The use of molecular markers for characterisation of spring barley for breeding to Fusarium head blight resistance

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ABSTRACT

Fusarium head blight (FHB) is a barley disease, which occurs every year in various areas of barley cultivation all over the world and the increasing incidence has been confirmed in the Czech Republic also during the last years. We aimed to emply AFLP (Amplified fragment length polymorphism) and SSR (Single sequence repeats) markers to describe diversity among breeding lines with a sufficient level of resistance towards FHB and to find marker(s) associated with the analysed traits. The number of eight accessions including five expected resistance donors and three sensitive lines were tested in the field and laboratory. The field values and the amount of deoxynivalenol were positively correlated (r = 0.92). The laboratory test and content DON manifested also a high correlation (r = 0.73). Several DH lines developed from androgenetic barley progenies of the F1 hybrids between the susceptible line PI 383933 and resistant line PEC 210 or the susceptible line PI 383933 and resistant cultivar Chevron were found resistant towards *Fusarium* infection in both the field and laboratory tests. Low infestation was found at line DH 37 from combination Chevron × PI 383933 and lines DH48, DH49, DH50 and DH55 from the combination PEC 210 × PI 383933. Cluster analyses based on 68 AFLP and 18 SSR markers demonstrate a genetic relationship among parental genotypes and DH lines. Some DH lines combined a sufficient degree of resistance against FHB and extract content (basic parameters malting quality). Statistically significant differences in malt-extract values were observed between groups of the DH lines possessing and not possessing the AFLP marker CAA/AGC 341bp. The markers will be further evaluated and optionally used for MAS.

Keywords: barley; *Hordeum vulgare* L.; Fusarium head blight; AFLP; SSR; dihaploid lines; malting quality

Fusarium head blight (FHB) is a barley disease, which occurs every year in various areas of barley cultivation all over the world (McMullen et al. 1997, Schwarz et al. 1997, Windels 2000). The increasing incidence of the disease has also been confirmed in Czech Republic during the last few years. Due to the particularly strong infection a great amount of malting barley was damaged in such a scale that only 20% of malting industry demands were covered from home production in the year 2000 (Psota 2000).

FHB can be observed on early dried spikelets or total parts of the spike. Strongly attacked grains are deformed, shrunken in various scales and they are getting white to rose pale color (Pekkarinen et al. 2000). The infestation of barley used to be caused by Fusarium fungus, mainly by *Fusarium graminearum*. Although FHB can reduce yield, the most significant effect is in reducing quality due to the accumulation of the mycotoxin, deoxynivalenol (DON), in the grain (McMullen et al. 1997, Windels 2000, Leege et al. 2001).

The reduction of infection by FHB can be partly eliminated by a direct chemical fungicide treatment or the breeding of varieties resistant to the fungus. Resistant varieties could be produced either by traditional breeding procedures or by the utilization of molecular markers (Steffenson 1998). PCR based methods AFLP and microsatelite (SSR-simple sequence repeats) analysis can be efficiently used for identification Fusarium head blight resistance in breeding programs (Qi and Lindhout 1997, Castiglioni et al. 1998, Armstrong et al. 2001, Zhu et al. 2001). The AFLP technique has been used previously as well to identify polymorphisms among genotypes (Mueller and Wolfenbarger 1999).

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Table 1. Characteristics of the barley cultivars and lines and their response to FHB

Cultivar/line	Pedigree	Origin	Row (numer)	Response FHB
PI 383933	Kanto Nijo2 = Ko.1018/Kyoto Nakate from Japan	USA	6	very susceptible
CI 4196	PI 64275 (Hang wang ta mai) Landrace from Being	China	2	resistant
Chevron	Clho 1111(PI 38061) = Landrace from Lucerne	Switzeland	2	resistant
Zhaoshu 3	Cultivar in East China, Zhejijang University, Hangzhou	China	2	middle resistant
Foster	Robust/3/Hazen//Glenn/Karl	USA	6	very susceptible
PEC 210	Released in Brazil as Embrapa 128	Brazil	2	resistant
Olbram	HVS 1703 × BR 2174	Czech Republic	2	susceptible
Victor	KM 341 × KM 788-1023	Czech Republic	2	resistant

We aimed to employ AFLP and SSR markers to describe diversity among breeding lines with a sufficient level of resistance towards Fusarium head blight and found putative AFLP marker(s) associated with the analysed traits.

