Ribotypes of *Polymyxa graminis* in Wheat Samples Infected with Soilborne Wheat Viruses in China

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Abstract

Polymyxa graminis is an obligate parasite and important vector of more than 14 soilborne plant viruses that pose a significant threat to cereal crops in Europe, North America, and Asia. Different ribotypes or formae speciales of *P. graminis* have been recognized and these may be associated with different cereal hosts or with transmission of different viruses. Two soilborne viruses infecting winter wheat in China have been reported and well studied (*Wheat yellow mosaic virus* [WYMV, genus *Bymovirus*] and *Chinese wheat mosaic virus* [CWMV, genus *Furovirus*]) but there has been no reported characterization of *P. graminis* isolates associated with them. In this study, the ribosomal DNA internal transcribed spacer (ITS) regions of *P. graminis* were examined from 63 wheat samples with apparent virus symptoms obtained from 16 sites within six Chinese provinces.

Their associations with soilborne viruses were investigated. Ribotype I (*P. graminis* f. sp. *temperata*) and ribotype II (*P. graminis* f. sp. *tepida*) were confirmed in winter wheat regions of China for the first time. All 63 wheat root samples were infected with ribotype I of *P. graminis* and 11 were also infected with ribotype II. There was no obvious association between the ribotypes and infection by either WYMV or CWMV (or double infection). Phylogenetic analysis of the *P. graminis* ITS1-5.8S-ITS2 sequences revealed that ribotype I in China belongs to previously reported subgroup Ib, whereas ribotype II belongs to IIa. There was considerable sequence variation (pairwise distances from 0.0219 to 0.0319) between Chinese ribotype I isolates of different regions and previously reported ribotype I isolate Ken5 (accession number HE860055.1).

The genus *Polymyxa* (Plasmodiophoridae, Cercozoa) contains two morphologically identical species, *Polymyxa graminis* and *P. betae*, which are soilborne, obligate intracellular parasites of plant roots (Biancardi and Tamada 2016; Bulman and Braselton 2014). These were formerly distinguished on the basis of host range: *P. graminis* mainly infects cereals and grasses, whereas *P. betae* is restricted to sugar beet and related plants in the family Chenopodiaceae (Barr 1979). Today, the sequence of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (rDNA) has been used to distinguish fungal isolates independent of morphology or host specificity (White et al. 1990). Restriction analysis of the ITS1-5.8S-ITS2

rDNA sequence was first successfully applied to distinguish *P. graminis* from *P. betae* (Ward et al. 1994). Further rDNA sequence analysis of samples from around the world has revealed the presence of six major subgroups (ribotypes) within *P. graminis* but not within *P. betae* (Cox et al. 2014; Smith et al. 2013; Ward and Adams 1998; Ward et al. 2005b). There was also evidence that ecological characteristics such as temperature requirements (Legrève et al. 1998) and host range (Legrève et al. 2000) correlated with these subgroups. Based on the ecological requirements and ITS analysis, five special forms (*P. graminis* f. sp. *temperata*, *P. graminis* f. sp. *tepida*, *P. graminis* f. sp. *subtropicalis*, *P. graminis* f. sp. *tropicalis*, and *P. graminis* f. sp. *colombiana*) were proposed by Legrève et al. (2002). These special forms clearly correspond to the ribotypes of Ward et al. (2005b): ribotype I (f. sp. *temperata*), ribotype II (f. sp. *tepida*), ribotype III (f. sp. *tropicalis*), ribotype IV (f. sp. *subtropicalis*), and ribotype V (f. sp. *colombiana*) (Neuhauser et al. 2010).

