Identification of the Phytoplasma Associated with Wheat Blue Dwarf Disease in China

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ABSTRACT

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Wheat blue dwarf disease (WBD) was first reported in China in the 1960s. It has caused severe losses on several occasions in winter wheat (*Triticum aestivum*) in northwestern China, and the nature of the pathogenic agent has been unknown. Here we have shown that WBD was caused by a 16SrI-C phytoplasma transmitted by *Psammotettix striatus*. This finding was based on molecular diagnostics, insect transmission trials, and host-range determination. Portions of the 16S rRNA and ribosomal protein (rp) genes, *rpsS* (*rps19*), *rplV* (*rpl22*), and *rpsC* (*rps3*), were amplified from DNA samples of WBD-infected wheat seedlings by polymerase chain reaction (PCR) utilizing phytoplasma specific primer pairs. The nucleotide sequences of these amplicons showed high identity to these genes from phytoplasma strains in the aster yellows group (16SrI). Pairwise nucleotide sequence identities of WBD 16S rDNA compared to representative genes of 16SrI group strains ranged from 98.9 to 99.9%, whereas compared to 17 other phytoplasma groups (16SrII to 16SrXVIII), sequence identity ranged from 88.6 to 96.0%. Similarly, the sequence identities of *rps19*, *rpl22*, and *rps3* between WBD and 16SrI group strains varied from 96.6 to 99.7%, but only 60.3 to 65% between WBD and other phytoplasma groups. Phylogenetic analyses were carried out on sequences from 16S rRNA and ribosomal protein genes (*rps19*, *rpl22*, and *rps3*), respectively, and both results indicated that WBD phytoplasma was a member of the 16SrI group and most closely related to subgroup 16SrI-C. WBD-infected *P. striatus* were present in wheat fields with WBD, and phytoplasma infection was verified by PCR detection followed by DNA sequencing. Insect transmission trials confirmed that *P. striatus* transmitted the WBD phytoplasmal agent from infected wheat to healthy wheat seedlings and seven other different plant species in the greenhouse. A survey of various weed species near WBD-infected wheat fields found 10 plant species in seven families to be positive for the presence of WBD phytoplasma.

Wheat blue dwarf disease (WBD) is one of the most important crop diseases affecting winter wheat (*Triticum aestivum* L.) in arid and semiarid areas of northwestern China, including Shaanxi, Shanxi, Gansu, and Ningxia provinces. This disease was first reported in the 1960s and was named wheat red dwarf disease because it exhib-

ited reddening and stunting symptoms in purple-stalked wheat varieties (27,44). Later, it was renamed wheat blue dwarf disease to distinguish it from wheat yellow dwarf disease caused by *Barley yellow dwarf virus* (BYDV) and *Cereal yellow dwarf virus* (CYDV) in China (42–45). Typically, WBD-infected wheat plants were severely stunted and dwarfed, with numerous short tillers developing at the base of the plants. Infected center leaves showed chlorotic mottling and curling or failed to expand. Lower leaves and stems were dark blue-green and gradually became stiff and thickened. In severely infected wheat plants, spikes were either deformed and sterile, or they did not develop at all (28,43). Epidemics of this disease have occurred more than 10 times in Shaanxi Province since 1960. Incidence of the disease was estimated to cover 900,000 ha and caused a total loss of about

50,000 metric tons of wheat yield during an average epidemic year (43).

WBD had been presumed to be a plant virus disease for many years. Subsequently, mycoplasma-like organisms (MLO) were observed in the phloem tissues of WBD-infected wheat (2,43), and in the saliva tissue of its leafhopper vector *Psammotettix striatus* under electron microscopy (39,43). *P. striatus* was the only leafhopper species that was identified and confirmed to transmit WBD in the field (43,44). WBD-infected *P. striatus* can persistently transmit the disease for the rest of its life, but it does not have transovarial transmission (39,43). Despite the progress of research on this disease, it had still not been confirmed that a phytoplasma was the cause of WBD.

Phytoplasmas are wall-less bacteria that are transmitted by phloem-feeding insects in nature, mainly leafhoppers and psyllids. They can inhabit and propagate in both plant phloem tissues and their insect vectors (16,20,33). Phytoplasmas cause complex disease syndromes with symptoms such as stunting, proliferation of auxiliary shoots, formation of sterile-deformed flowers, virescence, and phyllody in several hundred plant species (16). Since phytoplasmas cannot be cultured under axenic conditions, their identities and taxonomic positions were uncertain until recently, when molecular techniques were applied to the study of phytoplasmas. Cloned phytoplasma DNA probes, restriction fragment length polymorphism (RFLP), and sequences of genes, such as the 16S rRNA, ribosomal protein (9), and elongation factor TU (*tuf*) genes, are now widely used to differentiate various groups and subgroups of phytoplasmas (11,20,26,30,31,38). Most of the phytoplasmas that infect economically important crops have been classified phylogenetically using these techniques. Based on phylogenetic analysis of several gene sequences (16S rRNA, *rps/rpl*, and *tuf*), phytoplasmas were recently assigned to a provisional genus, “*Candidatus* (Ca.) Phytoplasma” within the class *Mollicutes*, which included 26 named species (9). More recently, Wei et al. (38) classified

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 appears in color in the online edition.

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phytoplasmas into 28 phylogenetic groups and 50 subgroups according to computer-simulated RFLP analysis of 16S rRNA gene sequences.

Although WBD has been present in China since the early 1960s, most research on WBD focused on symptom descriptions, vector transmission tests, and electron microscopy. The objectives of the present study were to identify the causal agent of WBD and reinvestigate its vector transmission and host range using molecular diagnostic techniques. Our investigation revealed that WBD is caused by a new phytoplasma belonging to the 16SrI-C phytoplasma group with a unique leafhopper vector and a special crop host range.