MATERIAL AND METHODS

Plant material

Varieties and lines used in the investigation are summarised and characterised in Table 1. DH (double haploid) lines used in the study were developed as described by Vagera and Ohnoutková (1993).

Evaluation of resistance

Field trials. Grain was cultivated using the standard agrotechnical procedure in two replications at 1 m^2 plots. The plots were artificially inoculated with spores of *Fusarium graminearum*, FG-S6/2 isolate, collected from farm field of Kroměříž vicinity to achieve sufficient disease severity. Inoculum was cultured on solid nutrient media (sterile wheat grain) and kept under NUV (Near Ultra Violet Light) light for 3–4 days following sporulation stimulation. Then the final substrate was dried and stored. Conidial concentration of the inoculum was adjusted under a microscope to 1 million conidia per ml suspension and put into plot at 30 ml on 1 m^2 by spraying using standardized technique. Inoculation was carried out at full anthesis. Disease grade was assessed visually on a six-score scale (Stack and McMullen 1995).

Evaluation DON content. Deoxynivalenol (DON) content was measured by the use of HPLC, Liquid Chromatograf PU 4100M (Philips) according to the methods of Langseth and Rundberget (1998) and Pfohl-Leszkowicz (1999). Analysis was performed from 10 g of homogenized grains. Each analysis was carried out in two replications.

Laboratory test (paper rollade). A hundred grains in two replications were tested per each accession as described previously by Tvarůžek et al. (2003). *Fusarium graminearum*, FG-S6/2 isolate was used for infections. The percentages of germinating and infected grains were checked.

DNA isolation. Genomic DNAs were extracted from bulked young leaves using Saghai-Maroof et al. (1984) protocol. DNA quality and quantity were estimated using GenQuant II Spectrophotometer.

AFLP assays. Restriction and pre-selective amplifications were carried out according to the Perkin-Elmer Protocol (Anonymus 1995) using *Eco*RI and *Mse*I restriction enzymes. Selective amplifications (Table 2) were performed as a multiplex reaction

Table 2. Survey of AFLP primers and number of different polymorphic products

AFLP primery Mse I/EcoRI	Number of polymorphic alell
CTA/ACA	11
CTA/AGG	23
CTA/ACC	17
CTA/ACT	19
CTA/AAG	15
CAA/ACA	8
CAC/ACA	9
CAC/AGG	5
CAC/AAC	5
Total	112

with minor modifications: The PCR reaction mix consisted of 1× Taq polymerase buffer (Qiagen), 2 pMol dNTPs (Gibco BRL), 10 pMol *Mse*I selective primer, 1 pMol *Eco*RI 6-FAM labelled primer, 1 pMol EcoRI-JOE labelled primer, 1 pMol *Eco*RI-TAMRA labelled primer (Applied Biosystem), 0.5 U Taq polymerase (Qiagen), 2.5 µl sterile water and 1 µl diluted preselective PCR product. Amplification products were separated by capillary electrophoresis using Perkin-Elmer Genetic Analyser ABI PRISM 310 and ROX-500 (Applied Biosystem) was used as an internal size standard. The results were evaluated by GeneScan and Genotyper software.

Microsatellite analysis. Microsatellite markers, which specifically flank to SSR loci (Becker and Henz 1995, Liu et al. 1996), were used in this study (Table 3). The PCR protocols have been optimised with respect to annealing temperature and termocycler apparatus type. Each reaction contained 100 ng DNA, 1 U Promega Taq polymerase and 1× corresponding buffer, 100µM dNTP (Gibco BRL), 1–3mM Mg^{++} (Promega), 6.25 pMol of both primers (Applied Biosystems), the forward primers were fluorescently labelled. The resulting PCR products were separated by capillary electrophoresis using the ABI PRISM 310 Genetic Analyser (Applied Biosystem).

Data analysis. For each accession, a binary matrix reflecting specific AFLP and SSR band presence (1) or absence (0) was generated. Pair-wise distances between the accessions based on Hamman similarity metrics (Armstrong et al. 1994) were calculated with using the Microsoft® Excel VBA (Visual Basic for Applications) macros. STATISTICA software (StatSoft, Inc.) was used for cluster analysis and two-way joining analysis. Quantitative data were standardized by range.

