SK(+), and was established as p*PWL1*-EC. p*PWL1*-EC was introduced into the genome of Ec-130U (an EC-I isolate) by cotransformation with pSH75 containing the hygromycin B phosphotransferase gene (Kimura and Tsuge 1993), resulting in seven transformants carrying *PWL1* (Supplementary Fig. S2). An infection assay revealed that four of the seven transformants gained avirulence on weeping lovegrass without changes of the virulence on finger millet (Fig. 2; Supplementary Fig. S2). This result indicates that the *PWL1* homolog in our clone is functional and, therefore, actually involved in the avirulence of the EC-II isolates on weeping lovegrass.

Molecular structure of *PWL1*-flanking regions in *Eleusine* and *Eragrostis* isolates.

Flanking regions of *PWL1*-ORF were cloned from genomic DNA of Ec-3J (EC-II), using cassette polymerase chain reaction (PCR) (discussed below), and were sequenced (DDBJ,

GenBank, and EMBL accession number AB480169). The left flank contained a hypothetical protein (MGG_00005, accession number EHA49556.1) and a portion (152 bp) of another hypothetical protein (OOU_Y34scaffold00593g1, accession number ELQ37450.1) (Fig. 4B). This peculiar structure

Fig. 1. Maximum parsimony phylogenetic tree of genotypes of *Pyricularia* isolates constructed from combined ribosomal DNA, actin, β -tubulin, and calmodulin sequences. Genotypes were defined by Hirata et al. (2007). The tree was rooted with INA-B-92-45 (*Bambusicularia brunnea*, genotype Ss) and HYZIM101-1-1-1 (*Proxipyrularia zingiberis*, genotype Zm1). Numbers at nodes show bootstrap values from 1,000 replications. Presence (+) or absence (-) of *PWL1* and the P structure was determined by Southern hybridization with the pPWL1-ORF probe and polymerase chain reaction with primers 239-3F and PWL1-1R, respectively.

(MGG_00005, the 152-bp portion of ELQ37450.1, and *PWLI*) (designated as P structure hereafter) was shared by all EC-II isolates (Supplementary Fig. S3). The 152-bp portion was flanked by a solo LTR of a LTR-retrotransposon, Inago1 (accession number AB334124). The right flank of *PWLI* also contained a solo LTR of Inago 1 (Fig. 4B).

To reveal a structure of the corresponding region in EC-I isolates (noncarriers of *PWLI*), the right flank of MGG_00005 was amplified from Ec-130U by cassette PCR with MGG_00005-specific primers. Sequence analysis revealed that Ec-130U carried a complete ORF (1,281 bp) of ELQ37450.1 in the right flank of MGG_00005 (Fig. 4A). This structure (designated as U structure hereafter) was also present in an *Eragrostis* isolate (ERc-2J). A comparison between the U and P structures led us to assume that their difference was attributable

Table 1. Pathogenicity of isolates from *Eleusine* spp. and *Eragrostis* spp. to finger millet and weeping lovegrass

Isolates	Infection type ^a			
	Group	Millet	Lovegrass	<i>PWLI</i> ^b
<i>Eleusine</i> isolates				
Ec-1J	EC-II	5	0	+
Ec-2J	EC-II	5	0	+
Ec-3J	EC-II	5	0	+
Ec-4J	EC-II	5	0	+
Ec-7I	EC-II	5	0	+
Ec-8I	EC-II	5	0	+
Ec-9N	EC-II	5	0	+
Ec-10N	EC-II	5	0	+
Ea-11J	EC-II	5	0	+
Eb-12J	EC-II	5	0	+
Ei-13I	EC-II	5	0	+
Ei-101J	EC-II	5	0	+
Ei-102J	EC-II	5	0	+
Ec-103J	EC-II	5	0	+
Ec-104J	EC-II	5	0	+
Ec-107C	EC-II	5	0	+
Ec-108C	EC-II	5	0	+
Ebb-110I	EC-II	5	0	+
Ebb-111I	EC-II	5	0	+
Ebb-112I	EC-II	5	0	+
Ec-113I	EC-II	5	0	+
Ec-114I	EC-II	5	0	+
Ec-115I	EC-II	5	0	+
Ec-116I	EC-II	5	0	+
Ec-118I	EC-II	5	0	+
Ec-119I	EC-II	5	0	+
Ec-120I	EC-II	5	0	+
Ec-121I	EC-II	5	0	+
Ec-122I	EC-II	5	0	+
Ec-123I	EC-II	5	0	+
Ei-124I	EC-II	5	0	+
Ec-125I	EC-II	5	0	+
Ec-126N	EC-II	5	0	+
Ec-127N	EC-II	5	0	+
Ec-5J	EC-I	5	4 to 5	—
Ec-6J	EC-I	5	4 to 5	—
Ec-105J	EC-I	5	4 to 5	—
Ei-128U	EC-I	5	4 to 5	—
Ec-129U	EC-I	5	4 to 5	—
Ec-130U	EC-I	5	4 to 5	—
<i>Eragrostis</i> isolates				
ER1-1J	—	0	5	—
ERc-2J	—	0	5	—
ERc-3J	—	0	5	—

^a Millet = finger millet (Iiya-zairai) and Lovegrass = weeping lovegrass (Yukijirushi 41-102). 0 = no visible evidence of infection; 1 = pinpoint spots, 2 = small lesions (<1.5 mm), 3 = lesions with an intermediate size (<3 mm), 4 = large, typical lesions, and 5 = complete blighting of leaf blades.