MATERIALS AND METHODS

Plants, insects, and source of disease materials. Winter wheat (*Triticum aestivum* cv. Xiaoyan 6) was used in this study. Wheat seedlings with or without WBD symptoms were collected from fields in Hancheng, Shaanxi Province, China and transplanted into pots in an insect-proof greenhouse. Leafhoppers (*Psammotettix striatus* and *Tettigella viridis*) and aphids (*Aphis gossypii*, *Macrosiphum avenae*, *Myzus persicae*, *Rhopalosiphum padi*, *R. maidis*, and *Schizaphis graminum*) that were frequently found in wheat fields during the spring wheat-growing season were collected from the fields. *P. striatus*, *T. viridis*, *R. padi*, *M. avenae*, and *S. graminum* were reared on healthy wheat seedlings, *R. maidis* on maize (cv. Cazorla), *M.*

persicae on *Brassica rapa*, and *A. gossypii* on watermelon. All insect species were maintained in growth chambers at 23°C (day) and 18°C (night) with a photoperiod of 14 h of light and 10 h of dark. The only exception was for *P. striatus* and *S. graminum*, which were cultured at a constant temperature of 14°C and a photoperiod of 14 h of light and 10 h of dark. Paulownia witches'-broom phytoplasma (PaWB), which is a representative strain of "Ca. Phytoplasma asteris" 16SrI-D (32), and chinaberry witches'-broom phytoplasma (CWB), an identified strain in the 16SrI-B subgroup (21), were used as a phytoplasma reference strains in this study.

Insect transmission. All insect species collected from the diseased fields were allowed to feed directly on healthy wheat seedlings (5 insects/seedling and 15 seedlings/trial) for 1 week to test for transmission of the disease. Pathogen-free, laboratory colonies of *P. striatus* and *T. viridis* were obtained originally from egg-hatched offspring on healthy wheat plants and were transferred on healthy wheat seedlings for several generations. Pathogen-free, as well as laboratory colonies, of all six aphid species were initiated from single viviparous aptera collected from healthy hosts. These hosts were wheat for *R. padi*, *S. graminum*, and *M. avenae*, maize for *R. maidis*, *Brassica rapa* for *M. persicae*, and watermelon for *A. gossypii*. For transmission trials, healthy insect larvae were fed for 2 or 3 days on WBD-symptomatic wheat plants for acquisition of the causal

agent, and then transferred to healthy wheat seedlings (5 insects/seedling, 15 seedlings/trial) for 7 days before insect eradication with ethyl acetate. The transmission experiments were repeated three times independently in insect-proof greenhouses.

Investigation of host ranges and the WBD causal agent in natural weeds. Healthy *P. striatus* larvae were fed for 6 to 7 days on severely infected WBD wheat for acquisition of the causal agent, and then used to inoculate 16 species of plants from eight plant families, including wheat (Table 1). Each plant was inoculated with seven WBD-infected *P. striatus* larvae for 7 days. Disease symptoms were observed over a period of 40 to 45 days, and a polymerase chain reaction (PCR) assay was used to detect the 16S rDNA of the WBD pathogenic agent.

Varieties of weeds with dwarfing and other abnormal growth symptoms were collected from areas close to WBD symptomatic wheat fields, and DNA was extracted, amplified by PCR, and analyzed by RFLP as described below for detection of possible phytoplasma infection. The weed species in the present investigation included goatgrass (*Aegilops squarrosa*), redroot amaranth (*Amaranthus retroflexus*), gmenlin's wormwood (*Artemisia gmelinii*), wild oat (*Avena fatua*), Japanese false bindweed (*Calystegia hederacea*), common cephalanoplos (*Cephalanoplos segetum*), aristate goosefoot (*Chenopodium aristatum*), oakleaf goosefoot (*Chenopodium glaucum*), flaxweed tansymustard (*Descurainia sophia*), goosegrass (*Eleusine indica*), stink grass (*Eragrostis cilianensis*), wormseed mustard (*Erysimum cheiranthoides*), madwoman's milk (*Euphorbia helioscopia*), tender catchweed bedstraw (*Gatium aparine* var. *tenerum*), corn gromwell (*Lithospermum arvense*), common purslane (*Portulaca oleracea*), pale pigeongrass (*Setaria glauca*), common chickweed (*Stellaria media*), white clover (*Trifolium repens*), veronica (*Veronica didyma*), volunteer wheat seedlings (*Triticum aestivum*), and maize (*Zea mays*).

Nucleic acid extraction and PCR amplification. Total DNA was extracted from plants using the standard cetyltrimethyl ammonium bromide (CTAB) method (14). Insect samples were stored at 4°C for up to 2 weeks, or dried with anhydrous calcium chloride, until processed for total DNA extraction. Following a DNA extraction procedure previously described for insects (3), samples were ground individually in Eppendorf tubes in 500 µl of preheated CTAB extraction buffer (2% wt/vol CTAB, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 100 mM Tris-HCl, pH 8.0) with 0.2% β-mercaptoethanol, followed by incubation at 60°C for 30 min, and extracted with chloroform-isoamyl alcohol (24:1). The aqueous DNA layer was precipitated with

Table 1. Occurrence of symptoms and phytoplasma infection in plant species inoculated by wheat blue dwarf disease (WBD)-infected *Psammotettix striatus* leafhoppers

