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Analysis of polymorphism of microsatellite markers linked to a long-term net form of net blotch resistance gene in winter barley varieties in the south of Russia

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Abstract

Net form of net blotch (NFNT) is the most dangerous and devastating disease of barley causing huge losses in Russia. Highly effective gene *rpt 5* determining resistance to net blotch pathogen in Russia was used in the study. A set of 95 winter barley indigenous and exotic varieties were studied to identify the genotypes with the alleles of 153 and 155 bp for the locus *bmag173* and the alleles of 186, 188, 192 bp for the locus *hvm74* exhibiting resistance to the net blotch pathogen (*Pyrenophora teres* Drechs. f. *teres*) occurring in North Caucasus region of Russia.

Key words: Barley, resistance, gene, alleles, net form of net blotch.

Introduction

Net blotch is widely occurring fungal disease of barley, incited by *Pyrenophora teres* Drechs. f. *teres*. Another form of net blotch i.e., the spot form (SFNB) caused by *Pyrenophora teresf* f. sp *maculata* is also commonly found across the world. Both the diseases are genetically distinct towards host, type of virulence(s) and different life cycles. The host range of the pathogen includes all cultivated and wild species of *Hordeum*. Under favorable conditions, net blotch can cause significant reductions in both yield and the quality of the crop. This disease is devastating in North Caucasus region of Russia, where yield losses on susceptible cultivars can reach up to 40% under epidemic condition. In the world, the estimates of yield loss due to net blotch range from trace to nearly 100%, and vary from 10% to 40% in average years (Mathre, 1997). Net blotch may reduce grain quality affecting kernel size and malt extract yield (in malting barley cultivars). The yield component most severely affected by net blotch is kernel weight, which is commonly reported in terms of 1000-kernel weight (Mathre, 1997).

Yield losses are typically more severe in regions with high humidity and precipitation (Ma et al. 2004). Yield losses from this disease on susceptible cultivars in Russia can reach up to 40% under epidemic condition. The poor grain quality rendered by the disease such as the reduction in 1000 kernel weight, sieving, protein, hectoliter weight making it unsuitable for obtaining malt for the brewing industry (Lashina 2015). Breeding suitable genotypes with high degree of resistance is most economical and effective way to increase yield. The use of resistant cultivars is an important part of an effective disease management program for net type of net blotch. Barley cultivars being grown throughout the world vary significantly in their resistance to the pathogen, ranging from highly resistant to highly susceptible (Sheridan 1997; Douiyssi et al. 1998). There are reports of considerable pathogenic variability between isolates of *P. teres* f. sp. *teres* (Wu et al. 2003).

High yielding varieties can be introduced for cultivation only when the improved traits determine the suitability of a genotype to specific conditions (Kuznetsova et al. 2006; Kostylev et al. 2014). The effectiveness of barley cultivation in the North Caucasus region in certain years decreases due to presence of high inoculum of the disease. The identification of novel sources of stabile resistance to leaf blotch is the prerequisite for breeding resistant

