Genetic associations in the detection of QTLs for wheat spike-related traits

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Abstract – The objective of this work was to assess the genetic diversity and population structure of wheat genotypes, to detect significant and stable genetic associations, as well as to evaluate the efficiency of statistical models to identify chromosome regions responsible for the expression of spike-related traits. Eight important spike characteristics were measured during five growing seasons in Serbia. A set of 30 microsatellite markers positioned near important agronomic loci was used to evaluate genetic diversity, resulting in a total of 349 alleles. The marker-trait associations were analyzed using the general linear and mixed linear models. The results obtained for number of allelic variants per locus (11.5), average polymorphic information content value (0.68), and average gene diversity (0.722) showed that the exceptional level of polymorphism in the genotypes is the main requirement for association studies. The population structure estimated by model-based clustering distributed the genotypes into six subpopulations according to log probability of data. Significant and stable associations were detected on chromosomes 1B, 2A, 2B, 2D, and 6D, which explained from 4.7 to 40.7% of total phenotypic variations. The general linear model identified a significantly larger number of marker-trait associations (192) than the mixed linear model (76). The mixed linear model identified nine markers associated to six traits.

Index terms: *Triticum aestivum*, genetic resources, microsatellites, population structure, spike-related traits.

Associação genética na detecção de QTLs relacionados a características da espiga de trigo

Resumo – O objetivo deste trabalho foi avaliar a diversidade genética e a estrutura de população de genótipos de trigo, para detectar associações genéticas significativas e estáveis, bem como avaliar a eficácia de modelos estatísticos para identificar as regiões cromossômicas responsáveis pela expressão de características da espiga. Foram determinadas oito importantes características durante cinco safras agrícolas na Sérvia. Uma série de 30 marcadores microssatélites, localizados próximos a locos agronomicamente importantes, foi utilizada para avaliação da diversidade genética, o que resultou num total de 349 alelos. As associações marcador-características foram analisadas com uso dos modelos linear generalizado e linear misto. Os resultados obtidos para número de variantes alélicas por loco (11,5), valor médio de conteúdo de informação polimórfica (0,68) e diversidade genética média (0,722) mostraram que o nível excepcional de polimorfismo nos genótipos é o principal requerimento para estudos de associação. A estrutura da população estimada pelo agrupamento com base no modelo distribuiu os genótipos em seis subpopulações, de acordo com o log da probabilidade dos dados. Associações significativas e estáveis foram detectadas nos cromossomos 1B, 2A, 2B, 2D e 6D, que explicaram de 4,7 a 40,7% do total das variações fenotípicas. O modelo linear generalizado revelou número significativamente maior de associações marcador-características (192) do que o modelo linear misto (76). O modelo linear misto identificou nove marcadores associados a seis características.

Termos para indexação: *Triticum aestivum*, recursos genéticos, microssatélites, estrutura da população, características da espiga.

Introduction

Wheat (*Triticum aestivum* L.) breeders worldwide invest a great deal of effort into creating cultivars able to challenge rising global issues, such as ongoing climate changes and a growing world population. A tendency in the breeding process is introducing

novel techniques and approaches that could improve current conventional breeding programs. Particularly, the advancement in the field of molecular biology by applying genetic marker technologies and new statistical approaches are powerful tools for indirect selection of valuable traits through marker-assisted selection (Landjeva et al., 2007). The detection of specific and precisely tagged chromosome regions responsible for the expression of certain agronomic traits could be an excellent contribution for the selection and generation of new high-yielding wheat varieties. Likewise, the knowledge of population diversity and structure is of major importance for an efficient use of elite lines and varieties in a breeding process (Laido et al., 2013).