Species tested	No. of symptomatic plants ^a	Detection of phytoplasmas by PCR ^b	Symptoms ^c
Apocynaceae			
<i>Catharanthus roseus</i>	13/35	4/5; +	St, Pr, V, Ph
Brassicaceae			
<i>Brassica oleracea</i> var. <i>capitata</i>	6/6	4/5; +	St, Y
<i>Brassica rapa</i> var. <i>akana</i>	0/4	0/4; –	
Compositae			
<i>Callistephus chinensis</i>	3/4	3/3; +	St, Y
Cucurbitaceae			
<i>Cucumis melo</i>	0/7	0/4; –	
Gramineae			
<i>Panicum miliaceum</i>	0/20	0/5; –	
<i>Triticum aestivum</i>	44/44	4/5; +	St, Y, Pr
<i>Secale cereale</i>	3/4	3/4; +	St, Y, Pr
Leguminosae			
<i>Phaseolus vulgaris</i>	0/5	0/5; –	
<i>Vigna unguiculata</i>	0/12	0/5; –	
<i>Medicago sativa</i>	15/20	3/5; +	St, Y
<i>Trifolium repens</i>	5/10	3/5; +	St, Y
Solanaceae			
<i>Capsicum annuum</i>	0/19	0/4; –	
<i>Lycopersicon esculentum</i>	15/31	3/5; +	St, Y, Ph
Umbelliferae			
<i>Apium graveolens</i> var. <i>dulce</i>	0/9	0/4; –	
<i>Daucus carota</i> subsp. <i>sativus</i>	0/30	0/4; –	

^a Total number of plants with symptoms/total number of plants inoculated.

^b Regular polymerase chain reaction (PCR) with primer pair R16mF2/R16mR1; total number of PCR-positive samples/total number of PCR-tested samples; +, PCR positive; –, PCR negative.

^c Symptoms: St, stunt; Y, yellows; Pr, proliferation; V, virescence; Ph, phyllody.

isopropanol, washed with 70% ethanol, and resuspended in 30 μ l of deionized water.

PCR reactions contained 50 to 100 ng of total DNA template, 2 mM $MgCl_2$, 75 mM Tris-HCl (pH 8.8, 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% (vol/vol) Tween 20, 200 μ M of each dNTP, 0.5 μ M of each primer, and 1.5 U *Taq* DNA polymerase in a total volume of 50 μ l. Regular PCR or nested PCR was used for phytoplasma 16S rRNA gene amplification. For regular PCR to detect phytoplasmas in insect materials, primer pair R16mF2/R16mR1 was used (10). For nested PCR amplifications of the phytoplasma 16S rRNA gene, two primer sets, R16mF1/R16mR1 and R16F2n/R16R2, were used (10). PCR amplification was cycled 35 times with the following parameters: 95°C for 1 min, 55°C for 0.5 min, and 72°C for 1 min in a Thermocycler (PTC-200, Cepheid, Hercules, CA, USA). For nested PCR, 1 μ l of diluted (1:50) PCR product from R16mF1/R16mR1 primer pair was used as the template. Primer pair rpF1/rpR1 (22,23) was used for PCR amplification of the phytoplasma ribosomal protein genes (*rps19*, *rpl22*, and *rps3*).

DNA sequencing and phylogenetic analysis. A 1.4-kb PCR product using primer pair R16mF2/R16mR1 and a 1.2-kb PCR fragment utilizing primer pair rpF1/rpR1 were separately amplified from DNA samples extracted from WBD symptomatic plants and WBD-infected leafhoppers (*P. striatus*). The amplicons were

purified and cloned. DNA sequence analysis was performed on both strands of the clones using an ABI Prism DNA sequencer (model 3730, ABI, Foster City, CA, USA). The nucleotide sequence data were analyzed by the Lasergene program (version 7.1.0, DNASTAR, Madison, WI, USA). The NCBI BLASTn program (version 2.2.19) was used to search the NCBI nucleotide collection (nr/nt) database and align the query nucleotide sequences with other phytoplasma 16S rRNA and *rps/rpl* gene sequences (1,25,41). A phylogenetic tree was constructed by the neighbor-joining method using MEGA software version 4 (29,34). The 16S rRNA gene sequence of *Escherichia coli* (GenBank accession no. V00348) was used as an outgroup to root a phylogenetic tree. Bootstrapping was performed 1,000 times to estimate the confidence values of the branches in the phylogenetic tree.

RFLP analysis of PCR products. A 1.2-kb 16S rDNA fragment was amplified by nested PCR from 10 naturally infected plant species, WBD-inoculated wheat, and *Catharanthus roseus*, as well as PaWB-infected *Paulownia fortunei* and CWB-infected *Melia azedarach*. After purification, PCR products were digested separately by four restriction endonucleases, *Sau3AI*, *RsaI*, *TaqI*, and *HhaI* (18), according to the manufacturer's instructions (Fermentas, China). The digested PCR products were then separated by electrophoresis in 2% agarose gels or 8% PAGE gels, and stained with ethidium bromide.

RFLP profiles were visualized and photographed over a UV transilluminator.

RESULTS

WBD symptoms in winter wheat.

Symptoms of the WBD disease appeared in winter wheat seedlings as early as the beginning of March in northwestern China (Shaanxi, Shanxi, and Gansu provinces). Initial symptoms included chlorotic mottling or curling of center leaves and slight dwarfing of the seedlings. Severe dwarfing with numerous short tillers developed at the base of the plants. Dark green or blue-green streaks on leaves along with yellowing at the tips usually appeared in late March or early April. Yellowing and streaking of leaves faded gradually from April to May, but severely diseased plants remained yellow and stunted (Fig. 1A and B). Other symptoms such as rotten rhizomes, root death, and stem distortion could also be observed in some susceptible wheat varieties, such as Xiaoyan 6. Most affected plants remained severely dwarfed and produced sterile flowers or heads without grains. An obvious symptom of WBD was that the most diseased plants were less than one-third to one-half the height of healthy wheat and had fewer tillers. All leaves gradually became yellow and some eventually died.