^b Presence (+) or absence (—) of *PWLI* homologs evaluated by Southern hybridization with pPWLI-ORF.

not to a deletion but to an insertion or translocation of a fragment containing *PWLI*. When genomic DNA of other EC-I isolates, other *Eragrostis* isolates, and EC-II isolates were subjected to PCR with the 239-1F primer on MGG_00005 and the 238-7F primer on ELQ37450.1 (Fig. 4), a fragment with the expected size (1,567 bp) was amplified from all these isolates (Fig. 3D). This result suggests that the U structure (Fig. 4A) is ubiquitous in *Eleusine* and *Eragrostis* isolates.

To reveal the mode of rearrangements in detail, two probes were produced. One probe, p239, contained a fragment of MGG_00005 amplified from Ec-3J with primers, 239-1F, and 239-1R (Fig. 4B). The other probe, p238, contained a fragment of ELQ37450.1 amplified from Ec-130U with primers 238-2R and 238-2F (Fig. 4A). When genomic DNA of Ec-3J (an EC-II isolate) was digested with *Clal* and probed with p239, four bands were recognized (Fig. 3B, lane 11). When the blot was reprobated, the first, third, and fourth bands hybridized with p238 (Fig. 3C), while the second band hybridized with pPWLI-ORF (Fig. 3A). Similar results were obtained with other EC-II isolates (Fig. 3, lanes 10 to 16). When genomic DNA of EC-I and *Eragrostis* isolates was probed with p239, three to six bands were produced (Fig. 3B, lanes 1 to 9). Reprobing of the blot showed that most of these bands hybridized with p238 (Fig. 3C, lanes 1 to 9). These results suggest that the U structure is commonly present in several copies in *Eleusine* and *Eragrostis* isolates and that, in EC-II isolates, a fragment carrying *PWLI* was inserted or translocated into one of these copies.

Distribution of *PWLI* homologs in members of the genus *Pyricularia*.

To reveal evolutionary processes of *PWLI* during speciation and differentiation in the genus *Pyricularia*, distribution of *PWLI* was examined using *Pyricularia* isolates from various hosts. Southern hybridization with pPWLI-ORF revealed that *PWLI* homologs were widely distributed in *Oryza*, *Panicum*, and *Lolium* isolates of *P. oryzae* (Figs. 1, 3A). *PWLI* homologs were also detected in cryptic species that are indistinguishable from *P. oryzae* in conidial morphology but distinguishable in multilocus phylogenetic analysis, e.g., *Pyricularia* sp. from *Leersia oryzoides* (LEo-1J), *P. pennisetigena* from *Cenchrus*

Fig. 2. Pathogenicity of EC-II (Ec-3J) and EC-I (Ec-130U) of *Eleusine* isolates, *Eragrostis* isolate (ERc-2J), and a transformant (UGt15) of Ec-130U carrying *PWLI* (Ec-130U+*PWLI*) toward finger millet (Iiya-zairai) and weeping lovegrass (JP No.3695), 4 days after inoculation.

ciliaris (Cc-1J), and *P. grisea* from *Digitaria* spp. (Figs. 1, 3A). This result suggests that *PWL1* homologs had already been present in the ancestral population of the *Pyricularia oryzae*–*P. grisea* species complex.

Molecular structure of *PWL1*-flanking regions in various isolates.

PCR with the *PWL1*-ORF-specific primers (PWL1-2F and PWL1-1R) resulted in amplification of a fragment with the expected size from *Panicum* isolates, *Lolium* isolates, an *Oryza* isolate (O-25V), and a *Cenchrus* isolate (Cc-1J) in addition to the EC-II isolates. Sequence analysis revealed that these amplicons from *Panicum*, *Oryza*, and *Cenchrus* isolates carried similar sequences with only two to four amino acid substitutions in their internal region when compared with the sequence of EC-II isolates.

Their flanks were cloned by using the same primers as used for the EC-II isolate (Ec-3J). Since Southern blot analysis with various restriction enzymes indicated that *Panicum* and *Lolium* isolates shared a very similar structure, a *Panicum* isolate (Pm-1J) was used as a representative of these two pathotypes. *PWL1*-flanks of O-25V and Cc-1J were cloned by cassette PCR. Those of Pm-1J were amplified by inverse PCR after self-ligation of *Hind*III or *Eco*RI digests. Nucleotide sequences obtained from the *Hind*III and *Eco*RI digests were combined in silico. These data are summarized in Figure 4C, D, and E. Pm-1J shared a highly homologous sequence spanning from 0.3 to 0.1 kb upstream of *PWL1* through approximately 1.3 kb downstream with Cc-1J and O-25V. The left portion of the homologous sequence in Cc-1J was flanked by a solo LTR of Inago1, while that in O-25V was flanked by the Pot3 transposon (Farman et al. 1996) and a solo LTR of another retrotransposon, Inago2 (accession number AB334125).