Spike-related traits are important yield components, which are less environmentally sensitive and exhibit higher heritability than yield per se (Cuthbert et al., 2008). The analyses of the genetic control of spike-related characteristics and of individual effects of different genes and quantitative trait loci (QTL) could provide specific information and be useful for indirect determination of yield improvement (Ma et al., 2007). In the last few years, association mapping has been considered one of the most promising methods for the exploration of the entire genome in the search of preferred chromosome regions, QTLs, and desired genes (Liu et al., 2010). The association mapping approach provides a greater potential for the identification of targeted QTLs and fine tuning and mapping of genes at a higher resolution than the previously used linkage mapping. Based on linkage disequilibrium, association mapping is applied directly to diverse genetic materials, resulting in a larger number of detected alleles per locus in a more representative genetic background. It also represents a higher resolution system due to the recombination events that have been accumulated during selection circles through evolution and historical breeding processes (Haseneyer et al., 2010). Cultivars genotyped with high-density markers and their associations show promise in resolving the genetic basis of complex traits of agronomic and economic importance (Wang et al., 2012). The analysis of complex traits by association mapping is required for breeders, since it facilitates even more the application of associated markers in the breeding process. One of the first association mapping studies in wheat aimed at identifying significant markers for kernel size and milling quality (Breseghello & Sorrells, 2006). Subsequently, a large number of works used genome-wide association studies (GWAS) to detect marker-trait associations (MTAs) for a large number of traits, including quality traits in soft wheat (Reif et al., 2011), yield and other agronomic traits in wheat (Liu et al., 2010), and seed longevity in hexaploid wheat (Rehman Arif et al., 2012). In bread wheat, a number of yield-component QTLs was associated with spike-related and adaptive traits (Neumann et al., 2011). The Tassel software (Bradbury et al., 2007) is one of the most sophisticated software programs with implemented algorithms and methods useful for association studies. The structure association analysis developed by Pritchard et al. (2000) first uses a set of random markers to estimate the population structure (Q matrix) and then incorporates this estimation into a general linear model (GLM) analysis. Yu et al. (2006) developed a new methodology, the mixed linear model (MLM) method, which incorporates both the population structure and the familial relatedness or the so-called "kinship" (K matrix), adapted for GWAS, to avoid false associations. This method is recommended in the absence of available pedigree data for clustering a large dataset into groups with improved statistical power (Zhang et al., 2010).

The objective of this work was to assess the genetic diversity and population structure of wheat genotypes, to detect significant and stable genetic associations, as well as to evaluate the efficiency of statistical models to identify chromosome regions responsible for the expression of spike-related traits.

Materials and Methods

A set of 283 wheat accessions originating from 24 countries was used for phenotype evaluation (Table 1). These varieties are part of the largest Wheat Core Collection in Serbia, which belongs to the Small Grains Department of the Institute of Field and Vegetable Crops in Novi Sad. The genotypes were sown in a randomized complete block design in a 1.2 m^2 plot, containing six rows, with a distance of 20 cm between rows. Field plots were cultivated at Rimski Šančevi (45°20'N, 19°51'E) in Novi Sad, Serbia, by applying standard agrotechnical practices (Malešević et al., 1994). The following spike-related traits were measured and recorded for association analysis, during five growing seasons, from 1995 to 1999: spike length,

Table 1. Wheat (*Triticum aestivum*) varieties and lines, origin, and distribution of subpopulations (genotype clusters, Q) obtained by the Structure software (Pritchard et al., 2000).