WBD transmission by *P. striatus*. To determine the WBD transmission vector, we tested leafhoppers *P. striatus* and *T. viridis*, and aphids *A. gossypii*, *M. avenae*, *M. persicae*, *R. padi*, *R. maidis*, and *S. graminum*. Among all insects tested, only *P. striatus* (Fig. 1C) transmitted the WBD agent from naturally infected wheat to healthy wheat seedlings. All 15 wheat seedlings showed typical WBD symptoms, such as dark blue streaks on leaves 2 weeks after infection and yellowish dwarfing and stunting 4 to 5 weeks after inoculation with *P. striatus* previously fed on WBD symptomatic wheat plants (Fig. 1D). Furthermore, about 70% of the WBD-infected *P. striatus* collected from diseased fields transmitted the disease agent to healthy wheat seedlings without an acquisition feeding procedure (data not shown).

Fig. 1. Wheat blue dwarf disease (WBD) and its leafhopper vector. **A**, A severely WBD-infected wheat field in Hancheng, Shaanxi Province, China. **B**, Wheat seedlings with yellow leaf tip and dwarfing symptoms after WBD infection. **C**, Leafhopper *Psammotettix striatus*, the only identified leafhopper species that transmits WBD. **D**, Wheat seedlings experimentally inoculated by WBD-infected (right) and non-WBD-infected *P. striatus* leafhoppers (left).

Fig. 2. Polymerase chain reaction amplification of 16S rRNA and *rps/rpl* gene fragments of wheat blue dwarf disease phytoplasma. **A**, 16S rRNA gene fragment. **B**, *rp19*, *rpl22*, and *rps3* gene fragment. M: DNA Marker DL2000 (Takara, Japan).

In contrast, the other insect species tested, *T. viridis*, *R. padi*, *S. graminum*, *M. avenae*, *R. maidis*, *M. persicae*, and *A. gossypii*, failed to transmit WBD, either directly or after an acquisition feeding procedure under controlled conditions (data not shown).

Identification of the WBD phytoplasma by sequence analysis of the 16S rDNA and ribosomal protein genes. To detect and identify phytoplasmas in the WBD-infected wheat samples and WBD-infected leafhoppers, PCR assays were conducted utilizing phytoplasma-specific primers targeting the 16S rRNA and ribosomal protein gene sequences (10,22). With primer pair R16mF2/R16mR1 targeted to the 16S rRNA gene, a PCR frag-

ment with the expected size of 1.4 kb was amplified from three DNA samples extracted from three different WBD-infected wheat samples that were collected from wheat fields in Hancheng, Shaanxi Province (Fig. 2A). No PCR amplicons were produced from control samples of healthy wheat plants (Fig. 2A). The same 1.4-kb DNA fragment was also amplified from 7 of 13 wheat seedlings that were inoculated with WBD-infected leafhoppers. These WBD-infected leafhoppers had been fed for 2 or 3 days on WBD symptomatic wheat plants for acquisition of the phytoplasma pathogen in the greenhouse, and the wheat seedlings subsequently displayed WBD symptoms (data not shown). These results indicated an intimate asso-

ciation between the phytoplasma and WBD disease. The 16S rDNA fragment was also amplified from 2 of 16 DNA samples extracted from individual *P. striatus* leafhoppers that were collected from wheat fields but not from the pathogen-free leafhopper colonies reared in the greenhouse (Fig. 2A).

For analysis of the ribosomal protein genes, a DNA fragment of approximately 1.2 kb, containing a partial S19 gene (*rps19*) and complete L22 (*rpl22*) and S3 (*rps3*) genes, was amplified by PCR with primer pair rpF1/rpR1 (22,23). This 1.2-kb amplicon was observed in the same DNA samples from the WBD-infected plants and insects as stated as above, including the field-infected wheat and field-collected

Table 2. 16S rDNA and *rps/rpl* gene sequence identities between the wheat blue dwarf disease (WBD) phytoplasma and related phytoplasmas