If the P structure was produced by an insertion or translocation of a fragment carrying *PWL1* into the U structure, the U structure should be ubiquitous in *Pyricularia* isolates whereas the P structure should be specific to the EC-II isolates. To check if this is the case, PCR and Southern analyses were

performed using various *Pyricularia* isolates. Fragments with an expected size were amplified with the U structure-specific primer pair from *Lolium*, *Triticum*, *Avena*, *Panicum*, and *Setaria* isolates of *P. oryzae* (Fig. 3D). Amplicons from *Oryza* isolates were larger than those from other isolates (Fig. 3D, lanes 30, 32, 33), suggesting that they have suffered from an insertion of an approximately 0.6-kb fragment. Furthermore, these *P. oryzae* isolates carried fragments that hybridized with both p239 and p238 (Fig. 3B and C). *Digitaria* isolates (Dsa-1J and Dsa-4J) belonging to *P. grisea*, a cryptic species different from *P. oryzae*, also had a fragment that hybridized with both p239 and p238 (Fig. 3B and C, lanes 37 and 38), although no fragment was amplified with the 239-1F/238-7F primer pair. These isolates may have the U structure that has suffered some nucleotide substitutions around regions corresponding to the primers. These results suggest that the U structure is ubiquitous in *Pyricularia oryzae* and had already been present in the ancestral population of the *Pyricularia oryzae*–*P. grisea* species complex, i.e., before the speciation of the cryptic species. On the other hand, the PCR analysis with 239-3F and PWL1-1R showed that the P structure was specific to EC-II isolates and not found in EC-I isolates, *Eragrostis* isolates (Supplementary Fig. S3), nor any other isolates of *Pyricularia* spp. (Fig. 1). In addition, isolates other than EC-II had no fragments that hybridized with both pPWL1-ORF and p239 (Fig. 3A and B), except one. The exceptional isolate (L-2J) (Fig. 3, lane 18) had one fragment that hybridized with both pPWL1-ORF and p239, but the fragment also hybridized with p238 (Fig. 3A, B and C), suggesting that this fragment has a different structure probably produced by another insertion event. Taken together, we suggest that the P structure was produced by an insertion or translocation of a fragment carrying *PWL1* into the U structure in a common ancestor of the EC-II isolates.

Detailed phylogenetic analysis using whole-genome sequences.

To clarify phylogenetic relationships of *P. oryzae* pathotypes in detail, 18 isolates (17 isolates of *P. oryzae* and one isolate of

Table 2. Primers used in this study^a

Target	Primer	Nucleotide sequence
ITS1-5.8S-ITS2	ITS4 ^b	TCCTCCGCTTATTGATATGC
	ITS5 ^b	GGAAGTAAAAGTCGTAACAAGG
β-tubulin	Btla ^c	TTCCCCCGTCTCCACTTCTTCATG
	Bt1b ^c	GACGAGATCGTTTCATGTTGAACCT
Actin	ACT-512F ^d	ATGTGCAAGGCCGGTTTCGC
	ACT-783R ^d	TACGAGTCCTTCTGGCCCAT
Calmodulin	CAL-228F ^d	GAGTTCAAGGAGGCCCTTCTCCC
	CAL-737R ^d	CATCTTCTGGCCATCATGG
ORF of <i>PWL1</i>	PWL1-2F	CGACCGCTGTAATCGCTGGTGGTCGC
	PWL1-pro-1F	CGTTGCATGTCCTGTCGCCATGCTC
	PWL1-1R	TTACATAATATGGCAGCCCTGATCTCCCTGACG
Construct for transformation	Fba-PWL1-2F	CTCTGCTCCGTGTCTTCAGATCAGC
	PWL1-3R	ATACGTGCGATTTCTGGCGTTATTGCG
Flanking regions of <i>PWL1</i>	PWL1-in1F	TTCAACTGTGGCCGCTTACCCTG
	PWL1-in2F	GGTGCGGGTTCATGAGGATAATGGGA
	PWL1-in1R	GCGACCACAGCGATTACAGCGGTG
	PWL1-in2R	CTTTGTTAGTCCATTTGCGACCACCAGC
MGG_00005	239-1F	GGGTACCCCTAGGCGCTCTGTAAATA
	239-3F	GTCACCCCTAGGCGCTCTGTAAATA
	239-1R	CTGGGAGAGGGAGGCAGGTATG
ELQ37450.1	238-2F	GCTGTTACCTGTAGGAGACGAACC
	238-7F	CGGAAAGCAAGGGTTACCCG
	238-2R	GTCGTATAGATTCAGGAGCGCCCTG

^a ITS = internal transcribed spacer, and ORF = open reading frame.

^b White et al. 1990.

^c Glass and Donaldson 1995.