Although *P. graminis* itself is not considered a plant pathogen (Kanyuka et al. 2003), it transmits agronomically important viruses to cereal crops, especially in temperate regions where ribotype I and ribotype II occur (Legrève et al. 1998). As a vector of more than 14 soilborne plant viruses belonging to the genera *Bymovirus* (*Potyviridae*) and *Furovirus* and *Pecluvirus* (*Virgaviridae*), *P. graminis* poses a significant threat to cereal crops, including wheat, barley, pearl millet, oat, rye, and sorghum (Adams and Jacquier 1994; Kühne 2009; Legrève et al. 2000). The viruses it transmits to wheat include the bymoviruses *Wheat yellow mosaic virus* (WYMV) in Asia and *Wheat spindle streak mosaic virus* in Europe and North America, and the furoviruses *Chinese wheat mosaic virus* (CWMV) in Asia, *Soilborne wheat mosaic virus* (SBWMV) mostly in the Americas, and *Soilborne cereal mosaic virus* (SBCMV) in Europe (Kanyuka et al. 2003; Kühne 2009). In studies of samples from the field, it has been shown that SBWMV and SBCMV occur on wheat with

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ribotype I, and that the bymoviruses *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) occur on barley with ribotypes I or II (Lebas et al. 2009; Smith et al. 2013). In more precisely controlled laboratory studies, transmission of BaMMV by ribotype I and SBCMV by ribotype II has been demonstrated (Adams et al. 1988; Vaianopoulos et al. 2007), whereas *Indian peanut clump virus* or *Peanut clump virus* (PCV) (genus *Pecluvirus*) was transmitted by ribotype III or IV isolates (Dieryck et al. 2011; Ratna et al. 1991; Thouvenel and Fauquet 1981).

Soilborne virus diseases of wheat have been reported in Shandong, Henan, Hubei, Jiangsu, Anhui, Sichuan, and Shanxi Provinces, China, since the 1970s (Chen 1993). Yield losses have been estimated typically at 10 to 30% but sometimes reaching 70% (Hou et al. 1985). WYMV and CWMV, both transmitted by *P. graminis*, have been identified as the disease agents (Chen 1993; Diao et al. 1999; Ye et al. 1999). WYMV is distributed widely in the winter wheat growing region of China but CWMV is restricted to areas of Shandong (cities: Yantai and Rongcheng) and Jiangsu (city: Dafeng) Provinces (Diao et al. 1999; Sun et al. 2013). *P. graminis* ribotype I was identified in two barley samples from Shanghai and Xiaoshan (Chen 1993; Legrève et al. 2002; Ward and Adams 1998) but the ribotypes present on wheat in China and responsible for transmitting WYMV or CWMV have not been determined. In this study, we determined the ITS region sequences of *P. graminis* isolates associated with WYMV or CWMV in wheat samples from different parts of the winter wheat growing region in China. The results provide further information on the interaction between *P. graminis* and the wheat viruses it transmits in China.

Materials and Methods

Wheat sample collection. Soilborne virus diseases of wheat are mainly distributed in the middle and lower valleys of the Yangtze and Huai Rivers in China (Chen 1993; Han et al. 2000). During March 2014 and 2016, 63 wheat samples of various cultivars displaying yellow mosaic symptoms on their leaves were collected from the fields of these regions, including six provinces (16 sites) in China where soilborne wheat disease has occurred severely for at least 5 years: Anhui (two locations, 2 samples), Hubei (two locations, 3 samples), Henan (five locations, 22 samples), Jiangsu (one location,

3 samples), Shandong (five locations, 32 samples), and Zhejiang (one location, 1 sample) (Fig. 1). Leaves were stored at -80°C while the corresponding root samples were washed in tap water to remove soil, parts were examined by light microscopy, and the remainder was stored at -80°C before DNA extraction.

Microscope observation. Small piece of roots were taken from naturally dried wheat samples and soaked in clean water for 1 h. Root pieces were mounted on microscope slides, and the presence of *P. graminis* sporosori (resting spores) were observed and photos were taken under a compound microscope (Axio Vert.A1; Zeiss) at magnifications of $\times 400$ and $\times 200$.

DNA extraction of wheat roots and polymerase chain reaction. Roots of each wheat plant were dried on a paper towel, and 50 mg of root was cut into small pieces and ground to a fine powder under liquid nitrogen using an automatic grinding machine. Total DNA was extracted using DNA secure Plant Kit (Tiangen). Extracted DNA was diluted with 50 μl of double-distilled (dd)H₂O prior to polymerase chain reaction (PCR). Primer pair PSP2/ITS4 was used to detect the presence of *Polymyxa* spp. (Legrève et al. 2003; White et al. 1990). Each 10- μl reaction mixture contained 1 μl of High Fidelity (HiFi) buffer I, 1 μl of 10 $\times$ dNTP (2.5 mM), 0.5 μl of DNA (100 ng/ μl), 0.25 μl of primer (10 μM), 0.25 μl of HiFi Taq DNA polymerase (5 U/ μl), and 6.75 μl of ddH₂O. PCR cycling conditions were 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 58°C for 50 s, and 72°C for 25 s; with a final extension at 72°C for 10 min. PCR products were observed on a 1% agarose gel under ultraviolet light.

