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Evaluation of diversity and resistance of maize varieties to *Fusarium* spp. causing ear rot in maize under conditions of natural infection

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Abstract: Fusarium ear rot in maize (*Zea mays* L.) is a serious disease in all maize-growing areas worldwide. A total of 454 fungal strains were isolated from 69 commercial maize hybrids grown in Harbin, China, and comprised *Fusarium subglutinans* (34.8%), *F. proliferatum* (31.3%), *F. verticillioides* (20%), *F. graminearum* (9.7%), and *F. equiseti* (4.2%). Among them, a complex of multiple species, *F. subglutinans*, *F. proliferatum*, and *F. verticillioides* are the dominant fungi causing ear rot. Among 59 commercial maize hybrids, eleven hybrids (18.6%) were found to be highly resistant to Fusarium ear rot. Simple sequence repeat (SSR) analysis using six pairs of primers resulted in 24 reproducible bands and cluster analysis separated the maize hybrids into eight groups. There was little genetic variation associated with disease resistance. No correlation was found between genetic diversity and disease resistance.

Keywords: disease resistance; Fusarium ear rot; genetic diversity; *Zea mays*

Maize (*Zea mays* L.) is one of the most important and widespread cereal crops in the world (Cho *et al.* 2014). Fusarium ear rot is a serious disease rampant in all maize growing areas worldwide, and is caused by multiple fungal species including *Fusarium temperatum*, *F. verticillioides*, *F. graminearum*, *F. andiyazi* and *F. proliferatum*, etc. (Presello *et al.* 2006; Borah *et al.* 2016; Venturini *et al.* 2017).

Control of Fusarium ear rot in maize using agronomic and chemical measures is not very effective. Therefore, development of maize genotypes with resistance to ear rot is important to reduce yield loss and most importantly to avoid mycotoxin contamination of food and feed (Kebebe *et al.* 2015, 2018), which is a problem especially under heavy natural infection with *Fusarium* spp. (Presello *et al.* 2008).

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However, few highly resistant maize varieties have been identified around the world (Zhang *et al.* 2012). Reid *et al.* (1992) suggested that one sufficiently aggressive isolate or a mixture of isolates should be used to screen for resistance in breeding programs.

Simple sequence repeat (SSR) markers have wide applicability for genetic analysis in crop improvement strategies (Inghelandt *et al.* 2010), using maize inbred lines and hybrids (Bedoya *et al.* 2017; Saiyad & Kumar 2018). However, the molecular processes and gene regulation of the defence system relevant to ear rot in maize remain poorly understood (Yuan *et al.* 2013).

Heilongjiang province is one of the major areas of maize production in China, which is one of the world's three gold corn belts. Our objective was to identify and analyse the *Fusarium* species causing ear rot in maize, assess the resistance and investigate the genetic diversity of commercial maize hybrids in this region.

MATERIAL AND METHODS

Diseased maize ears were collected from 69 commercial maize hybrids grown in the same location, which had continuous maize cropping for six years, in Harbin, Heilongjiang province, China, in 2015. To determine the species of pathogens and the nature of mixed infections, all diseased kernels (from one ear/per cultivar) were isolated using tissue isolation (Venturini *et al.* 2017). The pathogenicity of all isolates obtained was determined on a leading commercial maize hybrid (cv. Suiyu 10) using a silk channel inoculation method described by Kebebe *et al*. (2015) in the field according to Koch's postulates.

The pathogenic isolates were mainly identified according to morphological characteristics (Nelson *et al.* 1983). To select representative isolates, molecular identification was conducted using primer pair EF1-728F/EF1-986R (Carbone & Kohn 1999).

For evaluating the resistance of 59 commercial maize hybrids, the experiment was conducted twice at the same place, which had continuous maize cropping for six years, located in Harbin, China in May, 2015 and 2016. A split-plot design with three replications was laid out. Each sub-plot consisted of 10-m long rows with plant spacing of 0.20 and 0.45 m within and between rows, respectively. Conventional management and fertilization were conducted during the maize growth period.