^d Carbone and Kohn 1999.

P. grisea) were chosen as representatives. Whole genomes of nine of the 18 isolates were already sequenced by Yoshida et al. (2016) and Inoue et al. (2017). Those of the other nine isolates were sequences in the present study. A neighbor-joining tree was constructed using genomic sequences that could be aligned among the 18 isolates, including *P. grisea* (Fig. 5A). The tree

was rooted by using *Dsa-1J* (*P. grisea*) as an outgroup taxon. Its topology suggested that *P. oryzae* first differentiated into a group including *Oryza* and *Setaria* isolates and another group including *Lolium*, *Triticum*, *Eragrostis*, and *Eleusine* isolates. Then, *Dsa-1J* was excluded from the test isolates to increase the number of nucleotide sequences that could be aligned, and

Fig. 3. Analyses of the structure around MGG_00005 in representative *Pyricularia* isolates from various hosts. **A**, Genomic DNA was digested with *Cla*I and hybridized with pPWL1-ORF. **B**, p239, and **C**, p238, or **D**, amplified with primers 239-1F and 238-7F. Corresponding fragments in A and B are indicated by closed triangles and in B and C by closed circles. A fragment in lane 18 hybridized with all three probes, which was indicated by closed squares.

another neighbor-joining tree was constructed using genomic sequences that could be aligned among the 17 isolates of *P. oryzae* (Fig. 5B). Its topology suggested that, after the first differentiation mentioned above, the latter group differentiated into a subgroup including *Lolium* and *Triticum* isolates and another subgroup including *Eragrostis* and *Eleusine* isolates, which then further differentiated into a lineage consisting of *Eragrostis* isolates and another lineage consisting of *Eleusine* isolates. The latter lineage (the *Eleusine* pathotype lineage) further branched into EC-I and EC-II.

DISCUSSION

In the previous study, we suggested that the *Eleusine* pathotype was divided into two subgroups, EC-I and EC-II, using molecular markers such as rDNA and transposable elements (Tanaka et al. 2009). The present study showed that this grouping was correlated with the presence or absence of *PWL1*; all EC-II isolates were carriers of *PWL1*, while all EC-I isolates were noncarriers of *PWL1* (Table 1). This result suggests that the differentiation of the *Eleusine* pathotype into EC-I and EC-II occurred concomitantly with a loss or gain of *PWL1*. This raises a question about which event produced this contrastive distribution, a loss of *PWL1* in EC-I or a gain of *PWL1* in EC-II. All the results obtained in the present study strongly suggest that it was produced by a gain of *PWL1* in a common ancestor (founder) of EC-II. First, *PWL1* in all EC-II isolates was associated with the P structure (Supplementary Fig. S3), which represented itself to be produced by an insertion or translocation of a fragment carrying *PWL1* into the U structure (Fig. 4). Second, among *Pyricularia* isolates, the U structure and *PWL1* were ubiquitous or widely distributed, while the P structure was specific to the EC-II isolates (Figs. 1, 3). In addition, the P structure was not found even in whole-genome sequences of 140 *P. oryzae* isolates other than EC-II in the database. The gain of *PWL1* should have caused a

concomitant loss of pathogenicity on weeping lovegrass, since *PWL1* confers avirulence on weeping lovegrass (Fig. 2). Actually, all the EC-II isolates were avirulent on weeping lovegrass, while the EC-I and *Eragrostis* isolates were virulent (Table 1).

To reveal phylogenetic relationships of *Eragrostis*, EC-I, and EC-II isolates, a neighbor-joining tree was constructed using the whole-genome sequences. The resulting tree showed almost the same pattern of branching as those reported by Gladieux et al. (2018) and Valent et al. (2019). Their “*Eleusine* 1 lineage” and “*Eleusine* 2 lineage” are considered to correspond to the EC-I and EC-II lineages, respectively. When the host-range data were merged, its topology suggested that the loss of pathogenicity on weeping lovegrass in EC-II was preceded by a gain of pathogenicity on finger millet (Fig. 5).

As mentioned earlier, EC-I and EC-II can also be differentiated by the presence or absence of *grasshopper*. A Southern analysis in our previous study showed that *grasshopper* was present in all the EC-II isolates tested in the present study but absent in the EC-I or *Eragrostis* isolates (Tanaka et al. 2009). In addition, a sequence analysis in the present study revealed that the genome of Ec-3J contained approximately 17 copies of *grasshopper* but that those of EC-I (Ec-130U, Ec-5J, Ec-6J) and *Eragrostis* isolates (ER1-1J, ERc-2J, ERc-3J) contained no copies. It is unlikely that those copies of this transposable element were completely removed from the genome of a common ancestor of EC-I. It is more likely that *grasshopper* was inserted into the genome of a common ancestor (founder) of EC-II and then proliferated. We suggest that a significant genome arrangement, exemplified by the insertion of *PWL1* and *grasshopper*, occurred in a strain of the *Eleusine* lineage, which triggered the evolution into the *Eleusine*-specific subgroup EC-II.