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varieties. Despite the numerous studies on genetic control of barley to net blotch carried out world over in an existing genetic collection, only a small number of resistance genes have been identified to be highly effective against the disease (Anisimova 2006). A number of markers for resistance have been identified in barley, with one region on chromosome 6H producing highly significant association with a resistant phenotype (Cakir et al. 2003). Single dominant genes *viz*., *Rpt1a, Rpt1b, Rpt3d, Rpt2c* conferring resistance to net blotch have been reported out of which *Rpt1b*, *Rpt5* and *Rpt6* are recommended for use in breeding programmes in North Caucasus conditions (Afanasenko et al. 2000). Net blotch is a complex disease and controlled by several genes. Adawy et al. (2013) constructed a genetic linkage map and QTL analysis of net blotch resistance in barley. They have identified many genomic regions associated with net blotch resistance in barley. A total of 14 QTLs with a significance ranging from 0.01% to 5% were found on four linkage groups, the most significant QTL mapped on chromosome 6H. In addition to the genes mapped for NTNB resistance, a single gene *Rpt4* conferring resistance to spot type net blotch, was mapped to chromosome 7H. Among the above listed genes, the highly effective *Rpt5* that determines the resistance of the Ethiopian barley sample CI 9819 to net blotch was previously mapped on the 6H chromosome (Manninen et al. 2006). This dominant gene also proved to be highly effective against eight isolates of the fungus of various origins, including isolates from the USA, the UK, Finland and Canada (Afanasenko 2010). The genes, *Rpt1a, Rpt3d, Rpt1b* and *Rpt2c* have been assigned to chromosomes 3H, 2H, 3H and 5H, respectively using trisomic lines (Bockelmn et al. 1977). Konig et al al. (2014) working on doubled haploid population (DH) identified a single major gene on chromosome 7H and two other QTLs on chromosome 3H of barley. In same study they found that 4H and 5H chromosomes also carry QTLs that confer resistance against net form of net blotch but in another DH population. Similarly, Wonneberger et al. (2017) using a set of 589 polymorphic SNP markers mapped resistance loci in a population of 109 doubled haploid lines from a cross between Norwegian cultivars Arve and Lavrans. Three to four quantitative trait loci (QTL) associated with resistance to net blotch in the seedling stage were found per isolate used. A major, putatively novel QTL was identified on chromosome 5H (23-48% of the genetic variation). QTLs explaining between 12 and 16.5% were also found on chromosomes 4H, 5H, 6H and 7H, with the one on 6H being race-specific.

Koladia et al. (2017) also determined major resistance genes for *Pyrenophora teres* Drechs. f. *teres* in 3H and 7H chromosomes of CI5791 and Tifang genotypes of barley. However, Richards et al. (2016) mapped susceptibility locus (susceptibility to *P. teres* f. *teres*) in 6H chromosome for net form of net blotch through a high resolution map and demonstrated co-segregation of the *Spt1* and *Spt2.K* gene(s) tightly linked in repulsion on 6H chromosome of barley. Identification of several QTLs/genes in relation to susceptibility and resistance indicates that the genetic control of net form of net blotch resistance is complex in nature.

Molecular marker technology has facilitated marker assisted breeding and identification of genotypes with targeted traits. In 2011, a group of Australian scientists recommended ten linked microsatellite markers (*bmag0807, bmag173, hvm74, bmag0870, hvm65, bmag0496, bmag 0344a, ebmac0853, ebmac0806* and *ebmac0874*) for use in marker-assisted barley selection for net blotch resistance (Gupta et al. 2011). Among them, *bmag173 and hvm74* which are reliably associated with net blotch resistance and closely linked to *the rpt5 gene*, are very effectively used for marker-assisted selection of resistant varieties in Australia and Canada (Grewal et al. 2010). The present study was, therefore, aimed at identification of genotypes for net blotch resistance using *hvm74 and bmag0173* markers flanking the net blotch resistance gene *rpt5* and to study the effectiveness of this gene against pathogen prevailing in south of Russia.

Materials and methods

Materials and scale for scoring infection

An investigation was carried out to study 95 varieties of winter barley including 20 developed by the FSBSI Agricultural Research Center, Donskoy and 75 originating from different regions. The assessment of the genotypes for net blotch (*Pyrenophora teres* Drechs f. *teres*) resistance was carried out during 2015-2016 using the methodology as proposed by Afanasenko (1987).

The type of reactions and per cent infection were scored according to 0-4 scoring system:

0- immune (no symptoms, no infection);

1- highly resistant (tiny spots without chlorosis on the lower leaves);

2- relatively resistant (tiny spots like brown thin touches, with/without chlorosis; infection is more than 50% of the foliar surface of the lower leaves, single spots on the leaves of the 2nd level);

3- susceptible (typically net spots or brown stripes with chlorotic borders; the lower leaves die, infection is more than 50% of the foliar surface of the 2nd level, single spots on the upper leaves);

4- highly susceptible (net spots or brown stripes throughout all leaves, the foliar surface at all levels is infected by more than 50%, leaves die) reaction.

The variety 'Tigr' (FSBSI "ARC "Donskoy") was used as a standard susceptible control, the infection of which was more than 50% on the foliar surface of the second level (infection - 3 points according to a 4-point scale).