The natural occurrence of ear rot in maize was observed on 10 ears per plot at harvest time. Disease severities were scored visually, according to Kebebe *et al.* (2015) and slightly modified, measuring the percentage of disease area on full ears using a scale from 1 to 9 where 1 = no symptoms, 3 = 1 ~ to10%, 5 = 11 to ~25%, 7 = 26 to ~50%, 9 = 51 to ~100%. The percent disease index (PDI) for ear rot was calculated.

Twenty primer pairs from the maize genome (http://www.maizegdb.org/) were designed for genetic diversity analysis. Genomic DNA was extracted using the PlantGen DNA Kit (Qiagen, Beijing, P.R. China) and quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Each template was amplified using a 20-µl reaction volume that contained 0.1 ng of DNA, 2 mM $MgCl_2$, 200 µM of each dNTP, 200 nM of each primer, and 0.25 U Taq DNA polymerase (Invitrogen, Carlsbad, USA). The PCR was conducted as an initial denaturation at 95°C for 3 min, 40 cycles of 94°C for 30 s, 45°C for 30 s, 72°C 30 s, and final elongation at 72°C for 10 min. PCR products were loaded on a 6.5% v/v acrylamide gel with DNA Marker I, including 600, 500, 400, 300, 200 and100bp (Qiagen, Beijing, P.R. China). The resolution of the amplicons of their method is about 80 bp. Electrophoresis results were visualised by silver staining (Cutts *et al.* 2010). Gel images were scored visually and coded as "1"' for presence of a band "0" for absence of a band or "9" for missing a band for each variety and for each marker.

UPGMA cluster analysis was performed by using NTSYSpc (Ver. 2.11V, 2014). AMOVA analysis was conducted using GenAlEx statistical software (Ver. 6.502, 2012). Nei's genetic distance was calculated by means of PopGen32 software (Ver. 1.31, 1999).

RESULTS

Identification of isolates causing ears rot in maize. The PCR products of representative fungal isolates 24J, S7c, S15a, and S35e were sequenced directly and deposited in GenBank (accession numbers MF028813, MF445094, MF445099, and MF445097, respectively). MegaBLAST analysis revealed that they were 99% similar to *F. verticillioides* isolate DET-51 (KX385102.1), *F. equiseti* isolate YT2 (KX576659.1), *F. proliferatum* isolate 223_1_Cym (KX279453.1), and *F. subglutinans* strain S3.2 (MF045065.1), respectively.

A total of 454 fungal strains were isolated from diseased maize ears, of which 158 isolates (34.8%) were *Fusarium subglutinans*, 142 isolates (31.3%) , were *F. proliferatum*, 91 isolates (20%), were *F. verticillioides*, 44 isolates (9.7%), were *F. graminearum*,

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Table 1. Results of isolation of pathogens causing Fusarium ear rot in maize in Harbin, China

No.	*Fusarium* sp.	No. of isolates	Isolation rate (%)
1	*F. verticillioides*	91	20
2	*F. subglutinans*	158	34.8
3	*F. proliferatum*	142	31.3
4	*F. equiseti*	19	4.2
5	*F. graminearum*	44	9.7

and 19 isolates (4.2%) *F. equiseti* (Table 1). Among them, a species complex of *F. verticillioides*, *F. subglutinans*, and *F. proliferatum* species formed the primary pathogenic agent causing ear rot in maize.

Resistance determination. Among 59 maize hybrids, there were significant differences in the level of resistance to ear rot and no hybrids were found to be immune (Table 2). Eleven commercial hybrids were found to be highly resistant, including Longdan25, Hongyu415, and Fuer1. Five commercial hybrids were

Table 2. Evaluation of disease resistance of 59 commercial maize hybrids to Fusarium ear rot under conditions of natural infection in Harbin, China