Nº	Genotypes	Origin	Q	Nº	Genotypes	Origin	Q	Nº	Genotypes	Origin	Q	Nº	Genotypes	Origin	Q
1	Mironovska264	Ukraine	1	72	Pai Yu Pao	China	5	143	Sremica	Serbia	5	214	Produttore	Italy	4
2	Stepnjačka30	Russia	1	73	San Pastore	Italy	2	144	Fruškogorka	Serbia	5	215	Leone	Italy	5
3	Partizanka	Serbia	5	74	Biserka	Serbia	1	145	Banaćanka 1	Serbia	5	216	5263	-	5
4	Una	Serbia	5	75	NSR2	Serbia	5	146	Balkan	Serbia	5	217	NS 62-20	Serbia	6
5	Partizanka niska	Serbia	5	76	NS736	Serbia	1	147	Noe	France	6	218	NS 62-21	Serbia	6
6	Kolubara	Serbia	5	77	Rana Niska	Serbia	2	148	NSP 16	Serbia	5	219	NS 59-20	Serbia	6
7	Buckskin	USA	2	78	Italija	Serbia	1	149	NS 0.1081	Serbia	5	220	NS 59-23	Serbia	5
8	Tecumseh	USA	1	79	Kratka	Serbia	2	150	Žitnica	Serbia	1	221	Panonija	Serbia	5
9	Bersee0	France	2	80	Arg.80/5216	Argentina	1	151	Bolonjska	Italy	5	222	Košava	Serbia	5
10	M.Huntsman0	GBR[(1)]	2	81	Intro1066	USA	2	152	ZG195/7	Croatia	4	223	NS 625	Serbia	4
11	Bersee1	France	2	82	NS4/93	Serbia	5	153	ZG965	Croatia	4	224	NS 974/1	Serbia	5
12	M.Huntsman1	GBR	2	83	NS48/93	Serbia	5	154	ZG990	Croatia	4	225	NS 984/1	Serbia	5
13	Tanori71	Mexico	2	84	NS90/92	Serbia	2	155	ZG1008	Croatia	4	226	NS 12-77	Serbia	5
14	Lerma Rojo	Mexico	1	85	NS30/95	Serbia	1	156	ZG1020A	Croatia	4	227	NS 12-87	Serbia	5
15	Norteno67	Mexico	2	86	Riley	USA	5	157	ZG8056	Croatia	4	228	NS 55-30	Serbia	5
16	Condor	Australia	1	87	Knox 62	USA	6	158	ZG K 2A/82	Croatia	4	229	NS 55-32	Serbia	5
17	Banks	Australia	1	88	CombinationN	USA	3	159	ZG K 77/82	Croatia	4	230	NS 56-11	Serbia	5
18	Aobakomughi	Japan	1	89	Dimitrovska 5-12	Bulgaria	3	160	ZG K 146/82	Croatia	4	231	NS 63-15	Serbia	5
19	Galahad	GBR	2	90	Purdue 5752A-5-7-2	-	6	161	ZG K 172/82	Croatia	4	232	L-44/83	Serbia	5
20	Dwarf A[(2)]	GBR	2	91	7203-36	-	2	162	ZG K T 171/1/82	Croatia	4	233	NS 7003	Serbia	3
21	Bersee2	France	2	92	SI PV 63	-	2	163	ZG K 171/1/82	Croatia	4	234	NS 7007/3	Serbia	5
22	M. Huntsman2	GBR	2	93	Chinofuz	USA	2	164	ZG K 176/82	Croatia	4	235	ZG 884/73	Croatia	3
23	Hobbit	GBR	2	94	64209-77	USA	2	165	ZG K 242/82	Croatia	4	236	ZG 2396/73	Croatia	5
24	Bounty	GBR	2	95	Auburn	USA	6	166	ZG K T 178/82	Croatia	4	237	Resistente	Italy	4
25	Sentry	GBR	2	96	Purdue 79406-I-26-2	USA	6	167	Forlani	Italy	5	238	S.174/72	-	5
26	Wizard	GBR	2	97	NS1/92	Serbia	6	168	ZG K T 244/82	Croatia	4	239	L 60/71	Serbia	5
27	Norman	GBR	2	98	ND516	USA	3	169	INTRO 7	USA	4	240	Fisherect 4A	Mexico	6
28	Mithras	GBR	2	99	ND517	USA	3	170	INTRO 604	USA	4	241	Erect Raf E2	Mexico	2
29	Fenman	GBR	2	100	Lr1	USA	3	171	INTRO 509	USA	5	242	UC 66052	USA	6
30	Sandown	GBR	2	101	Lr3	USA	3	172	INTRO 613	USA	4	243	UC 66206	USA	6
31	Longbow	GBR	2	102	Lr10	USA	3	173	INTRO 29	USA	6	244	UC 67052	USA	6
32	Era	USA	3	103	Lr11	USA	5	174	GSN 17	USA	4	245	UC 64246	USA	6
33	Buckbuck	Mexico	1	104	Lr12	USA	3	175	NSP 54	Serbia	5	246	Pudue 6413	USA	6
34	Olesen Dwarf	Zimbabwe	1	105	Lr13	USA	3	176	NSP 40	Serbia	4	247	Sadovo "S"	Bulgaria	5
35	Bersee1+2	France	2	106	Lr14	USA	3	177	L-63/89	Serbia	5	248	Sadovo Super	Bulgaria	5
36	Yecora	Mexico	1	107	Lr15	USA	3	178	L-64/89	Serbia	5	249	M. Dwarf	GBR	2