Sequencing and phylogenetic analysis. The PCR products (PSP2/ITS4) of different samples were purified, ligated into the pMD18-T vector (TaKaRa), and sequenced (Tsingke) to obtain the ITS2 region of *P. graminis*. National Center for Biotechnology Information homology Blast using the sequences with primer pair (PSP2/ITS4) indicated that only ribotypes I and II were detected in all 63 samples. To distinguish these two ribotypes and detect samples jointly infected with ribotypes I and II, primer pair Pg.F2/Pg.R2 (Ward et al. 2005a) was used for the specific detection of ribotype II. In addition, because only ribotype I and II are present in the collected samples, we use primer pair NS7 (White et al. 1990) and ITS.CN (designed in this study) for the detection of ribotype I (although ribotype III and *P. betae* can also be detected by NS7/ITS.CN). The positions of the three primer pairs

Fig. 1. Map showing field locations of wheat samples collected in China. Sample locations used for this study are indicated with circles: 1 = Zhumadian, Henan; 2 = Nanyang, Henan; 3 = Dengzhou, Henan; 4 = Luohe, Henan; 5 = Puyang, Henan; 6 = Yantai, Shandong; 7 = Rongcheng, Shandong; 8 = Linyi, Shandong; 9 = Taian, Shandong; 10 = Qufu, Shandong; 11 = Yangzhou, Jiangsu; 12 = Lu'an, Anhui; 13 = Guoyang, Anhui; 14 = Jingzhou, Hubei; 15 = Zhongxiang, Hubei; and 16 = Lin'an, Zhejiang.

within rDNA and the product sizes are shown in Figure 2A. All primer sequences used for this study are listed in Table 1.

The sequences were assembled to obtain the complete ITS1-5.8S-ITS2 region (Legrève et al. 2002) and previously published sequences of this region from other *P. graminis* isolates were obtained from GenBank for use in phylogenetic analysis. ITS rDNA sequences of other plasmodiophorids, including *P. betae* (accession number Y12827), *Spongospora subterranea* (accession number Y12829), and *Plasmiodiophora brassicae* (accession number Y12831), were used as outgroups. Phylogenetic analysis was carried out using MEGA software (version 6) and the tree was generated using the neighbor-joining (NJ) algorithm (1,000 bootstrap replications) (Tamura et al. 2013). Trees were also constructed using the maximum-likelihood and maximum-parsimony algorithms (1,000 bootstrap replications) to evaluate the reliability of the tree. Pairwise distances between different samples were computed by MEGA using the maximum composite likelihood method (Tamura et al. 2004).

RNA extraction from leaves and reverse-transcription PCR. Total RNA was extracted from each leaf sample (100 mg) using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA quantity and quality was evaluated by denaturing agarose gel electrophoresis and a 2100 Bioanalyzer (Agilent). The purified RNA (approximately 2 µg) was used for reverse-transcription (RT)-PCR and cDNA was synthesized using a FastQuant RT Kit (Tiangen). The conditions used for PCR were same as described above. Primers specific to WYMV and CWMV were designed (Table 1) to detect the presence of the respective viruses.

Results

Observation of *P. graminis* in wheat roots. Direct microscopic observation of the infected root is the simplest way to determine

the presence of *P. graminis* (Rush 2003). Multiple mature sporosori of *P. graminis* were observed in nearly all wheat root segments of thoroughly washed samples (isolate SD01). The sporosori were variable in shape and size but most appeared globular in root cells (Supplementary Fig. S1), which is consistent with previous descriptions (Barr 1979). Most of the sporosori were mature and could be easily identified by their thick wall and dark brown color. Each sporosori contained numerous, individual resting spores, and sporosori were always restricted to cortical and epidermal cells.