SSR group	Variety	PDI			Type	SSR group	Variety	PDI			Type
		2015	2016	average				2015	2016	average	
I	Zao50	17.2	20.8	19.0	R	IV	Liangyu21	46.4	57.4	51.9	HS
	Zhongnong225	19.8	22.2	21.0	R		Jiulong17	31.3	33.5	32.4	S
	Zhongyu990	16.6	21.4	19.0	R		Badan5	17.6	28.2	22.9	R
	Yucheng1	18.4	29.2	23.8	R		Hetian1	27.6	42.8	35.2	S
II	Zhedan37	25.4	39.4	32.4	S		Jiudan57	20.4	21.6	21.0	R
	Weiyu1	17.3	18.9	18.1	R		Suiyu23	0.8	1.6	1.2	HR
	Xinmudan9	4.5	6.9	5.7	HR		Longdan44	11.5	14.9	13.2	R
	Suiyu10	48.9	71.5	60.2	HS		Longyu828	49.2	59.4	54.3	HS
	Suiyu19	27.9	33.1	30.5	S		Longdan69	16.4	22.6	19.5	R
	Longdan39	13.2	18.0	15.6	R		Jiulong9	26.8	39.8	33.3	S
	Xianyu696	17.8	20.2	19.0	R		Fengken008	21.5	26.1	23.8	R
	Keyu16	38.4	50.0	44.2	S		Jiudan48	6.1	9.1	7.6	HR
III	Zhongdan18	17.8	26.0	21.9	R		Fengtian27	7.3	9.1	8.2	HR
	Jiyu66	16.7	19.5	18.1	R		Anzao10	5.1	6.9	6.0	HR
	Jixiang1	14.7	17.7	16.2	R		Fengdan5	31.2	35.5	33.3	S
	Longdan25	6.4	12.6	9.5	HR		Fangyu3	20.4	25.3	22.9	R
	HF35	15.8	22.2	19.0	R	V	Nxblym	14.3	18.1	16.2	R
	Weiyu2	24.8	28.6	26.7	R		Jinghua8	19.2	22.8	21.0	R
	Tiannong9	17.6	26.2	21.9	R		Hongyu415	5.4	9.8	7.6	HR
	Jiulong19	9.2	11.8	10.5	R		Hetian8	26.4	36.4	31.4	S
IV	Xianyu335	81.3	90.2	85.7	HS		Hetian4	16.7	20.1	18.4	R
	Chengdan22	16.7	19.5	18.1	R		Hongchen968	12.4	16.2	14.3	R
	Longfuyu7	6.7	10.5	8.6	HR		Jiulong3	17.4	18.8	18.1	R
	Demeiya3	17.6	28.2	22.9	R	VI	Xiuyu518	11.5	15.0	13.3	R
	Hetian2	6.4	10.2	8.3	HR		Xinyu15	12.2	14.5	13.3	R
	Chunyu20	17.9	25.9	21.9	R	VII	Suiyu29	23.8	29.5	26.7	R
	HF257	16.4	23.6	20.0	R		Cunnuo	49.2	56.0	52.6	HS
	Fuer1	5.9	9.3	7.6	HR	VIII	Shengrui16	35.1	39.2	37.1	S
	Xianfeng38905	7.2	11.8	9.5	HR		Ping`an169	17.6	20.5	19.0	R
	Longfuyu9	16.3	26.5	21.4	R						

PDI – percent disease index; HR: PDI < 10% for highly resistant hybrids; R: 10% ≤ PDI < 30% for resistant hybrids; S: 30% ≤ PDI < 50% for susceptible hybrids; HS: 50% ≤ PDI for highly susceptible hybrids

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Table 3. Primers used in SSR analysis of 59 commercial maize hybrids

No.	SSR primer	Loci	Primer sequence 5'–3'	Repeat motif	Allelic No.	Allele size (bp)*
1	gst1	8.08	F:CACCCGATGCAACTTGCGTAGA R:TCGTCACGTTCCACGACATCAC	AGGAG	4	181, 175, 164, 155
2	umc1149	8.06	F:TACAGTAGGGATTCTTGCAGCCTC R:GTGGGACCTTGTTGCTTCCTTT	(AG)10	6	198, 179, 164, 157, 150, 133
3	umc108	5.07	F:GCAAACCTTGCATGAACCCGATTGT R:CAAGCGTCCAGCTCGATGATTTC	ATCG	2	187, 177
4	bnlg2291	4.06	F:CCTCTCGATGTTCTGAAGCC R:GTCATAACCTTGCCTCCCAA	(AG)17	5	388,361, 267, 162, 142
5	gln4	5.06	F:AGCAGAACGGCAAGGGCTACT R:TTTGGCACACCACGACGA	AACGC	4	265, 257, 236, 209
6	bnlg1152	8.06	F:CGCTACCGATTGTTGAATTG R:AAAGTCGTCCGGTCAAATTG	AG(18)	3	191, 183, 170

*Markers with only one scored allele were dominant loci (presence/absence)

identified as highly susceptible, including Suiyu10 and Longyu828 (Table 2).