37	Bersee3	France	2	108	Lr16	USA	5	179	L-152/89	Serbia	5	250	Stephens	USA	4
38	M. Huntsman3	GBR	2	109	Lr17	USA	3	180	L-154/89	Serbia	5	251	Multibraun	Austria	2
39	Bersee2+3	France	2	110	Lr20	USA	3	181	L-156/89	Serbia	5	252	ST 924 (Selekta)	Russia	4
40	Bersee7	France	2	111	Lr22	USA	6	182	L-159/89	Serbia	5	253	Hilgendorf 61	Austria	4
41	Akakomughi	Japan	1	112	Lr30	USA	3	183	L-160/89	Serbia	5	254	NS 112/92	Serbia	5
42	Fortunato2D	Italy	2	113	Tiha	Serbia	6	184	30-SC.Smoc.88/89	Czech Republic	5	255	Sutjeska	Serbia	5
43	Talent	France	1	114	NS116/95	Serbia	5	185	NS 56/90	Serbia	5	256	Zvezda	Serbia	5
44	Cap.dep./Mara	Italy	2	115	Purdue composite	USA	6	186	NS 3/90	Serbia	6	257	Zelegora	Serbia	5
45	Mara	Italy	2	116	Purdue 5565 C-4-1-3-3	USA	6	187	Vitka	Croatia	5	258	Szegedi 60	Hungary	6
46	D6899	USA	1	117	Agent	USA	3	188	Skopljanka	Macedonia	5	259	WSTGP 91-2	Hungary	6
47	Cap./Bez.5A	Italy	2	118	ABE	USA	6	189	Nova Skopljanka	Macedonia	5	260	A.dw.20/6/Ciano 3-5	-	4
48	S13	Italy	1	119	Sap "S"-Mon "S"	USA	6	190	Radika	Macedonia	5	261	NSP 192	Serbia	6
49	NS322	Serbia	1	120	OK 75R 3645	USA	6	191	Szegedi 5	Hungary	4	262	NS 114/90	Serbia	5
50	NS603	Serbia	1	121	T 734-145	-	6	192	Lovrin 24	Romania	5	263	NS 1/94	Serbia	5
51	NS732	Serbia	1	122	Purdue 77249-RCI-133	USA	6	193	Huequen	Chile	6	264	NS 2/94	Serbia	5
52	NS900	Serbia	2	123	Caldwell	USA	6	194	Napo 63	Colombia	6	265	NS 36/91	Serbia	5
53	NS 51-11	Serbia	1	124	Pesma	Serbia	5	195	NO 5519	China	5	266	NS 7/94	Serbia	4
54	NS 54-52	Serbia	1	125	Sreća	Serbia	5	196	SST 101/A	South Africa	6	267	NS 9/93	Serbia	5
55	Bezostaja Dwarf	Russia	5	126	NS7/93	Serbia	5	197	Zarija	Russia	5	268	NS 18/93	Serbia	5
56	Pitikul	Moldavia	5	127	NS83/92	Serbia	5	198	PPG-186	Russia	5	269	NS 23/94	Serbia	5
57	Szegedi7610	Hungary	2	128	NS22/93	Serbia	5	199	Kunčevska	Russia	5	270	NS 57/92	Serbia	5
58	Szegedi765	Hungary	1	129	NS38/93	Serbia	5	200	Nemčinovskaja 110	Russia	5	271	L 165/94	Serbia	5
59	Timson Sun	Australia	1	130	Adder	USA	6	201	*Triticum spelta*[(3)]	-	6	272	NS 97/95	Serbia	5
60	F5 5065-2	Romania	1	131	NSP88	Serbia	5	202	WWMCB 339	-	5	273	NS 98/95	Serbia	6
61	BCD 1186/83	Moldavia	1	132	Dina	Serbia	5	203	WWMCB 338	-	2	274	NS 124/95	Serbia	5
62	BCD 1286/83	Moldavia	5	133	NSP199	Serbia	5	204	Campodoro Cont.	Italy	5	275	Danica	Serbia	5
63	BCD 1295/83	Moldavia	1	134	NS135/90	Serbia	5	205	CR-8	-	5	276	Proteinka	Serbia	5
64	BCD 1304/83	Moldavia	1	135	NS10/94	Serbia	5	206	CR-10	-	5	277	NSP 51	Serbia	5
65	NSP187	Serbia	1	136	NS39/93	Serbia	5	207	2004	-	5	278	NSA 89-5126	France	5
66	L69/92	Serbia	1	137	Jubilejnaja50	Russia	5	208	2005	-	5	279	L 131/94	Serbia	5
67	Norin50	Japan	1	138	Odeska 51	Ukraine	5	209	2017	-	5	280	L 351/94	Serbia	5
68	Norin61	Japan	1	139	Pavlovska 102	Russia	5	210	3015	-	5	281	NS 50-14	Serbia	5
69	Peking 1-38	China	1	140	Dnjestrovskaja 25	Russia	5	211	3017	-	5	282	Mironovska 10	Ukraine	5
70	No4	China	2	141	Elkohorn	-	6	212	3020	-	5	283	Raduša	Serbia	6
71	Hang Chou	China	1	142	Atlas 66	USA	6	213	3002	-	5	284	Chinese Spring	Control	