***P. graminis* ribotype detection.** In an initial PCR that should detect any ribotype of *P. graminis*, primer PSP2 (forward) and fungal universal primer ITS4 (reverse) were used to amplify the complete ITS2 region (Legrève et al. 2003; White et al. 1990) (Fig. 2A). The expected band of around 250 bp (252 bp) was obtained from all 63 samples.

Each of the samples was then amplified with primer pairs NS7/ITS.CN (amplifying a band of approximately 800 bp) and Pg.F2/Pg.R2

Table 1. Primer sequences used in this study

Primer	Primer sequence (5'–3')	Reference
PSP2 (F)	TTGCGCTTTCGAGAGCCCT	Legrève et al. 2003
ITS4 (R)	TCCTCCGCTTATTGATATGC	White et al. 1990
Pg.F2 (F)	ATGTGGATCGTCTCTGTTGCTGGA	Ward et al. 2005a
Pg.R2 (R)	CCTCATCTGAGATCTTGCCAAGT	Ward et al. 2005a
NS7 (F)	GAGGCAATAACAGGTCTGTGATG	White et al. 1990
ITS.CN (R)	GCGGATTATCGTTCCAAGAA	Present study
WYMV-F	CCGCCACCAAAGAGAAATGG	Present study
WYMV-R	TCGGAGGTGAGCATGGTATT	Present study
CWMV-F	TGAGACATGGCAGAGTACGA	Present study
CWMV-R	AGCCTCGAAAGAATCCTGTG	Present study

Fig. 2. Amplification of nuclear ribosomal DNA (rDNA) fragments from *Polymyxa graminis*. **A**, Positions and product sizes obtained using three primer pairs: NS7/ITS.CN, Pg.F2/Pg.R2, and PSP2/ITS4. **B**, Agarose gel showing polymerase chain reaction products obtained using primer pairs PSP2/ITS4 (a), NS7/ITS.CN (b), and Pg.F2/Pg.R2 (c). Lane M: DNA ladder; sample I: wheat root singly infected with *P. graminis* ribotype I; sample II: wheat root jointly infected with ribotypes I and II; sample III: wheat root free of *P. graminis*; and sample IV: no-template control.

Table 2. *Polymyxa graminis* ribotypes (formae speciales) and viruses detected in 63 wheat samples from different sites in China