Based on these considerations, we propose two models that illustrate possible evolutionary pathways of EC-I, EC-II, and *Eragrostis* isolates (Fig. 6). In one model (Fig. 6A), the

Fig. 4. A, Structure around the null allele in Ec-130U (EC-I isolate) and ERc-2J (*Eragrostis* isolate) and B, *PWL1* in Ec-3J (EC-II isolate), C, Cc-1J (*Cenchrus* isolate), D, Pm-1J (*Panicum* isolate), and E, O-25V (*Oryza* isolate). Shaded areas represent highly homologous regions. Arrows and bold lines represent primers and probes used in the present study.

common ancestor of these three groups is assumed to have been pathogenic on *Eragrostis* spp. but nonpathogenic on *Eleusine* spp. because it carried one or more avirulence genes on *Eleusine* spp. (hereafter referred to as AVR on Ele). An individual gained pathogenicity on *Eleusine* spp. through a loss of the

AVR on Ele (host jump), resulting in the prototype of EC-I. This prototype retained the pathogenicity on its original host, i.e., weeping lovegrass, and, therefore, could survive either host or use weeping lovegrass as an alternate host when finger millet was not available. Then, a member of this type happened to

Fig. 5. Phylogenetic relationships of representative isolates inferred from whole-genome sequences. **A**, A neighbor-joining tree constructed from multiple alignments of conserved genomic sequences shared by *Pyricularia oryzae* and *P. grisea*. The tree was rooted using *P. grisea* Dig41 as an outgroup taxon. **B**, A neighbor-joining tree constructed from multiple alignments of conserved genomic sequences shared by *P. oryzae* isolates. The arrow indicates the node to the outgroup taxon (*P. grisea* Dig41) inferred from A. Original hosts of the isolates are color-coded as shown in A. Host ranges of the isolates are shown by colored ovals. The numbers on the nodes indicate bootstrap values from 1,000 replications.

gain a DNA or chromosomal fragment carrying *PWL1* through sexual hybridization or a parasexual recombination (Chuma et al. 2011; Noguchi et al. 2006; Zeigler et al. 1997). This caused a concomitant loss of pathogenicity on weeping lovegrass, which drove evolution into a specialist on finger millet, resulting in the EC-II type, i.e., a genuine *Eleusine* pathotype. In the other model (Fig. 6B), the common ancestor of EC-I, EC-II, and *Eragrostis* isolates is assumed to have been pathogenic on both *Eragrostis* spp. and *Eleusine* spp. already. An individual in this ancestral population lost its pathogenicity on *Eleusine* spp. through a gain of the AVR on Ele, resulting in a common ancestor of *Eragrostis*-specific isolates. Then, an individual in the remaining population lost its pathogenicity on *Eragrostis* spp. through a gain of *PWL1*, resulting in a common ancestor of EC-II (*Eleusine*-specific isolates). In this model

EC-I is assumed to maintain the ancestral characteristic—pathogenicity on both genera.

In either model a key event for evolving into a specialist on *Eleusine* spp. is a gain of an avirulence gene. We suggest that, in addition to a loss of avirulence genes exemplified in the wheat blast fungus (Inoue et al. 2017), a gain of avirulence genes may have played a role in the evolution of pathotypes or parasitic specialization of *P. oryzae* at the plant genus level. Further studies are needed to reveal the evolutionary process of EC-I, EC-II, and *Eragrostis* isolates in detail. It will be vital to clone the avirulence gene of *Eragrostis* isolates on finger millet identified by Yaegashi (1978). This should be the AVR on Ele in Figure 6. A comparison of its corresponding locus in EC-I and EC-II isolates may reveal critical molecular events that enabled them to parasitize finger millet.

Fig. 6. Hypothetical models for the evolution of *Eragrostis* isolates and EC-I and EC-II isolates based on an assumption that their common ancestor was **A**, pathogenic on *Eragrostis* spp. but nonpathogenic on *Eleusine* spp. or **B**, pathogenic on both *Eragrostis* spp. and *Eleusine* spp. The blue oval indicates a loss of an avirulence gene while the red ovals indicate gains of an avirulence gene. AVR on Ele = an avirulence gene on *Eleusine* spp.

MATERIALS AND METHODS

Fungal materials.

Finger millet-infecting *Pyricularia* strains tested were 30 isolates from *Eleusine coracana*, five isolates from *Eleusine indica*, one isolate from *Eleusine africana*, one isolate of *Eleusine boranensis*, and three isolates from *Bambusa* sp. collected in five countries (Japan, China, Nepal, India, and Uganda) (Supplementary Table S1). The three *Bambusa* isolates (Ebb-110I, Ebb-111I, Ebb-112I), which had been collected in a finger millet-cultivating area in India, were considered to be members of the *Eleusine* pathotype that had colonized bamboo leaves by opportunistic infection, because they showed specific pathogenicity to finger millet in artificial inoculation tests (Kato et al. 2000). *Eleusine* isolates in this paper include these *Bambusa* isolates. *Eragrostis* isolates tested were one from *Eragrostis lehmanniana* and two from *Eragrostis curvula*, provided by N. Hayashi, National Institute of Agrobiological Sciences, Japan. In addition, 70 isolates were employed to construct phylogenetic trees.