Determining the genetic diversity of maize hybrids. Among the 20 pairs of SSR primers tested, six pairs produced 24 reproducible polymorphic bands, ranging from 133 to 388 bp in size (Table 3). Genetic similarity coefficients among the 59 maize hybrids ranged from 0.58 to 0.96, with a mean of 0.77. When the genetic similarity coefficient was set to 0.69, UPGMA (Unweighted pair group method)

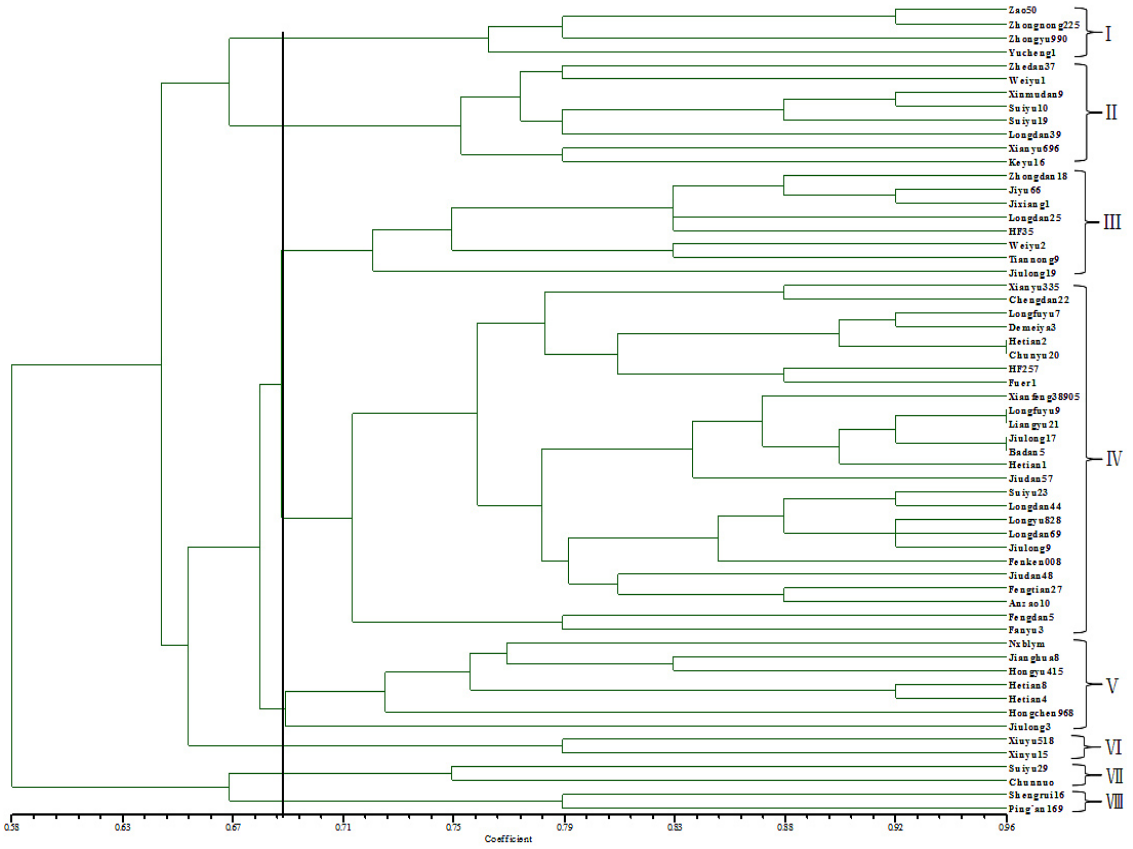

Figure 1. UPGMA tree of 59 commercial maize varieties grown in Heilongjiang province, China based on polymorphic SSR markers