Molecular analysis

For microsatellite analysis, genomic DNA was isolated from barley leaves by a standard procedure using CTAB-buffer (Saghai-Maroof et al. 1984). Amplification of the *hvm74* and *bmag0173* loci was carried out by PCR using the published primers: *hvm74* - 5'-AGGAAGTCATTGCGTGAG-3' and 5'-TGATCAAG AATGATAACATGG-3'; *bmag173*- 5'-CATTTTTGTT GGTGACGG-3' and 5'-ATAATGGCGGGAGAGACA-3' (GrainGenes: A Database for *Triticeae* and *Avena*, wheat.pw.usda.gov). The expected size of the PCR fragment for *the bmag173* locus was 150 bp and for *hvm74* it was 190 bp.

PCR was performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA). The alleles of *the hvm74* and *bmag173* loci were identified following the protocol of PCR in a 20 μl reaction containing 50-100 ng of DNA, 1xbuffer for Taq polymerase (pH 8.6, 2.5mM Mg2 +) (Sileks), 200 ìmol dNTPs, 0.25 μmol of each primer and 2.5 units of Taq polymerase (Sileks). An automatic station of capillary electrophoresis of high resolution QIAxcel, was used method previously developed by the researchers at the N.I. Vavilov Institute.

The visualization of PCR products was carried out in 1% agarose gel, then by high-resolution electrophoresis in 6% polyacrylamide gel in 1 × TBE buffer (voltage of 10V, 3h). Ethidium bromide was used as the intercalating agent for DNA visualization in the ultraviolet. The size of alleles of microsatellite loci *hvm74* and *bmag173* were determined using an automatic station of high-resolution capillary electrophoresis QIAxcel System Capillary Electrophoresis (Qiagen). When using QIAxcel, the length of the fragments was calculated using internal standards, in which markers of fragments' length (QX DNA Size Marker 25-500 bp) were used. The assessment of the validity of the effect of a combination of alleles of the analyzed microsatellite loci on the resistance to the net blotch was carried out using the nonparametric Kruskal-Wallis test using Statistica 7.0 software.

Results and discussion

The genotypes were screened for the presence of *Rpt* gene linked to net form of net blotch resistance with the help of molecular markers associated with the disease. To identify the allelic diversity of loci *bmag173* and *hvm74,* PCR fragments containing these tandem double-nucleotide repeats were amplified in indigenous and exotic varieties taken for analysis. In a set of 95 varieties, the PCR fragments of microsatellite loci *bmag173* and *hvm74* were analyzed using vertical electrophoresis in 6% acrylamide gel (PAAG) (Figs. 1 and 2).