Isolate	Location	Wheat cultivar	Ribotype (formae speciales)		Viruses			
			I (<i>temperata</i>)	I (<i>temperata</i>) + II (<i>tepida</i>)	WYMV ^a	CWMV ^a	WYMV+CWMV	Virus free
AH01	Lu'an, Anhui	Yangmai20	...	+	+	...	...	...
AH02	Guoyang, Anhui	Unknown	...	+	+	...	...	...
HB01	Jingzhou, Hubei	Unknown	+	...	+	...	...	...
HB02	Jingzhou, Hubei	Unknown	+	...	+	...	...	...
HB03	Zhongxiang, Hubei	Ningmai15	+	...	+	...	...	...
HN01	Dengzhou, Henan	Unknown	+	...	+	...	...	...
HN02	Luohe, Henan	Unknown	+	...	+	...	...	...
HN03	Nanyang, Henan	Lankao198	+	...	+	...	...	...
HN04	Puyang, Henan	Zhoumai16	...	+	+	...	...	...
HN05	Puyang, Henan	Luomai9	...	+	+	...	...	...
HN06	Puyang, Henan	Luomai10	...	+	+	...	...	...
HN07	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN08	Zhumadian, Henan	Hengguan35	...	+	+	...	...	...
HN09	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN10	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN11	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN12	Zhumadian, Henan	Hengguan35	+	...	...	...	...	+
HN13	Zhumadian, Henan	Aikang58	+	...	+	...	...	...
HN14	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN15	Zhumadian, Henan	Zhongluo1	+	...	+	...	...	...
HN16	Zhumadian, Henan	Huayu198	+	...	+	...	...	...
HN17	Zhumadian, Henan	Aikang58	+	...	+	...	...	...
HN18	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN19	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN20	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN21	Zhumadian, Henan	Unknown	+	...	+	...	...	...
HN22	Zhumadian, Henan	Unknown	+	...	+	...	...	...
JS01	Yangzhou, Jiangsu	Yangmai158	...	+	+	...	...	...
JS02	Yangzhou, Jiangsu	Unknown	+	...	+	...	...	...
JS03	Yangzhou, Jiangsu	Unknown	...	+	+	...	...	...
SD01	Linyi, Shandong	Linmai4	+	...	...	...	...	+
SD02	Linyi, Shandong	Unknown	+	...	+	...	...	...
SD03	Linyi, Shandong	Linmai4	+	...	+	...	...	...
SD04	Linyi, Shandong	Changwu134	+	...	...	...	...	+
SD05	Linyi, Shandong	Weidong4	+	...	+	...	...	...
SD06	Linyi, Shandong	Linmai4	+	...	+	...	...	...
SD07	Linyi, Shandong	Linmai4	+	...	+	...	...	...
SD08	Linyi, Shandong	Linmai4	+	...	+	...	...	...
SD09	Linyi, Shandong	Luyuan502	+	...	...	...	+	...
SD10	Qufu, Shandong	Unknown	...	+	+	...	...	...
SD11	Rongcheng, Shandong	Changwu134	+	...	N/A	N/A	N/A	N/A
SD12	Rongcheng, Shandong	LYT3204	+	...	N/A	N/A	N/A	N/A
SD13	Rongcheng, Shandong	Lantian23	+	...	N/A	N/A	N/A	N/A
SD14	Rongcheng, Shandong	Funong50002	+	...	N/A	N/A	N/A	N/A
SD15	Rongcheng, Shandong	Unknown	+	...	N/A	N/A	N/A	N/A
SD16	Rongcheng, Shandong	10CA23	+	...	N/A	N/A	N/A	N/A
SD17	Rongcheng, Shandong	Yannong22	+	...	...	...	+	...
SD18	Rongcheng, Shandong	Unknown	+	...	N/A	N/A	N/A	N/A
SD19	Rongcheng, Shandong	Weidong4	+	...	N/A	N/A	N/A	N/A
SD20	Rongcheng, Shandong	Yumai18	+	...	N/A	N/A	N/A	N/A
SD21	Tai'an, Shandong	Linmai4	+	...	N/A	N/A	N/A	N/A
SD22	Tai'an, Shandong	Linmai4	+	...	...	...	+	...
SD23	Tai'an, Shandong	Linmai4	...	+	...	...	+	...
SD24	Tai'an, Shandong	Linmai4	+	...	...	...	+	...
SD25	Yantai, Shandong	Yannong22	+	...	...	...	+	...
SD26	Yantai, Shandong	Unknown	...	+	...	...	+	...
SD27	Yantai, Shandong	LYT3204	+	...	N/A	N/A	N/A	N/A
SD28	Yantai, Shandong	Yannong22	+	...	...	+	...	...
SD29	Yantai, Shandong	Changwu134	+	...	N/A	N/A	N/A	N/A
SD30	Yantai, Shandong	Unknown	+	...	...	...	+	...
SD31	Yantai, Shandong	Unknown	+	...	...	...	+	...
SD32	Yantai, Shandong	Unknown	+	...	...	...	+	...
ZJ01	Lin'an, Zhejiang	Unknown	+	...	...	...	...	+

^a WYMV and CWMV indicate a single infection in the samples. N/A = not applicable.

(amplifying a band of 430 bp). All 63 samples gave clear positive bands of approximately 800 bp, and 11 samples also yielded a band of 430 bp. Examples of these results are shown in Figure 2B. DNA sequencing and blast results of the PCR products indicated that all of the samples were infected with ribotype I *P. graminis* and 11 samples (from Anhui, Henan, Jiangsu, and Shandong Provinces) were jointly infected with ribotypes I and II (Table 2). No samples contained only ribotype II.