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Table 4. Hierarchical partitioning of variance among and within population groups from genetic diversity of commercial maize hybrids based on analysis of molecular variance (AMOVA)

Source of variation	DF	SS	Variance component	Total (%)	F_{ST}	*P*-value
Disease resistance[a]						
Among groups	3	11.95	0.011	0.294	0.003	0.418
Within groups	55	211.65	3.848	99.706		
Genetic group[b]						
Among groups	7	86.87	1.545	36.566	0.366	0.001
Within groups	51	136.73	2.681	63.434		

[a]Percent disease index of ears rot in maize indicates four groups: highly resistant hybrids, resistant hybrids, susceptible hybrids, highly susceptible hybrids; [b]eight SSR groups separated using UPGMA cluster analysis; DF – degree freedom; SS – sum of squares; F_{ST} – forced swimming test

cluster analysis placed the hybrids into eight SSR groups (Figure 1).

AMOVA (analysis of molecular variance) indicated that over 99% of the genetic variation in disease resistance was distributed within populations (Table 4). No correlation ($P = 0.418$) was found between genetic diversity and disease resistance. Based on Wright's theory, the genetic difference associated with disease resistance ($F_{ST} = 0.003$) was considered to be low (Hartl & Clark 1997). Pairwise comparison between types of resistance indicated significant differences among all the four types ($P < 0.05$ and 0.01) with *F*ST values ranging from 0.04 to 0.08 (Table 5).

DISCUSSION

Ear rot caused by *Fusarium* spp. affects maize production and kernel quality in many countries. The dominant species causing ear rot in maize differ widely in different geographic regions, such as *F. verticillioides* and *F. graminearum* in Nepal (Desjardins & Proctor 2011), *F. verticillioides* or *F. subglutinans* in Mexico (Madania *et al.* 2013), and *F. verticillioides, F. proliferatum*, and *F. meridionale* in the Chongqing areas (Zhou *et al.* 2018). We found in this study that the dominant fungi causing ear rot in maize in Harbin, China were the species complex *F. subglutinans*, *F. proliferatum*, and *F. verticillioides*.

Table 5. Pairwise fixation index between different types of resistance calculated from the SSR data set

Type[a]	HR	R	S	HS
HR (11)	–	–	–	–
R (34)	0.04*	–	–	–
S (9)	0.07*	0.05**	–	–
HS (5)	0.07**	0.06**	0.08**	–

[a]The number of hybrids in each group is indicated in parentheses; HR –highly resistant hybrids; R – resistant hybrids; S – susceptible hybrids; HS – highly susceptible hybrids; *,**values significantly differ at $P < 0.05$ and $P < 0.01$

Cultivation of resistant varieties is the most cost effective and practical means of reducing the damage from ear rot (Chen *et al.* 2012). There are some reports about disease resistance to ear rot in some maize cultivars, such as CO272, CO325, and Pride K127 (Reid *et al.* 1993), and Monafound (Pascale *et al.* 2002). The above-mentioned evaluations of resistance to maize ear rot were based on inoculation with a single pathogenic fungus. However, complexes of multiple *Fusarium* species were the dominant fungi causing ear rot in commercial hybrids under conditions of natural infection (Dorn *et al.* 2009) and our results confirmed this. Therefore, it is insufficient to screen resistance of commercial maize varieties to ear rot using artificial inoculation with single strains of pathogenic fungus (Zhang *et al.* 2012; Duan *et al.* 2016). Rather, natural infection as a screening method for disease resistance is likely to be more representative and have practical significance for selecting resistant material in other maize growing areas.

Using SSR analysis in this study, 59 maize hybrids could be placed into eight SSR groups. This observation was similar to Nikhou *et al.* (2013) and Xu *et al.* (2013) indicating that SSRs are efficient markers to classify closely-related maize lines. In other words, SSR markers were able to detect the extent and considerable level of genetic diversity in maize hybrids.

This study provides the basis for an economical and effective way to screen for resistance and aid in

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the genetic control of maize ear rot. The results will have great practical significance and reference value for global maize production and research

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