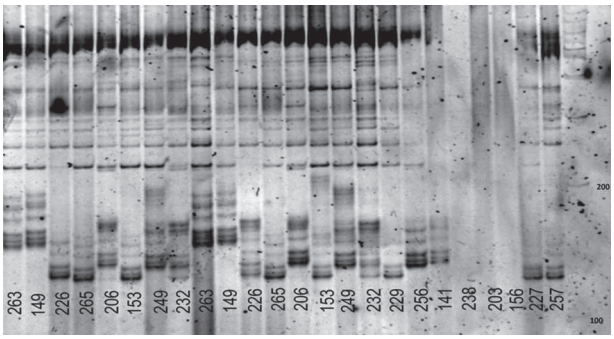

Fig. 1. Polymorphism of the SSR marker *bmag173* in polyacrylamide gel

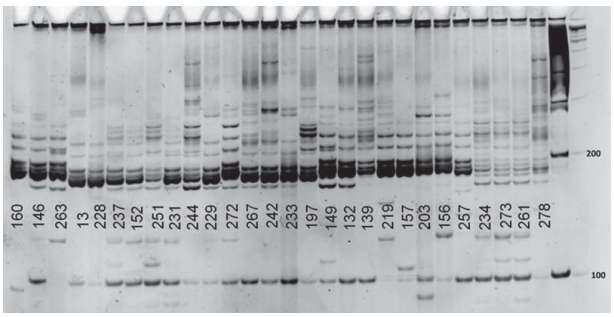

Fig. 2. Polymorphism of the SSR marker *hvm74* in polyacrylamide gel

The *Rpt5* gene which controls the net blotch resistance and is localized on the short arm of the chromosome 6H, is one of the main determinants of resistance to the no-type of this pathogen (Manninen et al. 2006; Qamar et al. 2008). Gupta et al. (2010) analyzed this fragment of the chromosome 6H in the varieties 'Pompadur' and 'Stirling' using SSR markers, as a result of which, it was found that the markers *hvm74* and *bmag173* are closely related to the resistance to the net blotch resistance. A number of markers for resistance have been identified in barley, with one region on chromosome 6H producing highly significant association with a resistant phenotype (Cakir et al. 2003). Adawy et al. (2013) constructed a genetic linkage map and QTL analysis of net blotch resistance in barley and mapped several genomic regions associated with net blotch resistance in barley. A total of 14 QTLs with a significance ranging from 0.01% to 5% were found on four linkage groups, the most significant QTL mapped on chromosome 6H. In addition to the genes mapped for NTNB resistance, a single gene *Rpt4* conferring resistance to spot type net blotch, was mapped to chromosome 7H. Koladia et al. (2017) developed a mapping population (RIL) from CIho 5791 × Tifang and used to identify major dominant resistance genes on barley chromosomes 6H and 3H in CI5791 and on 3H in Tifang. The barley line CIho 5791 confers high levels of resistance to net form of net blotch (*Pyrenophora teres* f. *teres*) with few documented isolates overcoming this resistance.

Lebedev et al. (2016) envisaged the application of DNA fragment markers directly into the analyzed sample, which significantly improves the accuracy of the alleles size estimation. Thus, the alleles of microsatellite loci *bmag173* and *hvm74* linked to the net blotch resistance gene *rtp5* were identified in 95 barley varieties using the nonparametric Kruskal-Wallis test (Table 1). The results of the statistical analysis (Kruskal-Wallis test) showed that the presence of alleles of *bmag173* with a size of 153 and 155 bp and alleles of *hvm74* with dimensions of 186, 188 and 192 bp significantly correlate with high resistance to net blotch (p=0.0000) (Figs. 3 and 4). It should be noted that extremely favorable weather conditions for the development of net blotch disease prevailed in 2015-2016. Therefore, an abundant amount of precipitations in the spring-summer period contributed to the massive spread of net blotch among winter barley crops. In 2016, there was a larger infection with the pathogen than in 2015, due to the accumulation of high load of pathogen. However, the varieties with the alleles 153 and 155 amplified by *bmag173* and the alleles 186, 188 and 192 of *the hvm74* locus were less infected with the net blotch, even under these conditions, compared to the other varieties under the study and control.

Based on the results obtained, the studied varieties were divided into groups according to the allele sizes of microsatellite loci *bmag173* and *hvm74*. It was noted that among the variety-carriers of the desired alleles *hvm74*, both stable and susceptible samples were found. It was concluded that for the detection of donors of net blotch resistance, the variety is necessary to be the carrier of the desired allele(s) for both markers, as they are flanking with respect to the *Rpt5* gene (Table 2). It is worth noting that the most effective combination of alleles of two microsatellite loci was found mainly in the exotic varieties originating from different regions.

The study established that the gene *Rpt5* is effective in barley resistance to net blotch in the Southern Federal District. The system of microsatellite markers (*hvm74, bmag173*) recommended as a marker-auxiliary tool for selection of stable forms in Canada and Australia is also effective in the south of

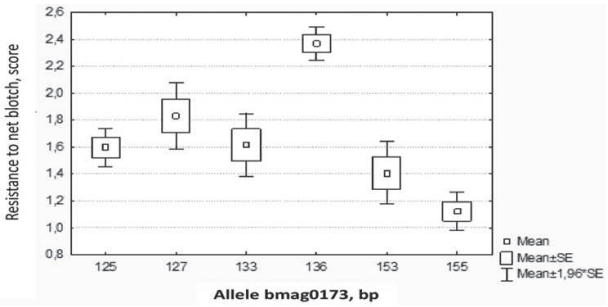

Fig. 3. Variability of varieties according to resistance-susceptibility to net blotch, depending on the alleles of microsatellite loci *bmag173*