These isolates were maintained on barley seed media (Hayashi and Kato 1988) at 4°C in the Laboratory of Plant Pathology, Kobe University. They were transferred to a potato dextrose agar slant just before use and were grown at room temperature. One-week to three-month-old cultures were used for experiments.

Construction of a multilocus phylogenetic tree.

Mycelial plugs were transferred to 50 ml of complete medium (3 g of yeast extract, 3 g of casamino acid, and 5 g of sucrose per liter) and were grown at 25°C for 4 days. Total DNA was extracted from mycelia as described previously (Nakayashiki et al. 1999). A portion was amplified from rDNA, *ACT*, *TUB*, and *CAL* with primers ITS5 and ITS4 (White et al. 1990), ACT-512F and ACT-783R (Carbone and Kohn 1999), Bt1a and Bt1b (Glass and Donaldson 1995), and CAL-228F and CAL-737R (Carbone and Kohn 1999), respectively, in a 50-μl reaction containing 1 unit KOD-plus (Toyobo), 1× PCR buffer provided by the manufacturer, 200 μM each dNTP, 0.2 μM each primer, 1.0 mM MgSO₄, and 10 ng of template DNA. Nucleotide sequences of these primers are listed in Table 2. This reaction was run by Mastercycler (Eppendorf) programmed for 5 min at 96°C, 30 cycles of 10 s at 96°C, 5 s at 56°C, and 30 s at 68°C, followed by 5 min at 68°C. PCR products were purified by ethanol precipitation and were sequenced with the same primers as in the amplification, using Big Dye terminator cycle sequencing ready reaction kit 3.1 and ABI 3100 genetic analyzer (Applied Biosystems). Sequences obtained were combined, were aligned with Clustal W (Thompson et al. 1994), and, finally, were optimized manually.

Multilocus phylogenetic trees were constructed with the MP method, using MEGA7 (Kumar et al. 2016). Alignment gaps were treated as missing data. To assess the robustness for each branch, bootstrap analysis (Felsenstein 1985) was performed with 1,000 replications.

Construction of a phylogenetic tree using whole-genome sequences.

A total of 18 representative isolates were selected to construct a phylogenetic tree based on whole-genome sequences. Whole-genome sequences of GFS11-7-2 (Si-1J), Ina168 (O-6J), Z2-1 (Ec-2J), Br48 (T-4B), and Dig41 (Dsa-1J) were obtained from Yoshida et al. (2016), while those of Br116.5 (T-7B), Br118.2 (T-8B), TP2 (L-2J), and MZ5-1-6 (Ec-3J) were obtained from Inoue et al. (2017). Whole genomes of PO12-7301-2 (O-23IN), TorideI-1-7 (O-31J), TP1 (L-1J), FSECu1-1-1 (ERc-2J), SZECu1-1-1 (ERc-3J), NI986 (ERI-1J), GFEC1-5-1 (Ec-6J),

SZEC1-1-1 (Ec-5J), and UG77-15-1-1 (Ec-130U) were sequenced using Illumina MiSeq. Genomic DNAs were extracted from mycelia using the plant genomic DNA extraction miniprep system (Viogene). Sequence libraries were generated using NEBNext DNA library prep master mix set for Illumina (New England Biolabs) and NEBNext multiplex oligos for Illumina (New England Biolabs) and were sequenced with MiSeq reagent kit v3 (Illumina), following the supplied protocols. Genome contigs were assembled on CLC Genomics Workbench 6.8.1 software (Qiagen Bioinformatics).

Genome sequences obtained were aligned with mauve-Aligner algorithm in MAUVE software (Darling et al. 2004). Neighbor-joining trees were constructed using MEGA version X software (Kumar et al. 2018) with p-distance method. Alignment gaps were completely removed. The trees were visualized in FigTree 1.4.4.

Pathogenicity assay.

Seeds of *Eragrostis curvula* (Yukijirushi 46-102 or JP No.3695) and *Eleusine coracana* (Iiya-zairai) were sown in plastic pots (15 × 6 × 10 cm) filled with Sakata Supermix soil (Sakata Co.) and were grown in a controlled-environment room with natural light at 25°C for 30 and 14 days, respectively. Conidial suspension (2 × 10⁵ per milliliter), prepared as described previously (Murakami et al. 2000), was sprayed onto the seedlings with 0.01% Tween 20. Inoculated seedlings were incubated in a dark, moistened box for 24 h at 25°C and were then transferred to a controlled-environment room with fluorescent lighting regulated at 26°C. Four to five days after inoculation, symptoms were evaluated based on the color of lesions and the affected leaf area. The affected area was rated using six progressive grades from 0 to 5: 0 = no visible evidence of infection, 1 = pinpoint spots, 2 = small lesions (<1.5mm), 3 = lesions with an intermediate size (<3mm), 4 = large, typical lesions, and 5 = complete blighting of leaf blades. A disease score is composed of a number denoting the affected area and a letter or letters indicating the lesion color: 'B' for brown and 'G' for green. All inoculation tests were conducted at least three times.