[(1)]GBR, Great Britain. [(2)](Maris Hobbit). [(3)]var. *duhamelianum.*

peduncle length, number of spikelets per spike, number of sterile spikelets per spike, spike index, spike weight, grain weight per spike, and grain number per spike.

Genomic DNA from all varieties (approximately ten plantlets per genotype) was isolated from fresh young leaves using the CTAB protocol described by Doyle & Doyle (1990). Wheat genotype population was profiled with 30 microsatellite markers out of 41 initial markers, excluding 11 with non-specific PCR products. The sequences of SSR markers were taken from the GrainGenes database (GrainGenes, 2014) (Table 2). The additional variety Chinese Spring was used as a positive control. Microsatellites were positioned along almost all three genomes and located near previously detected important QTLs. PCR amplifications were carried out according to the protocols given by Röder et al. (1998). The reaction in 10 µL volume contained 30 ng of DNA template, 1x buffer solution, 2 mmol L^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$, 10 pmol of fluorescently labeled forward and unlabeled reverse primers, and 1 unit of *Taq* polymerase. PCR started with an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 52–62°C for 45 s, and 72°C for 45 s. The final extension was 10 min at 72°C. The PCR amplicons were separated by size using capillary electrophoresis on an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The reaction volume of 10 µL consisted of 2 µL of mixed differently-labeled PCR products, 0.2 µL of GeneScan 500 LIZ size standard (Applied Biosystems, Foster City, CA, USA), and 7.8 µL of Hi-Di formamide. The dye-labeled products were identified by fluorescence detection, and microsatellite analysis was performed using the GeneMapper software, version 4.0 (Applied Biosystems, Foster City, CA, USA).

The parameters of genetic diversity were calculated with the PowerMarker software, version 3.25 (Liu & Muse, 2005). The population structure based on genetic data was estimated by the Bayesian algorithm implemented in the Structure software, version 2.3.4 (Pritchard et al., 2000). The hypothetical number of clusters was set ranging from 1 to 20, whereas the length of the burn-in and the Markov chain Monte Carlo (MCMC) were determined at 100.000. The real number of subpopulations was obtained by comparing log probabilities of data Pr [X|K], and corrections were done according to Evanno et al. (2005). The selection of the most appropriate number of subgroups was a critical step for further association analysis. Determination of internal genetic structure was done by additional analysis through principal coordinate analysis (PCoA).

The marker-trait associations were analyzed in the Tassel software, version 2.1. (Bradbury et al., 2007) using two models: GLM and MLM (Yu et al., 2006). The Q matrix for further association analysis was determined based on the average value of three iterations of log probability of data obtained by the Structure software (Pritchard et al., 2000). In order to define the level of genetic covariance between pairs of individuals, a kinship (K) analysis was carried out by molecular data, converting the distance matrix to a similarity matrix using the Tassel software (Bradbury et al., 2007). The magnitude of QTL effects was explained by the R^2 parameter. The descriptive statistics of all phenotypic data was performed in the Statistica software, version 10 (Statsoft, Tulsa, OK, USA).