RESULTS AND DISCUSSION

The number of eight accessions including five expected resistance donors and three sensitive lines were tested in field tests. Cultivars Olbram and Victor originated in Czech Republic and represent well adopted plant material. The lines CI 4196, PEC 210 and cvs. Chevron and Victor were characterised as resistant with an average infection below 2%. According to the convention (EBC 2000) this value is an upper limit for malting barley. Line Zhaoshu 3 exhibited medium resistance. We certified, that resistance degree of investigated foreign varieties responded to declared resistance/susceptibility (Prom et al. 1997, Steffenson 1999) also in climatic conditions of the Czech Republic. The finding was affirmed by field tests and also by laboratory tests and deoxynivalenol (DON) content measurement. The field values and the amount deoxynivalenol had positively correlated (r = 0.92). The laboratory test and content DON manifested high correlation (r = 0.73) as well. Partial correlation between the two data sets was significant. These data are in accordance with the results of other authors (Lemmens et al. 1997, Buerstmayr et al. 2004). Buerstmayr et al. 2004 assessed that FHB severity in the field and the amount DON in the harvested lines was positively correlated (r = 0.87).

Table 3. Survey of microsatellite markers and number of different polymorphic products

Microsatelites	Chromozome	Number of polymorphic allel
Bmac0181	4(4H)	5
Bmag0105	5(1H)	5
EBmac0541	3(3H)	5
HvABA		1
HvLTPPB	3(3H)	6
HVMO3	4(4H)	6
HVM27	3(3H)	6
HVM40	4(4H)	8
Total		42

To assess the diversity within the evaluated set of breeding material (Table 1) AFLP and SSR markers were employed. Both AFLP and SSR were proved to be suitable for gene-pools richness estimation differing in their ability to score a different number of alleles. Whereas AFLP is biallelic, SSR can identify alelic series on the same loci (Powell et al. 1996). The number of alleles on one SSR loci varied from 2 to 8, with an average mean 5.37 ± 1.25. The number of 112 AFLP alleles was amplified by nine selective primer combinations across analysed accessions (Tables 2 and 3). This is a much higher number in comparison with the number of alleles found in a set of Czech spring malting barley cultivars (Poláková et al. 2001). Overall diversity (Hamman similarity metrics) within the set was estimate to be 0.800 by SSR and 0.860 by AFLP markers. Genetic distance between the most distant accessions reached 1.78, and 1.50 as assessed by SSR and AFLP, respectively. Both AFLP and SSR markers thus revealed a sufficient degree of genetic diversity within the set of tested accessions. Due to higher number of the marker scored by AFLP higher degree of diversity was revealed by AFLP. This phenomenon was described by other authors as well (Hedryk 1999, Baloux et al. 2000). The degree of diversity within the studied set of lines indicates, that available plant material can be efficiently used in breeding and new allele combinations can be

Table 4. Evaluation of barley DH lines in comparison to check varieties (field and laboratory test on paper rolades)