PCR cloning of *PWL1*.

PWL1 was amplified with primer pairs PWL1-2F/PWL1-1R (for the 383-bp fragment of *PWL1* ORF) and PWL1-pro-1F/PWL1-1R (for the entire ORF) (Table 2) in a 50-μl reaction containing the same components as mentioned above. This reaction was run by Mastercycler (Eppendorf) programmed for 5 min at 96°C, 30 cycles of 10 s at 96°C, 5 s at 63°C, and 30 s at 68°C, followed by 5 min at 68°C. Amplified fragments were purified by ethanol precipitation and were inserted into the *EcoRV* site of BluescriptII SK(+), using DNA ligation kit ver.2 (Takara). The ligation product was transformed into *Escherichia coli* DH5-α, and three colonies were recovered from each ligation reaction. The recombinant plasmid was extracted from each of the three cultures (colonies) with QIAprep spin miniprep kit (Qiagen) and were sequenced, using the BigDye terminator cycle sequencing ready reaction kit with M13 forward and reverse primers and ABI 3100 genetic analyzer (Applied Biosystems). The authentic DNA sequence in the genomic DNA was determined by comparing sequences of three clones derived from the three colonies.

Southern blot analysis.

Southern hybridization was performed using the ECL direct nucleic acid labeling and detection system (GE Healthcare). Total DNA (1 μg) was digested with restriction enzymes (Takara) and was fractionated on a 0.9% agarose gel in Tris-acetate-EDTA (TAE) buffer. The fractionated DNA fragments

were transferred to a nylon membrane (Hybond-N+; GE Healthcare) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and were fixed by UV irradiation.

Plasmids used for probes were pPWL1-ORF containing a fragment of *PWL1* amplified from Ec-3J with the PWL1-2F/PWL1-1R primer pair, p239 containing a fragment of MGG_00005 amplified from Ec-3J with the 239-1F/239-1R primer pair, and p238 containing a fragment of ELQ37450.1 amplified from Ec-130U with the 238-2R/238-2F primer pair. pPWL1-ORF was one of the recombinant plasmids described above. p239 and p238 were produced through the same procedures as pPWL1-ORF, except for the program for amplification of target fragments, 3 min at 94°C, 30 cycles of 10 s at 94°C and 30 s at 68°C, followed by 5 min at 68°C.

The membrane was hybridized overnight with labeled probes in Gold hybridization buffer (GE Healthcare) containing 0.5 M NaCl at 42°C. After hybridization, the membrane was washed twice in the primary wash buffer (0.4% sodium dodecyl sulfate and 0.5× SSC) for 10 min at 55°C, followed by two washes in 2× SSC for 5 min at room temperature. Detection of target DNA was performed according to the manufacturer's instructions.

Transformation of *P. oryzae* with a *PWL1* homolog.

A 4.2-kb fragment containing *PWL1* was amplified from Ec-3J with the Fba-PWL1-2F/PWL1-3R primer pair in a 50-μl reaction containing the same components as mentioned above. This reaction was run by Mastercycler (Eppendorf) programmed for 3 min at 94°C, 30 cycles of 10 s at 94°C and 3 min at 68°C, followed by 5 min at 68°C. Amplified fragments were purified by ethanol precipitation and were inserted into the *EcoRV* site of BluescriptII SK(+) using DNA ligation kit ver.2 (Takara). The ligation product was transformed into *Escherichia coli* DH5-α and was established as pPWL1-EC. An EC-I isolate, Ec-130U, was transformed with pPWL1-EC as described by Tosa et al. (2005).

Cloning of *PWL1* flanking regions.

Flanking regions of *PWL1* were cloned by cassette PCR (Isegawa et al. 1992) or inverse PCR (Table 3). Cassette PCR was performed using oligonucleotide cassettes and cassette primers included in an LA PCR in vitro cloning kit (Takara). Total DNA (5 μg) was digested with the restriction enzymes (Takara) shown in Table 3 for 3 h at 37°C. The digested DNA was purified by ethanol precipitation and was ligated with oligonucleotide cassettes carrying corresponding restriction sites by using Ligation Kit ver.2 (Takara). The ligation product was purified by ethanol precipitation and was used as a template for the first PCR. The target fragment was amplified in a 50-μl reaction containing 1 unit KOD-plus (Toyobo), 1× PCR buffer provided by the manufacturer, 200 μM each dNTP, 0.2 μM each primer (Table 3), 1.0 mM MgSO₄, and 10 ng of the template

DNA. The reaction was run by Mastercycler (Eppendorf) programmed for 5 min at 96°C, 30 cycles of 10 s at 96°C and 3 min at 68°C, followed by 5 min at 68°C. The product of the first PCR was diluted 100 to 1,000 times with water and was then used as a template for the second (nested) PCR. The second PCR was performed in the same conditions as the first PCR, with the primer sets shown in Table 3.