Group and subgroup	Acronyms	Disease caused	16S rDNA		<i>rps19</i> , <i>rpl22</i> and <i>rps3</i> gene	
			GenBank accession no.	Nt identity (%) to WBD (GenBank DQ078304)	GenBank accession no.	Nt identity (%) to WBD (GenBank EF417591)
16SrI-A	TBB	Tomato big bug	L33760	99.2		
16SrI-A	HYDP	Hydrangea phyllody			AY264868	96.6
16SrI-A	OatP	Oat proliferation	AF453416	99.2		
16SrI-B	HP	Hydrangea phyllody	AY265207	99.4		
16SrI-B	AY	Aster yellows	AY180952	99.4		
16SrI-B	MBS	Maize bushy stunt	AY265208	99.4	AY264858	96.9
16SrI-B	BaDef	Barley deformation	AY734453	99.4	AY735448	97.3
16SrI-B	SAY	Western aster yellows SAY	M86340	99.2		
16SrI-C	CPhA	Clover phyllody strain rrnA	AF222065	99.9	AY264862	99.6
16SrI-C	CPhB	Clover phyllody strain rrnB	AF222066	99.7		
16SrI-C	KVE	Clover phyllody strain KVE	AY265217	99.7	AY264861	99.5
16SrI-C	KVG	Clover phyllody strain KVG	AY265218	99.7	AY264860	99.7
16SrI-D	PaWB	Paulownia witches'-broom	AY265206	99.2	AY902910	96.8
16SrI-D	PaWB	Paulownia witches'-broom			AY662673	96.8
16SrI-E	BBS	Blueberry stunt	AY265220	99.2	AY264863	96.9
16SrI-F	ACLR-AY	Apricot chlorotic leaf roll	AY265211	98.9		
16SrI-B	CWB-HN	Chinaberry witches'-broom			EU348781	96.9
16SrI-B	CWB-WSh	Chinaberry witches'-broom			DQ648460	96.7
16SrI-B	CWB-GZh	Chinaberry witches'-broom			DQ648461	96.8
16SrI	MDNY	Mulberry dwarf			FJ154858	97.3
16SrII	PEY	Picris echioides phyllody			EF186802	63.9
16SrII	PnWB	Peanut witches'-broom	L33765	88.6		
16SrIII	CX	Peach X-disease			EF186813	60.9
16SrIII	SP	Spirea stunt			EF186806	61.2
16SrIII	MWI	Milkweed yellows			EF186808	63.4
16SrIII	WX	Western X phytoplasma	L04682	90.0		
16SrIV	CLY	Coconut lethal yellowing	U18747	90.1		
16SrV	EY	Elm yellow	AF189214	90.2		
16SrVI	CPS	Catharanthus phyllody			EF183494	62.4
16SrVI	PWB	Potato witches'-broom			EF183487	63
16SrVI	PWB	Potato witches'-broom			AY197683	63
16SrVI	CP	Clover proliferation			EF183486	63
16SrVI	CP	Clover proliferation	AY390261	90.3		
16SrVII	AshY	Ash yellows	X68339	89.6	EF183492	61.9
16SrVIII	LfWB	Loofah witches'-broom	AF353090	90.8		
16SrIX	AlmWB-A112	Almond witches'-broom			EF186803	65
16SrIX	KAP	Knautia arvensis			EF186801	63.8
16SrIX	GLLhon	Gliricidia little leaf			EF186800	62.4
16SrIX	PPWB	Pigeon pea witches'-broom	AF248957	88.7	EF193383	64.8
16SrX	AP	Apple proliferation	X68375	91.6		
16SrXI	RYD	Rice yellow dwarf	D12581	89.4		
16SrXII	STOL	Stolbur	X76427	96.0		
16SrXIII	GRI	Goldenrod yellows			EF186810	61.3
16SrXIII	WWB	Walnut witches'-broom			EF186812	60.3
16SrXIII	MPV	Mexican periwinkle virescence	AF248960	96.0		
16SrXIV	BGWL	Bermuda grass white leaf	AJ550984	90.0		
16SrXV	HWB	Hibiscus witches'-broom	AF147708	88.9		
16SrXVI	ScYL	Sugarcane yellow leaf	AY725228	93.5		
16SrXVII	PBT	Papaya bunchy top	AY725234	92.3		
16SrXVIII	PPT	Nebraska potato purple top	DQ174122	95.2		

WBD-infected *P. striatus*, but not from the corresponding controls (healthy wheat plants and pathogen-free *P. striatus* colonies) (Fig. 2B).

To confirm that the PCR fragments described above were derived from phytoplasmas, four of the 1.4-kb (16S rDNA) and four of the 1.2-kb (*rps19/rpl22/rps3*) PCR fragments from two field-infected wheat samples and two WBD-infected *P. striatus*, respectively, were cloned and sequenced. Nucleotide sequence analysis revealed that the entire 1,432 nucleotides of the 1.4-kb fragments from field WBD wheat samples and WBD-infected *P. striatus* with primer pair R16mF2/R16mR1 were identical in sequence (GenBank ac-

cession no. DQ078304). A BLASTn search showed that the WBD 1,432-bp fragment shared highest nucleotide identities with 16S rDNAs from aster yellows group (16SrI) phytoplasmas. The pairwise identities of the 16S rDNA genes between WBD and 14 selected strains of the 16SrI group ranged from 98.9 to 99.9%, whereas nucleotide identities between WBD and 17 other phytoplasma groups (16SrII to 16SrXVIII) ranged from 88.6 to 96.0% (Table 2).

The 1.2-kb PCR fragments from field-collected WBD wheat samples and WBD-infected *P. striatus* with primer pair rpF1/rpR1 consisted of the same 1,240 nucleotides (GenBank accession no.

EF417591). Sequence analysis showed that pairwise nucleotide identities ranged from 96.6 to 99.7% between WBD and nine selected strains in the aster yellows group (16SrI), but only from 60.3 to 65% between WBD and other phytoplasma groups (Table 2). Therefore, both 16S rDNA and *rps/rpl* gene sequence comparisons suggest that the WBD phytoplasma is a member of the aster yellows group (16SrI) (18,19,26,38).

In the phytoplasma 16SrI group, the WBD 16S rRNA gene nucleotide sequence has 99.7 to 99.9% identity to four clover phyllody (CPh) phytoplasmas, the representative strains of the 16SrI-C subgroup (Table 2). The nucleotide identities within

Fig. 3. Phylogenetic tree constructed by parsimony analysis of 16S rDNA sequences from wheat blue dwarf disease (GenBank accession no. DQ078304) and 28 other phytoplasmas using the neighbor-joining method. Bootstrap values (expressed as percentages of 1,000 replications) are shown, and branch lengths are proportional to the number of inferred character state transformations. Bar = substitutions per base. The 16S rRNA sequence of *Escherichia coli* (GenBank accession no. V00348) was used as an outgroup. Abbreviations of phytoplasmas, with GenBank accession numbers, are defined as follows: barley deformation (BaDef, AY734453), western aster yellows SAY (SAY, M86340), Paulownia witches'-broom (PaWB, AY265206), hydrangea phyllody (HP, AY265207), maize bushy stunt (MBS, AY265208), aster yellows (AY, AY180952), blueberry stunt (BBS, AY265220), apricot chlorotic leaf roll (ACLR-AY, AY265211), tomato big bug (TBB, L33760), oat proliferation (OatP, AF453416), wheat blue dwarf (WBD, DQ078304), clover phyllody strain rnaA (CPhA, AF222065), clover phyllody strain KVG (KVG, AY265218), clover phyllody strain rnaB (CPhB, AF222066), clover phyllody strain KVE (KVE, AY265217), Mexican periwinkle virescence (MPV, AF248960), Nebraska potato purple top (PPT, DQ174122), stolbur (STOL, X76427), sugarcane yellow leaf (ScYL, AY725228), papaya bunchy top (PBT, AY725234), apple proliferation (AP, X68375), western X phytoplasma (WX, L04682), Bermuda grass white leaf (BGWL, AJ550984), pigeon pea witches'-broom (PPWB, AF248957), coconut lethal yellowing (CLY, U18747), loofah witches'-broom (LfWB, AF353090), elm yellow (EY, AF189214), clover proliferation (CP, AY390261), ash yellows (AshY, X68339).