DH lines, variety	Origin	Pedigreé DH male × DH female	Evaluation (0–5)* 13 June	% of infected grains by FHB		
				1st replication	2nd replication	average
DH34	1628ch × 1618a	Chevron × PI 383933	2	25	12	18.5
DH35	1628ch × 1618a	Chevron × PI 383933	3	35	33	34
DH36	1628ch × 1618a	Chevron × PI 383933	2	27	20	23.5
DH37	1628ch × 1618a	Chevron × PI 383933	0	0	0	0
DH38	1628ch × 1618a	Chevron × PI 383933	3	21	21	21
DH39	1628k × 1618a	Chevron × PI 383933	2	32	30	31
64		Chevron = K	0	18	20	19
66		PI 383933 = K	3	91	96	93.5
DH40	1614c × 1618c	PEC 210 × PI 383933	2	26	27	26.5
DH41	1614c × 1618c	PEC 210 × PI 383933	3	29	29	29
DH42	1614c × 1618c	PEC 210 × PI 383933	0	25	22	23.5
DH43	1614c × 1618c	PEC 210 × PI 383933	1	15	9	12
DH44	1614c × 1618c	PEC 210 × PI 383933	2	17	22	19.5
DH45	1614c × 1618c	PEC 210 × PI 383933	0	20	16	18
DH46	1614c × 1618c	PEC 210 × PI 383933	1	16	30	23
DH47	1614b × 1618c	PEC 210 × PI 383933	2	27	14	20.5
DH48	1614b × 1618c	PEC 210 × PI 383933	1	20	6	13
DH49	1614b × 1618c	PEC 210 × PI 383933	1	13	12	13
DH50	1614b × 1618c	PEC 210 × PI 383933	0	14	17	15.5
DH51	1614b × 1618c	PEC 210 × PI 383933	2	92	85	88.5
DH52	1614b × 1618c	PEC 210 × PI 383933	3	98	95	96.5
DH53	1614b × 1618c	PEC 210 × PI 383933	2	95	71	83
DH54	1614b × 1618c	PEC 210 × PI 383933	2	93	93	93
DH55	1614b × 1618c	PEC 210 × PI 383933	1	8	4	6
DH56	1614b × 1618c	PEC 210 × PI 383933	2	94	97	95.5
DH57	1614b × 1618c	PEC 210 × PI 383933	1	14	18	16
DH58	1614b × 1618c	PEC 210 × PI 383933	2	11	18	14.5
DH59	1614b × 1618c	PEC 210 × PI 383933	2	12	13	12.5
DH60	1614b × 1618c	PEC 210 × PI 383933	2	32	36	34
DH61	1614b × 1618c	PEC 210 × PI 383933	1	28	26	27
DH62	1614b × 1618c	PEC 210 × PI 383933	3	56	49	52.5
DH63	1614b × 1618c	PEC 210 × PI 383933	3	12	18	15
65		PEC 210 = K	1	8	14	11
66		PI 383933 = K	3	91	96	93.5

*0 = non infected ear, 5 = strongly infected ear

expected (Franckowiak et al. 2000). Specific DNA profiles were generated for each line, which can make further evaluation of marker segregation possible. Especially SSR profiles of individual lines can be further used to monitor the share of parental genomes by backcrossing to select most promising lines (Ovesná et al. 2002).

Based on preliminary screening susceptible line PI 383933, resistant line PEC 210 and resistant cultivar Chevron were used for DH lines development. The number of 24 DH lines resulting from the combination PI 383933 × PEC 210 and 6 DH lines resulting from the combination PI 383933 × Chevron were further studied.

Several developed DH lines were found resistant towards *Fusarium* infection in both the field and laboratory tests. Low infestation was found at line DH 37 from combination Chevron × PI 393933 and lines DH48, DH49, DH50 a DH55 from the combination PEC 210 × PI 383933. In laboratory tests the mean susceptibility value of the DH lines (Chevron × PI 383933) was 21.3 ± 8.1% which is an acceptable value in comparison with susceptible parental line: 93% for sensitive parent and 19% for resistant one (Table 4). The DH line 37 was even fully resistant. Higher differences among DH lines in resistance degree was recorded in lines resulting from the combination PEC 210 × PI 383933, however other three lines DH48, DH49, DH50 exceeding resistance of the resistant parent in the field trials were revealed.

Molecular markers were used to reveal genetic structure among developed lines. Cluster analyses based on 68 AFLP and 18 SSR markers demonstrate a genetic relationship among parental lines and DH lines. As it was possible to expect slightly different clustering was observed when AFLP and SSR data sets were used. That reflects different position of AFLP and SSR markers in the barley linkage maps (Qi et al. 1996) and nature of the markers (Nybom 2004).

When the marker number AFLP based data were used to draw dendrogram, two main clusters were identified (Figure 1). DH lines resulting from combination Chevron × PI 383933 were clustered together. The second cluster was formed by three sub-clusters of DH lines resulting from crosses between PEC210 × PI 383933. The susceptible parent PI 383933 was an out-layer towards both clusters. The clustering reflects the pedigree of the DH lines. It has to be noted, that the DH lines production depends on regeneration ability of individual genotypes and some genotypes posses better androgenic ability (Machii et al. 1998). It means that genetic basis of the DH lines may not represent the whole scale of possible gene combinations.