For inverse PCR, total DNA (1 μg) was digested with *HindIII* or *EcoRI* (Takara) for 5 h at 37°C. The digested DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), was purified by ethanol precipitation, and was self-ligated in a 500-μl reaction with 10 U T4 ligase (Takara). The ligation product was purified by ethanol-precipitation and was used as a template for the first PCR. The target fragment was amplified in a 50-μl reaction containing 1 unit KOD-plus (Toyobo), 1× PCR buffer provided by the manufacturer, 200 μM each dNTP, 0.2 μM each primer (Table 3), 1.0 mM MgSO₄, and 10 ng of the template DNA. This reaction was run by Mastercycler (Eppendorf) programmed for 5 min at 96°C, 30 cycles of 10 s at 96°C, and 3 min at 68°C, followed by 5 min at 68°C. The product of the first PCR was diluted 100 to 1,000 times with water and was used as a template for the second (nested) PCR. The nested PCR was performed in the same conditions as the first PCR, with primers shown in Table 3.

The PCR products were purified by ethanol precipitation and were inserted into the *EcoRV* site of BluescriptII SK(+) using DNA Ligation Kit ver.2 (Takara). The ligation product was transformed into *Escherichia coli* DH5-α, and three colonies were recovered from each ligation reaction. The authentic sequence in the genomic DNA was determined by comparing DNA sequences of three clones derived from the three colonies as described above.

Detection of the MGG_00005 - ELQ37450.1 structure by PCR.

The presence or absence of the U structure (Fig. 4) in *Pyricularia* isolates was surveyed by PCR. Genomic DNAs of 39 representative isolates from various hosts were amplified with primers 239-1F and 238-7F (Table 2) in a 10-μl reaction mixture containing 0.25 U rTaq DNA polymerase, 1× PCR buffer (+Mg), 200 μM dNTPs, 0.5 μM of each primer, and 2 ng of template DNA. This reaction was run by Mastercycler (Eppendorf) programmed for 10 min at 94°C, 10 cycles of 1 min at 94°C, 30 s at 55°C, and 100 s at 72°C, followed by 20 cycles of 1 min at 94°C, 30 s at 55°C, and 2 min at 72°C, and a final extension of 10 min at 72°C. PCR products were separated on a 0.9% agarose TAE gel and were detected by staining with ethidium bromide.

The presence or absence of the P structure (Fig. 4) was checked by PCR, with primers 239-3F and PWL1-1R and Quick Taq HS DyeMix (Toyobo), using a program of 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 62°C.

Table 3. Restriction enzymes and primers used for cloning of *PWL1*-flanking regions^a

Isolate	Target	Enzyme	Primers for	
			First PCR	Second PCR
Ec-130U, ERc-2J	Null allele	<i>EcoRI</i>	239-1F/C1 ^b	239-3F/C2 ^b
Ec-3J	5' flank	<i>FbaI</i>	PWL1-in2R/C1	PWL1-in1R/C2
	3' flank	<i>HindIII</i>	PWL1-in1F/C1	PWL1-in2F/C2
Cc-1J	5' flank	<i>HindIII</i>	PWL1-in2R/C1	PWL1-in1R/C2
	3' flank	<i>Sall</i>	PWL1-in1F/C1	PWL1-in2F/C2
O-25V	5' flank	<i>HindIII</i>	PWL1-in2R/C1	PWL1-in1R/C2
	3' flank	<i>Sall</i>	PWL1-in1F/C1	PWL1-in2F/C2
Pm-1J	5' and 3' flanks	<i>HindIII</i> and <i>EcoRI</i>	PWL1-in1F/PWL1-in2R	PWL1-in2F/PWL1-in1R

^a Cassette PCR was used on all isolates except Pm-1J, for which inverse PCR was used.

^b C1 and C2 are primers provided by the manufacturer.

ACKNOWLEDGMENTS

We are grateful to N. Hayashi, National Institute of Agrobiological Sciences, Japan, for providing the weeping lovegrass isolates, to R. Honkura, Miyagi University, for providing a rice isolates for whole genome sequencing, to S. Takumi, Kobe University, for valuable suggestions on phylogenetic analyses, to H. Tendo, Kobe University for technical supports, and to M. Kusaba, Saga University, for critically reading the manuscript. Special thanks are due to T. Ishii, professor at the Laboratory of Plant Breeding, Kobe University, N. Mori, professor at the Laboratory of Crop Evolution, Kobe University, and I. Chuma, assistant professor at the Laboratory of Plant Pathology, Kobe University (present affiliation, Obihiro University of Agriculture and Veterinary Medicine), for monitoring the experiments, and B. Valent, professor at the Department of Plant Pathology, Kansas State University, for giving valuable advice on how to solve the problems that occurred in the previous version of this article.

AUTHOR-RECOMMENDED INTERNET RESOURCES

FigTree software: <http://tree.bio.ed.ac.uk/software/figtree>
Qiagen Bioinformatics Genomics Workbench software:
<https://www.qiagenbioinformatics.com>

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