Fig. 4. Phylogenetic tree constructed by parsimony analysis of *rpl19*, *rpl22*, and *rps3* gene sequences from wheat blue dwarf disease (GenBank accession no. EF417591) and 28 other phytoplasmas using the neighbor-joining method. Bootstrap values (expressed as percentages of 1,000 replications) are shown, and branch lengths are proportional to the number of inferred character state transformations. Bar = substitutions per base. Abbreviations of phytoplasmas, with GenBank accession numbers, are defined as follows: potato witches'-broom (PWB, EF183487 and AY197683), clover proliferation (CP, EF183486), mulberry dwarf (MDNY, FJ154858), Catharanthus phyllody (CPS, EF183494), ash yellows (AshY, EF183492), almond witches'-broom (AlmWB-A112, EF186803), Gliricidia little leaf (GLLhon, EF186800), pigeon pea witches'-broom (PPWB, EF193383), Picris echioides phyllody (PEY, EF186802), Knautia arvensis (KAP, EF186801), goldenrod yellows (GRI, EF186810), milkweed yellows (MWI, EF186808), spirea stunt (SP, EF186806), walnut witches'-broom (WWB, EF186812), peach X-disease (CX, EF186813), clover phyllody strain KVG (KVG, AY264860), clover phyllody strain KVE (KVE, AY264861), clover phyllody strain rnaA (CPhA, AY264862), wheat blue dwarf (WBD, EF417591), hydrangea phyllody (HYDP, AY264868), blueberry stunt (BBS, AY264863), barley deformation (BaDef, AY735448), Paulownia witches'-broom (PaWB, AY902910 and AY662673), maize bushy stunt (MBS, AY264858), chinaberry witches'-broom (CWB, EU348781, DQ648460, and DQ648461).

the 16SrI-C isolate phytoplasmas, including CPhA, CPhB, CPh-KVG, and CPh-KVE, are also between 99.7 to 99.9%. The homology of the WBD phytoplasma to strains from the other 16SrI subgroups was $\leq 99.4\%$ (Table 2). The WBD *rps19*, *rpl22*, and *rps3* gene nucleotide sequences had 99.5 to 99.7% identity to three clover phyllody (CPh) phytoplasmas, whereas the homology of WBD phytoplasma to isolates in other 16SrI subgroups was less than 97.3% (Table 2). These results indicate that the WBD phytoplasma is most closely related to the 16SrI-C subclade. Phylogenetic analysis of 16S rRNA genes and *rps19*, *rpl22*, and *rps3* genes was also performed, using nucleotide sequence alignments of the WBD phytoplasma with phytoplasma strains from the other 18 phylogenetic groups. As shown in Figures 3 and 4, the phylogenetic trees revealed that the WBD phytoplasma grouped with strains from the 16SrI group in one monographic cluster, distinct from strains of other phytoplasma groups. In the 16SrI cluster, the WBD phytoplasma was localized in the subcluster 16SrI-C, suggesting that it is evolutionarily closest to the four clover phyllody phytoplasma strains (CPhA, CPh-KVG, CPhB, and CPh-KVE).

Host range of the WBD phytoplasma.

We inoculated 16 plant species from eight families through transmission trials of WBD-infected *P. striatus* under controlled conditions in a greenhouse. Symptom development was observed over a period of 40 to 45 days post-WBD inoculation. Three to five plants of each species were tested to confirm WBD phytoplasma infections by PCR assays of the 16S rRNA gene using primer pair R16mF2/R16mR1 (10). The results are summarized in Table 1. We observed disease symptoms and positive PCR amplification of the phytoplasmal 16S rRNA gene in eight species of seven families, including *Callistephus chinensis*, *Catharanthus roseus*, *Brassica oleracea* var. *capitata*, *Lycopersicon esculentum*, *Medicago sativa*, *Secale cereale*, *Trifolium repens*, and *Triticum aestivum* (Table 1). The main symptoms of infected plants were stunting and yellowing in *C. chinensis*, *B. oleracea* var. *capitata*, *M. sativa*, and *T. repens*; stunting, yellowing, and proliferation of shoots in *L. esculentum*, *S. cereale*, and *T. aestivum*; and stunting, yellowing, proliferation, and virescence in *C. roseus* (Table 1). However, no WBD phytoplasmas were detected in the other eight plant species tested, including *Bras-*

sica rapa var. *akana*, *Capsicum annuum*, *Cucumis melo*, *Panicum miliaceum*, *Phaseolus vulgaris*, *Vigna unguiculata*, *Apium graveolens* var. *dulce*, and *Daucus carota* subsp. *sativus* (Table 1).