DNA sequence assembly and homology analysis. rDNA sequences including the complete ITS1-5.8S-ITS2 for the 63 ribotype I of *P. graminis* were obtained by assembling the overlapping sequences obtained with primer pairs NS7/ITS.CN and PSP2/ITS4. These were deposited in GenBank with accession numbers KX961536 to KX961598 (Supplementary Table S1). In total, 11 ribotype II sequences obtained with primers Pg.F2 and Pg.R2 and covering most of the ITS1-5.8S-ITS2

region (Fig. 2) were also deposited in GenBank with accession numbers KX961599 to KX961609.

The 63 sequences of ribotype I (ITS1-5.8S-ITS2, 440 bp) are highly similar and have pairwise distances between them varying from 0.000 to 0.0167, while the most similar previously reported isolate Ken5 (ribotype I, accession number HE860055.1) has pairwise distances ranging from 0.0219 to 0.0319 with the Chinese isolates (Supplementary File S1). The ribotype II sequences (partial ITS1-5.8S-ITS2, 409 bp) were identical to each other and to the previously reported isolate SP2 (ribotype II, accession number KF535920.1). All of the sequences used in this analysis are listed in Supplementary File S2.

Association of ribotypes with soilborne wheat viruses. Field investigations for the occurrence of vector- and soilborne viruses can provide indirect evidence for an association between them (Dieryck et al. 2011). In this study, the presence of WYMV and CWMV in

Fig. 3. Phylogenetic analysis of *Polymyxa graminis* based on the internal transcribed spacer (ITS)1-5.8S-ITS2 sequences of 21 isolates from GenBank and 5 from this study (shown in bold) using other plasmodiophorids (*P. betae*, *Spongopora subterranea*, and *Plasmodiophora brassicae*) as an outgroup. Tree branches were bootstrapped with 1,000 replicates using the neighbor-joining method. The scale bar represents a genetic distance of 0.05 for horizontal branch length.

wheat leaves was determined by RT-PCR for 51 wheat samples (the remaining 12 samples were excluded due to insufficient leaves or poor quality of RNA). Most of wheat samples (36 of 51) were singly infected with WYMV (Table 2), which is consistent with the wide distribution of WYMV in China (Han et al. 2000). Both ribotype I (27 of 36 samples) and mixed ribotypes (9 of 36 samples) were associated with WYMV infection (Table 2). Infection by CWMV alone was detected in only one sample (SD28), which is associated with ribotype I. Ten samples were jointly infected with WYMV and CWMV, and the associated *P. graminis* was mostly ribotype I (8 of 10 samples) (Table 2). No virus was detected in the leaves of four samples (HN12, SD01, SD04, and ZJ01), perhaps because the yellow leaf symptoms had been caused by phytotoxicity or freezing injury, and ribotype I of *P. graminis* was detected in the roots of all these samples. There was also no evidence that ribotypes were consistently associated with geographic regions or wheat cultivars (Table 2). Although ribotype II was found less frequently and only in mixed infections, it was found in widely distributed samples, including Anhui (Lu'an, Guoyang), Henan (Puyang, Zhumadian), Jiangsu (Yangzhou), and Shandong (Qufu, Tai'an, Yantai) (Table 2).

Phylogenetic analysis. The isolates of ribotype I determined in this study can clearly be divided into four groups based on their similarity to the isolate Ken5. In all, 14 isolates are identical to each other and have a pairwise distance of 0.0219 to Ken5, 32 isolates have a distance of 0.0244, 13 isolates have a distance of 0.0269, and 4 isolates have a distance of 0.0318 to 0.0319. Isolates AH01, SD01, HB01, and HN04, representing these four groups, were selected for phylogenetic analysis. AH01-2 was selected as the ribotype II isolate. Detailed information regarding all of the isolates used in phylogenetic analysis is listed in Supplementary Table S2 and the sequences are listed in Supplementary File S3. The phylogenetic tree generated by the NJ method is shown in Figure 3. Within *P. graminis*, five clades representing the five previously recognized ribotypes were discriminated with strong bootstrap support (Legrève et al. 2002; Neuhauser et al. 2010; Ward and Adams 1998). Ribotype I, II, and III can be further separated into subclades (a and b) based on the diversity of each clade (Fig. 3). Isolates AH01, SD01, HN04, and HB01 obtained in this study were obviously clustered in Pg-Ib though they differ from the previously reported isolates SP1, Ag, and Ken5. AH01-2 clearly grouped with Pg-IIa (Fig. 3). Within the Pg-Ib subgroup, single-nucleotide polymorphism was found (A to G) in positions 11 and 373 of the ITS1-5.8S-ITS2 region. High diversities between the Chinese isolates and the previously published ones were also identified in positions 114 to 117 and 307 to 351 (Supplementary Fig. S2). Trees generated by maximum-likelihood (Supplementary Fig. S3) and maximum-parsimony (tree not shown) algorithms had very similar topology to that produced by the NJ method, reflecting the reliability of the data.