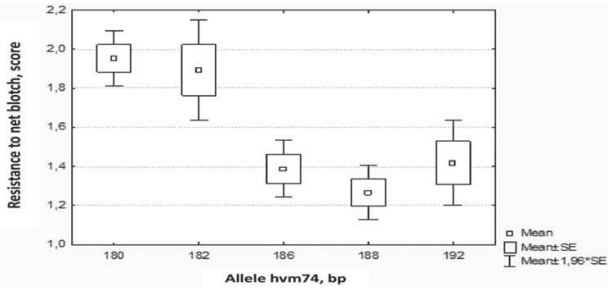

Fig. 4. Variability of varieties according to resistance-susceptibility to net blotch, depending on the alleles of micro satellite loci *hvm74*

Table 1. The analyzed barley varieties with identified alleles of *bmag173* and *hvm74* loci and phenotypic evaluation data for net blotch resistance

Acc.No.	Variety	Origin	Alleles *hvm74*, (bp)	Alleles *bmag173* (bp)	Average resistance score (0-4 scale) over two years	Acc.No.	Variety	Origin	Alleles *hvm74*, (bp)	Alleles *bmag173,* (bp)	Average resistance score (0-4 scale) over two years
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
263	Tiffani	Germany	188	153	1.25	229	Pattern	SRIA, RF	180	125	1.88
247	Vanessa	France	192	153	1.25	272	Parallelum 1913	ARC Donskoy, RF	186	125	1.88
163	Explorer 4/2	France	192	153	1.38	250	Docile	France	188	125	1.88
252	HVW 1427	Germany	192	153	1.75	257	Ushi	Germany	182	125	2.13
162	Explorer 3/2	France	186	155	0.80	251	Caprice	France	182	125	2.13
146	KWS-Scala	Germany	188	155	1.00	1	Parallelum 1904	ARC Donskoy, RF	186	125	2.13
161	Wintwalt	Germany	186	155	1.00	12	Foks 1	ARC Donskoy, RF	180	125	2.25
151	KWS-Hiskory	Germany	186	155	1.00	266	Willis	USA	180	125	2.38
160	Explorer 8	France	188	155	1.05	147	KWS-Meredian	Germany	180	133	0.93
154	Explorer 3	France	186	155	1.13	139	Samson	KRIA, RF	188	133	1.13
157	Explorer 5	France	186	155	1.25	138	Kondrat	KRIA, RF	188	133	1.13
156	Explorer 4	France	188	155	1.38	203	Sekret	KRIA, RF	186	133	1.38
149	KWS-234	Germany	188	155	1.50	148	KWS-117	Germany	188	133	1.38
135	Erema	ARC Donskoy, RF	188	127	1.00	141	Platon	KRIA, RF	182	133	1.50
143	Romans	KRIA, RF	192	127	1.00	246	Nectaria	France	188	133	1.50
159	Explorer 7	France	180	127	1.38	275	Parallelum 1917	ARC Donskoy, RF	180	133	1.63
264	Arkona	Germany	192	127	1.50	199	Skorokhod	KRIA, RF	180	133	1.63
261	Nixe	Germany	180	127	1.63	249	18513 EH11	France	180	133	1.63
274	Pallidum 1916	ARC Donskoy, RF	180	127	1.75	248	6577 ÑÍ	France	192	133	1.63
255	Cita	Germany	180	127	1.75	271	Parallelum 1911	ARC Donskoy, RF	180	133	1.75
230	Espada	SRIA, RF	180	127	1.88	197	Premier	KRIA, RF	180	133	1.88
267	Callao	USA	180	127	1.88	232	Prikumsky 85	SRIA, RF	182	133	2.50
258	Tokyo	Germany	180	127	1.88	256	Rocca	Germany	180	133	2.63
234	Trudivnik	Ukraine	180	127	1.88	244	Capten	France	180	135	2.00
205	Radikal	KRIA, RF	180	127	1.88	206	Avans	KRIA, RF	180	135	2.00
281	Pallidum 1925	ARC Donskoy, RF	182	127	2.13	279	Parallelum 1923	ARC Donskoy, RF	180	135	2.13
226	Derzhavny	SRIA, RF	182	127	2.25	280	Parallelum 1924	ARC Donskoy, RF	180	135	2.13
237	Sinelnikovsky 56	Ukraine	180	127	2.75	236	Metelitsa	Ukraine	180	135	2.38
231	Dostoyny	SRIA, RF	180	127	2.75	276	Parallelum 1920	ARC Donskoy, RF	180	135	2.38
150	KWS-Casino	Germany	188	125	1.00	278	Parallelum 1922	ARC Donskoy' RF	182	135	2.50
158	Explorer 6	France	182	125	1.13	282	Pallidum 1926	ARC Donskoy, RF	182	135	2.50