We also surveyed various weed species near WBD-infected wheat fields for the presence of the WBD phytoplasma by directed PCR or nested PCR, and subsequent PCR-RFLP analysis. In total, 22 weed species from 13 families were subjected to molecular diagnosis of the WBD phytoplasma (Table 3). These selected weed plants showed abnormal growth symptoms, including shortened internodes, proliferation of shoots, stunting, leaf distortion, virescence, and other typical symptoms that would usually be caused by phytoplasmas. Results by direct PCR using primer pair R16mF2/R16mR1, or a nested PCR using a second primer pair R16F2n/R16R2 are summarized in Table 3. Ten plant species in seven families were positive for the presence of the WBD phytoplasma, including redroot amaranth (*Amaranthus retroflexus*), corn gromwell (*Lithospermum arvense*), flaxweed tansymustard (*Descurainia sophia*), wormseed mustard (*Erysimum cheiranthoides*), goatgrass (*Aegilops squarrosa*), wild oat (*Avena fatua*), stink grass (*Eragrostis cilianensis*), volunteer wheat seedlings (*Triticum aestivum*), white clover (*Trifolium repens*), and veronica (*Veronica didyma*) (Table 3). *Lithospermum arvense* and *Erysimum cheiranthoides* were negative for the presence of phytoplasmas by directed PCR, but positive by nested PCR analysis, suggesting they contain lower levels of phytoplasmas than the other eight positive weed species. Although displaying abnormal growth symptoms, 12 other weed species were found to be negative for phytoplasmas by PCR, indicating the disease symptoms might be caused by other pathogenic agents and not phytoplasmas (Table 3).

To determine if phytoplasmas in the 10 PCR-positive weed species were the WBD phytoplasma, we performed RFLP analysis following nested PCR amplification of the 1.2-kb 16S rDNA fragments. Restriction endonuclease digestion with *Sau3AI*, *RsaI*, and *TaqI*, which distinguish 16SrI phytoplasmas from those belonging to other phylogenetic groups (18), produced RFLP patterns that were the same among all samples with the fragments predicated from the WBD and PaWB phytoplasmic 16S rDNA sequences (Fig. 5). The results suggested that the 10 weed species were naturally infected by a 16SrI group phytoplasma. Apart from being identical to PaWB, the RFLP profiles obtained with *Sau3AI*, *RsaI*, and *TaqI* are also the same as those reported previously for aster yellows (AY) phytoplasma (17,18), providing additional evidence that WBD phytoplasma is closely related to the phytoplasma strains in the 16SrI group. Enzyme

Table 3. Polymerase chain reaction (PCR) detection of the wheat blue dwarf disease (WBD) phytoplasma in weed species growing in or adjacent to WBD-infected wheat fields

Weeds	PCR ^a	
	Directed-PCR	Nested-PCR
Amaranthaceae		
<i>Amaranthus retroflexus</i>	4/5; +	5/5; +
Apocynaceae		
<i>Calystegia hederacea</i>	0/4; –	0/4; –
Boraginaceae		
<i>Lithospermum arvense</i>	0/3; –	2/3; +
Brassicaceae		
<i>Erysimum cheiranthoides</i>	0/4; –	3/4; +
Caryophyllaceae		
<i>Stellaria media</i>	0/5; –	0/5; –
Chenopodiaceae		
<i>Chenopodium aristatum</i>	0/4; –	0/4; –
<i>Chenopodium glaucum</i>	0/4; –	0/4; –
Compositae		
<i>Artemisia gmelinii</i>	0/4; –	0/4; –
<i>Cephatanoplos segetum</i>	0/4; –	0/4; –
<i>Descurainia sophia</i>	3/5; +	3/5; +
Euphorbiaceae		
<i>Euphorbia helioscopia</i>	0/3; –	0/3; –
Gramineae		
<i>Aegilops squarrosa</i>	2/3; +	3/3; +
<i>Avena fatua</i>	3/4; +	3/4; +
<i>Eleusine indica</i>	0/3; –	0/3; –
<i>Eragrostis cilianensis</i>	3/4; +	4/4; +
<i>Setaria glauca</i>	0/4; –	0/4; –
<i>Triticum aestivum</i>	1/3; +	3/3; +
<i>Zea mays</i>	0/3; –	0/3; –
Leguminosae		
<i>Trifolium repens</i>	3/5; +	4/5; +
Portulacaceae		
<i>Portulaca oleracea</i>	0/4; –	0/4; –
Rubhceae		
<i>Gatium aparine</i> var. <i>tenerum</i>	0/5; –	0/5; –
Scrophulariaceae		
<i>Veronica didyma</i>	2/4; +	3/4; +

^a Total number of PCR-positive samples/total number of PCR-tested samples; +, PCR positive; –, PCR negative.

HhaI can be used to distinguish subgroup 16SrI-C phytoplasmas from other AY phytoplasmas (18). *HhaI* digestion of 16S rDNA fragments amplified from the 10 field-collected weed samples all revealed a similar pattern to those from WBD-infected *C. roseus*, but different from chinaberry witches'-broom (CWB, 16SrI-B) (21) and PaWB (16SrI-D) (18,32) (Fig. 5). The RFLP profile obtained with *HhaI* was also the same as those reported previously for 16SrI-C phytoplasmas (18). These results indicate that the weeds collected from the WBD-infected wheat field areas were most likely infected with the WBD phytoplasma.

DISCUSSION

In the present study, we have provided evidence that WBD, which frequently affects winter wheat in northwestern areas of China, is caused by a phytoplasmal agent transmitted by the leafhopper *Psammotettix striatus*. Phylogenetic analy-

ses based on both 16S rRNA and ribosomal protein gene sequences revealed that WBD phytoplasma is a member of the aster yellows (AY) group (16SrI) and most closely related to CPh phytoplasmas in subgroup 16SrI-C. The AY group (16SrI) is the most diverse and widespread phytoplasma group and causes more than 100 plant diseases, making this disease economically important throughout the world. Over 100 isolates of AY phytoplasmas have been examined and classified into 15 subgroups based on RFLP analysis of their 16S rDNA sequences (9,19,26). The phytoplasmas of subgroup 16SrI-C have been reported to infect many herbaceous plant species, including clovers, buttercup, common meadow grass, tall fescue, and strawberry; as well as woody plant species, including grapevines, apple trees, olive, lilies, eucalyptus, and European hackberry (6,7,14,37). However, there are no previous reports of subgroup 16SrI-C strains infecting small grain crops, including

wheat. The 16SrI-C phytoplasmas employ a number of insect vectors including *Aphrodes bicincta*, *Euscelidius variegatus*, *Euscelis lineolatus*, *Macrosteles cristata*, *Macrosteles viridigriseus*, and *Paraphlepsius irroratus* (www.ipwgn.net.org) (8), but *P. striatus* has not previously been shown to transmit any phytoplasmas from this subgroup. It was previously reported in China (39,43) that *P. striatus* was an essential leafhopper vector for transmission of WBD, but until our present study it had not been proven that this disease was caused by a phytoplasma.