Discussion

P. graminis has been known to be the vector of soilborne cereal viruses in China since the 1980s (Chen 1993). In this study, 63 wheat samples were collected from sites within the main winter wheat production region of China where soilborne wheat virus disease has been severe, and the ribotypes of *P. graminis* on the plants were identified based on their ITS sequences. The presence of *P. graminis* in the root samples was confirmed by PCR and microscopic observation. All 63 wheat root samples were infected with ribotype I of *P. graminis*, and 11 samples were jointly infected with ribotypes I and II. Previous studies indicated that barley is more frequently infected with ribotype I and wheat with ribotype II in Europe (Legrève et al. 2002; Vaianopoulos et al. 2007; Ward et al. 2005a). However, our results showed that wheat is clearly more associated with ribotype I of *P. graminis* (Table 2) and it's still not clear for the association of *Polymyxa* ribotypes with barley in China. Host specificity of the ribotypes (formae speciales) is probably complex and various factors may affect the host preference of *P. graminis*, such as the host cultivar and environmental elements (Smith et al. 2013; Vaianopoulos et al. 2007). The predominance of *P. graminis* ribotype I in wheat might be due to the particular wheat cultivars grown in China (Table 2) or the nucleotide differences between Chinese isolates and those previously

reported. In temperate regions, it is common to detect both ribotype I and ribotype II together in soil or infected plant roots (Cox et al. 2014; Smith et al. 2013; Vaianopoulos et al. 2007; Ward et al. 2005a; Ziegler et al. 2015). It will be of interest to investigate the ribotype preference of *P. graminis* on barley and different Chinese wheat varieties in the future. In addition, it was unexpected that no plants singly infected with ribotype II were detected in our study, perhaps due to the limited number of wheat samples collected.

Experimental transmission by zoospores has only been convincingly demonstrated for a few soilborne viruses: *Beet necrotic yellow vein virus* (Tamada et al. 1975), BaYMV (but using virus species since reclassified as BaMMV) (Adams et al. 1988), SBWMV (Rao and Brakke 1969), and PCV (Dieryck et al. 2011). For this reason, studies on the transmission of different virus isolates by different ribotypes of *P. graminis* have usually been indirect, relying on associations discovered by studying naturally infected plants. The results reported here are the first attempt to associate the ribotypes of *P. graminis* with CWMV and WYMV, the two viruses responsible for wheat yellow mosaic disease in China (Chen 1993; Diao et al. 1999; Ye et al. 1999). Our results show that the virus-infected samples had ribotype I or a mixture of ribotype I and ribotype II (Table 2). More work needs to be done using *P. graminis* zoospores as inoculum under controlled conditions to investigate the transmission preferences (if any) of the different ribotypes for WYMV and CWMV on wheat in China.

Previous research showed that there was considerable sequence variation among the ribotype I isolates of *P. graminis* in temperate regions (Smith et al. 2013), and our results confirm and expand this known variation. By contrast, the sequences of ribotype II isolates obtained were identical to that of a previously known isolate. Phylogenetic analysis of *P. graminis* ITS1-5.8S-ITS2 sequences indicates that the isolates transmitting soilborne wheat disease in China belong to a distinct subclade of ribotype Ib (Pg-Ib) and perhaps also to ribotype IIa (Pg-IIa) (Fig. 3). It is not yet known whether the Chinese subclade of Pg-Ib has a particular significance in respect to the soilborne viruses they transmit.

Our results provide a starting point for research into *P. graminis* isolates in China. It would be particularly interesting to investigate the ribotypes (formae speciales) associated with soilborne viruses in other cereal crops such as barley, oat, and rice in China.

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