Results and Discussion

A total of 349 alleles was detected in 30 SSR loci, and the mean number of alleles per loci was 11.5 (Table 3). This result was higher than the diversity (7.2) found among USA wheat accessions (Chao et al., 2007). Chen et al. (2003) reported extremely low values of mean alleles per locus and other polymorphism parameters as a result of the genotype's specific region of origin, which led to a narrowing of genetic diversity. The sufficient genetic variation observed in the material evaluated in the present study was confirmed in other studies with the materials from the same core collection (Kobiljski et al., 2002). However, since the previous analysis was performed on only 96 genotypes, the mean average number of alleles per locus (7.96) was lower than in the present study. The average number of polymorphic information content (PIC) value was 0.688, representing a highly significant level of genetic polymorphism. Considering the cosmopolitan origin of the studied varieties (Table 1), the breeding material indicates a broad genetic diversity that proved to be an excellent base for further research.

The population structure distributed genotypes into six subpopulations using log probability of data obtained by the Structure software (Figure 1), whereas the corrections of the number of clusters (ΔK) according to Evanno et al. (2005) indicated the distribution of genotypes into three existing subpopulations

(Figure 2). Evanno's corrections generally predicted the existence of two or three subpopulations regardless of the number and diversity of the investigated materials (Vigouroux et al., 2008), which was confirmed in the present study. The classification of 283 genotypes was more effective in discriminating the genotypes toward log probability of data. The largest group (Q5) consisted of 114 genotypes, mainly originating from Serbia, whereas the smallest group (Q3) included 18 cultivars, mostly from the USA. The other subpopulations consisted of 37 genotypes (Q1), with diverse geographic origin; 45 genotypes (Q2),

Table 2. Microsatellite markers, sequences of forward and reverse primers, annealing temperature (Tm), repeated motif, and expected amplicons in the Chinese Spring variety of wheat (*Triticum aestivum*), used as a positive control.