Several phytoplasmas that affect cereal crops have been investigated previously, including Western severe aster yellows (SAY) (15,20), Eastern aster yellows (NAY) (20), maize bushy stunt (MBS) (12,18), oat proliferation (OatP) (13), oat yellows (OatY) (36), barley deformation (BaDef) (36), and *Triticosecale* stunt (TrSt) (36). Interestingly, these all belong to the AY group (16SrI). SAY and NAY

Fig. 5. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA sequences amplified by nested polymerase chain reaction (PCR) with primer pairs R16mF2/R16mR1 followed by R16F2/R16R2 from 10 naturally infected plant species. PCR products were digested with *Sau3AI*, *RsaI*, *TaqI*, and *HhaI*. Digestion products were then separated by electrophoresis in 2% agarose gels (for *Sau3AI*, *RsaI*, and *TaqI*) or 8% PAGE gel (for *HhaI*). Lanes 1 and 18, DNA marker, marker 1: DNA 100-bp ladder marker (Takara, Japan), marker 2: DNA Marker DL2000 (Takara, Japan), marker 3: DNA Marker I (Biotek, China); 2 (for *Sau3AI*, *RsaI*, and *TaqI*), PaWB (Paulownia witches'-broom) infected Royal Paulownia (*Paulownia fortunei*); 2 (for *HhaI*), CWB (chinaberry witches'-broom phytoplasma) infected *Melia azedarach*; 3 and 16, wheat inoculated with wheat blue dwarf disease (WBD) in the laboratory; 4 and 15, *Catharanthus roseus* inoculated with WBD in the laboratory; 5, goatgrass (*Aegilops squarrosa*); 6, volunteer wheat seedlings (*Triticum aestivum*); 7, candygrass (*Eragrostis cilianensis*); 8, veronica (*Veronica didyma*); 9, flixweed tansymustard (*Descurainia sophia*); 10, clover (*Trifolium repens*); 11, amaranth pigweed (*Amaranthus retroflexus*); 12, wild oat (*Avena fatua*); 13, wormseed mustard (*Erysimum cheiranthoides*); 14, corn gromwell (*Lithospermum arvense*); 17, PaWB (Paulownia witches'-broom) infected Royal Paulownia (*Paulownia fortunei*).

were reported to cause severe yield losses in wheat and barley in Canada (7). MAS affects corn varieties in North America and Brazil (5). OatP and OatY are phytoplasma-associated diseases occurring in oat crops in Lithuania (13,36). BaDef and TrSt are two phytoplasma diseases affecting barley and *Triticosecale* (*Triticum* × *Secale*) in Lithuania (35,36). Based on RFLP and phylogenetic analyses of 16S rRNA and *rps19*, *rpl22*, and *rps3* gene sequences, NAY and OatP have been identified as 16SrI-A phytoplasmas, while SAY, MAS, OatY, BaDef, and TrSt are classified in the 16SrI-B subgroup (13,35,36). We compared available 16S rRNA and *rps19*, *rpl22*, and *rps3* gene sequences between WBD and these phytoplasmas and found that the pairwise identity of 16S rDNA between WBD and the other cereal phytoplasmas were 99.2 to 99.4%, which is less than the 99.7 to 99.9% identity of WBD to the 16SrI-C subgroup strains (Table 2). A similar relationship was obtained from comparison of the *rps19*, *rpl22*, and *rps3* genes. Nucleotide sequence identities of the *rps19*, *rpl22*, and *rps3* genes were 96.9 to 97.3% between WBD and the other cereal phytoplasmas, which was significantly lower than the identity of 99.5 to 99.7% between WBD and 16SrI-C phytoplasmas (Table 2). Phylogenetic trees based on these sequence comparisons also indicated that WBD is phylogenetically closer to the 16SrI-C subgroup than to any other 16SrI subgroups, which include the other cereal phytoplasmas (Figs. 3 and 4).

P. striatus was previously reported to be infected by the grapevine Stolberg (STOL) 16SrXII phytoplasma (4). However, except for the WBD phytoplasma, *P. striatus* was not reported to transmit other cereal crop-associated phytoplasmas. *P. striatus* is a predominant Cicadellidae in wheat fields in Shaanxi and Gansu provinces of northwestern China. It can produce four generations a year and is able to adapt to 18 species of gramineous crops and weeds (40). A previous survey from our laboratory found that the severity of WBD in the field was correlated with high populations of *P. striatus* (24). The present study demonstrated that WBD is exclusively transmitted by *P. striatus*. PCR assays, with both 16S rDNA and *rps/rpl* gene primers, successfully identified the WBD pathogen as a phytoplasma and detected it in infected wheat, weeds, and WBD-infected *P. striatus*. Therefore, PCR-based molecular techniques can provide a sensitive and useful diagnostic tool to detect, monitor, and predict the occurrence of WBD epidemics.

In summary, the present investigation demonstrated that WBD is caused by a novel phytoplasma strain belonging to the 16SrI group. Based on phylogenetic analysis of the 16S rRNA and *rps19*, *rpl22*, and *rps3* gene sequences, the WBD phytoplasma is most closely related to subgroup

16SrI-C phytoplasmas. However, the WBD phytoplasma differs significantly from other phytoplasmas in the 16SrI-C subclade because it has a very different host range, and transmission occurs by *P. striatus* leafhopper vectors.

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