Some quality parameters of studied parental and DH lines were measured (data not shown here). Quality parameters are significant for further genetic improvement in backcross hybridization in the frame of breeding programs (Ruckenbauer et al. 2001). DH lines combining sufficient degree of resistance against FHB and high quality values are requested. Using two-way joining results analysis we visualised the relation between the quality parameter values and genetic structure of the evaluated set of DH lines (Figure 2). Significant correlations were found between extract content

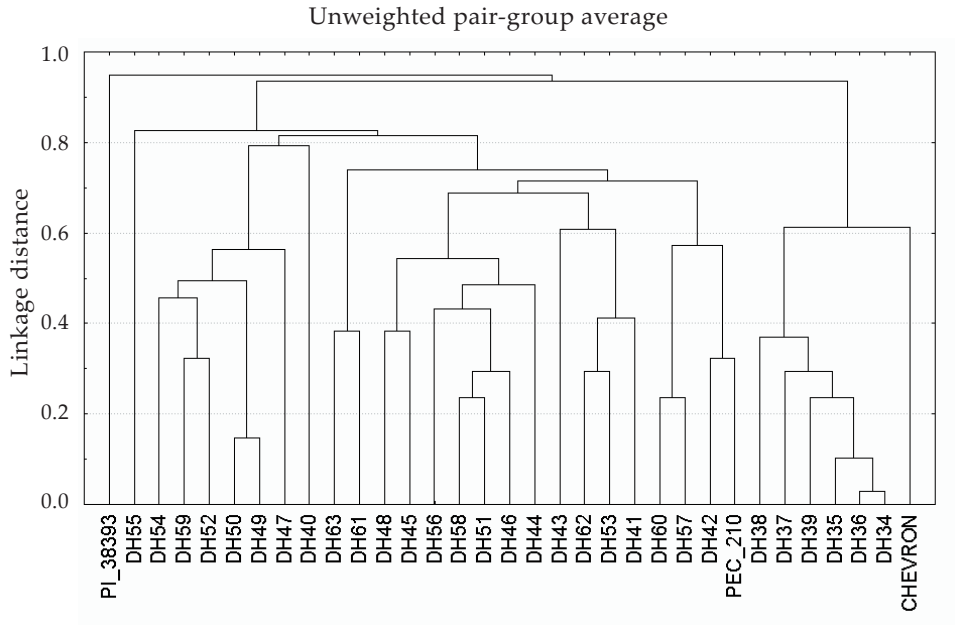

Figure 1. Dendrogram of parental varieties and DH lines polymorphism

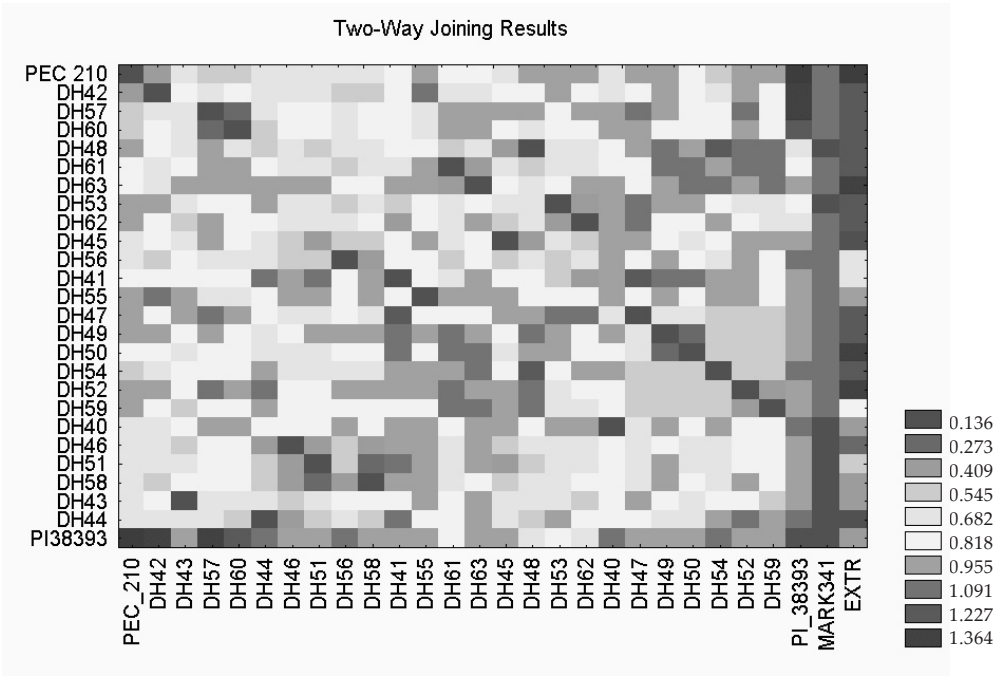

Figure 2. Two-Way Joining Analysis of quality parameters of DH lines

measured as amount of soluble substances obtained from starch by acting of enzymes and genetic distances among accessions. It is apparent, that DH lines: DH54, DH59, DH52 and DH50 forming one sub-cluster and group of lines DH51, DH58, DH46 clustered also together have high value of extract. Coincidence between extract content and the AFLP markers Mse-CAA/Eco-AGC 341 bp and Mse-CAA/EcoRI-ACT 162 was found. Statistically significant differences in malt-extract values (malt-extract value 77.9, respective 76.1) were observed between group of the DH lines possessing the AFLP marker CAA/AGC 341bp (DH41, DH42, DH45, DH47, DH49, DH50, DH52, DH54, DH55, DH56, DH57, DH59, DH60, DH61, DH62, DH63) and DH lines without marker (DH40, DH43, DH44, DH46, DH48, DH51, DH53, DH58). High malt-extract lines are included in clusters I – DH42, DH57, DH60 and II – DH47, DH49, DH50, DH52, DH 54, low malt-extract lines are mainly presented in cluster III – DH44, DH46, DH51, DH56, DH58. Thus we showed, that two-way joint analysis is a useful tool for visualisation of coincidence of putative markers with selected traits. The putative marker will be further verified, cloned, sequenced, evaluated and optionally used for MAS.

SSR markers were not evaluated in this way, because an association of some of them with phenotype and field parametrs is known. Study has been carried to find QTLs associated with FHB resistance using SSR markers (Canci et al. 2004). Only indirect association with FHB might be thus found.