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
131	Zhiguli	ARC 'Donskoy', RF	188	125	1.13	277	Parallelum 1921	ARC Donskoy, RF	180	135	2.5
132	Timofey	ARC 'Donskoy', RF	188	125	1.13	242	Esterel	France	180	135	2.5
153	Explorer 2	France	180	125	1.25	13	Parallelum 1923	ARC Donskoy, RF	180	135	2.5
219	Khutorok	KRIA, RF	186	125	1.25	243	Azurel	France	180	135	2.63
269	Khobbit	Germany	182	125	1.38	133	Tigr	ARC Donskoy, RF	182	135	2.63
152	Explorer 1	France	180	125	1.38	225	Dobrynya 3	KRIA, RF	180	nd	1.25
265	Cornelia	Germany	180	125	1.38	204	Kozyr	KRIA, RF	186	nd	1.38
195	Mikhalko	KRIA, RF	186	125	1.38	259	Blanka	Germany	186	nd	1.38
142	Gordey	KRIA, RF	186	125	1.38	198	Vavilon	KRIA, RF	186	nd	1.5
196	Fakir	KRIA, RF	186	125	1.38	235	SelenaStar	Ukraine	182	nd	1.63
136	Vivat	ARC 'Donskoy', RF	182	125	1.50	273	Pallidum 1915	ARC Donskoy, RF	182	nd	1.63
253	Cotanici	Germany	180	125	1.50	201	Bastion	KRIA, RF	186	nd	1.63
254	Trasco	Germany	180	125	1.50	202	Kordon	KRIA, RF	186	nd	1.63
262	Punch	Germany	186	125	1.50	268	Tatu	Germany	180	nd	2
245	Broinskaily	France	180	125	1.63	233	Aborigen	Ukraine	180	nd	2.5
238	Bezosty	France	182	125	1.75	228	Zhavoronok	SRIA, RF	180	nd	2.63
227	Andryusha	SRIA, RF	180	125	1.88						

Acc. Accession; bp= base pair; nd= Not detected

Table 2. The identified donors for net form of net blotch resistance in barley

Variety	Origin	Size of the allele *hvm74*	Size of the allele *bmag 173*	Average resistance score (0-4 scale) over two years
Explorer 3/2	France	186	155	0.80
Wintwalt	Germany	186	155	1.00
KWS-Hiskory	Germany	186	155	1.00
Explorer 8	France	188	155	1.05
Explorer 3	France	186	155	1.13
Explorer 5	France	186	155	1.25
Tiffani	Germany	188	153	1.25
Explorer 4	France	188	155	1.38
Explorer 4/2	France	192	153	1.38
KWS-234	Germany	188	155	1.50

Russia. The barley genotypes with alleles of 153 and 155 bp for the *bmag173* locus, as well as 186, 188, 192 bp for the *hvm74* locus are resistant to net blotch. The varieties Explorer 3/2, Explorer 8, Explorer 3, Explorer 5, Vanessa, Explorer 4, Explorer 4/2 (France), Winwalt, 'KWS-Hiskory, Tiffani, KWS-234, HVW 1427 (Germany) are the carriers of the desirable alleles.

Authors' contribution

Conceptualization of research (AAD, EKP); Designing of the experiments (AAD, EKP); Contribution of experimental materials (AAD, AVA, EKD); Execution of field/lab experiments and data collection (AAD, EKP, MVL); Analysis of data and interpretation (AAD, EKP, MVL); Preparation of manuscript (AAD, EKP).

Declaration

The authors declare no conflict of interest.

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