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ABSTRAKT

Použití AFLP a SSR analýz pro charakteristiku a šlechtění jarních ječmenů na rezistenci vůči klasovým fuzáriím

Fusarium head blight (FHB) je choroba ječmene, která se vyskytuje každoročně v různých oblastech pěstování ječmene na celém světě a její zvýšený výskyt byl potvrzen v posledních letech rovněž v ČR. Cílem studie bylo ověřit AFLP a SSR markery ke sledování diverzity mezi šlechtitelskými liniemi s různou úrovní rezistence k FHB. K hodnocení odolnosti vůči fuzáriím bylo v polních a laboratorních pokusech použito 8 odrůd (linií) jarního ječmene, které vykázaly náchylnost nebo rezistenci v infekčních testech. Bylo zjištěno, že úroveň napadení fuzárii je v kladné korelaci (r = 0,92) s obsahem deoxynivalenolu (DON) v zrně a rovněž i laboratorní test s obsahem DON vykazoval vysokou korelaci (r = 0,73). Na základě screeningu byly náchylné linie PI 383933, rezistentní linie PEC 210 a rezistentní odrůda Chevron použity pro tvorbu dihaploidních linií z F1 hybridů. Byly nalezeny některé DH linie, které vykazovaly rezistenci vůči fuzáriové infekci jak v polních, tak v laboratorních testech. Nízký stupeň napadení byl zjištěn u linie DH37 z kombinace Chevron × PI 383933 a linií DH48, DH49, DH50 a DH55 z kombinace PEC 210 × PI 383933. Shluková analýza založená na 68 AFLP a 18 SSR markerech demonstrovala genetický vztah mezi rodičovskými genotypy a DH liniemi. V rámci studovaných výchozích materiálů a DH linií byly měřeny i některé kvalitativní parametry. DH linie kombinovaly dostatečný stupeň rezistence proti FHB a vyznačovaly se i hodnotami hlavního ukazatele sladovnické kvality (extraktu sladu). Dvojrozměrná analýza ukázala statisticky průkazné diference v hodnotách extraktu sladu mezi skupinou DH linií nesoucích AFLP marker CAA/AGC 341bp. Prokázalo se, že markery mohou být dále hodnoceny a druhotně využity pro selekci šlechtitelských materiálů.

Klíčová slova: ječmen; *Hordeum vulgare* L.; Fusarium head blight; AFLP; SSR; dihaploidní linie; sladovnická kvalita

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