SSR markers	Forward primer	Reverse primer	Tm (ºC)	Repeated motif	Expected amplicons (bp)
CFD65 (1A)	5' AGA CGA TGA GAA GGA AGC CA 3'	5' CCT CCC TTG TTT TTG GGA TT 3'	62	(CT)32	199
WMC333 (1A)	5` TCA AGC ATA GGT GGC TTC GG 3'	5' ACA GCA GCC TTC AAG CGT TC 3'	62	(GT)15	174
CFA2086 (2A)	5' TCT ACT TTC AGG GCA CCT CG 3'	5' TCT CTC CAA ACC TCC CTG TAA 3'	62	(CA)21	220
WMC170 (2A)	5` ACA TCC ACG TTT ATG TTG TTG C 3'	5` TTG GTT GCT CAA CGT TTA CTT C 3`	62	(CA)19	230
GWM294 (2A)	5' GGA TTG GAG TTA AGA GAG AAC CG3'	5' GCA GAG TGA TCA ATG CCA GA 3'	62	(GA)9TA(GA)15	-
WMC407 (2A)	5` GGT AAT TCT AGG CTG ACA TAT GCT C 3'	5' CAT ATT TCC AAA TCC CCA ACT C 3'	62	(GA)16	135
CFD71 (4A)	5' CAA TAA GTA GGC CGG GAC AA 3'	5' TGT GCC AGT TGA GTT TGC TC 3'	62	(CA)10(GA)30	216
GWM160 (4A)	5' TTC AAT TCA GTC TTG GCT TGG 3'	5' CTG CAG GAA AAA AAG TAC ACC C 3'	62	(GA)21	-
CFA2114 (6A)	5' ATT GGA AGG CCA CGA TAC AC 3'	5' CCC GTC GGG TTT TAT CTA GC 3'	62	(CA)32	209
WMC333 (6A)	5` TCA AGC ATA GGT GGC TTC GG 3'	5' ACA GCA GCC TTC AAG CGT TC 3'	62	(GT)15	174
CFA2257 (7A)	5' GAT ACA ATA GGT GCC TCC GC 3'	5' CCA TTA TGT AAA TGC TTC TGT TTG A 3'	60	(TG)28	167
WMC83 (7A)	5` TGG AGG AAA CAC AAT GGA TGC C 3'	5` GAG TAT CGC CGA CGA AAG GGA A 3`	62	(GT)28	160
GWM11 (1B)	5` GGA TAG TCA GAC AAT TCT TGT G 3'	5` GTG AAT TGT GTC TTG TAT GCT TCC 3`	52	(TA)6CATA(CA)19(TA)6	196
WMC44 (1B)	5` GGT CTT CTG GGC TTT GAT CCT G 3'	5` TGT TGC TAG GGA CCC GTA GTG G 3`	52	(GT)35	242
GWM148 (2B)	5' GTG AGG CAG CAA GAG AGA AA 3'	5' CAA AGC TTG ACT CAG ACC AAA 3'	62	(CA)22	-
BARC101 (2B)	5' GCT CCT CTC ACG ATC ACG CAA AG 3'	5' GCG AGT CGA TCA CAC TAT GAG CCA ATG 3'	55	(TAA)9	-
WMC154 (2B)	5` ATG CTC GTC AGT GTC ATG TTT G 3'	5' AAA CGG AAC CTA CCT CAC TCT T 3'	62	(GT)34	147
GWM181 (3B)	5' TCA TTG GTA ATG AGG AGA GA 3'	5' GAA CCA TTC ATG TGC ATG TC 3'	52	(GA)28	-
GWM368 (4B)	5' CCA TTT CAC CTA ATG CCT GC 3'	5' AAT AAA ACC ATG AGC TCA CTT GC 3'	62	(AT)25	-
WMC28 (5B)	5` ATC ACG CAT GTC TGC TAT GTA T 3'	5` ATT AGA CCA TGA AGA CGT GTA T 3`	51	(CA) (T) (CA) (GT) 29	188
GWM271 (5B)	5' CAA GAT CGT GGA GCC AGC 3'	5' AGC TGC TAG CTT TTG GGA CA 3'	62	(CT)4imp(GA)10	-
GWM219 (6B)	5' GAT GAG CGA CAC CTA GCC TC 3'	5' GGG GTC CGA GTC CAC AAC 3'	60	(GA)35imp	-
WMC166 (7B)	5` ATA AAG CTG TCT CTT TAG TTC G 3'	5` GTT TTA ACA CAT ATG CAT ACC T 3'	55	(GA)8(GT)8(GT)8	305
WMC216 (1D)	5` ACG TAT CCA GAC ACT GTG GTA A 3'	5` TAA TGG TGG ATC CAT GAT AGC C 3`	55	(GT)22	123
WMC167 (2D)	5` AGT GGT AAT GAG GTG AAA GAA G 3'	5` TCG GTC GTA TAT GCA TGT AAA G 3`	52	(CA)22(CA)8(CA)8	185
WMC18 (2D)	5` CTG GGG CTT GGA TCA CGT CAT T 3'	5` AGC CAT GGA CAT GGT GTC CTT C 3'	62	(CA)(CT)	230
GWM157 (2D)	5' GTC GTC GCG GTA AGC TTG 3'	5' GAG TGA ACA CAC GAG GCT TG 3'	62	(CT)14	-
WMC144 (2D)	5` GGA CAC CAA TCC AAC ATG AAC A 3'	5' AAG GAT AGT TGG GTG GTG CTG A 3'	62	(CA)14	143
GWM292 (5D)	5' TCA CCG TGG TCA CCG AC 3'	5' CCA CCG AGC CGA TAA TGT AC 3'	62	(CT)38	-
PSP3200 (6D)	5` GTT CTG AAG ACA TTA CGG ATG 3'	5` GAG AAT AGC TGG TTT TGT GG 3`	62	(AAG)16	170
Markers with non-specific products					
BARC124 (2A, 2B)	5' TGC ACC CCT TCC AAA TCT 3'	5' TGC GAG TCG TGT GGT TGT 3'	52	(CT)19	-
CFD38 (6D)	5' TGG CCA TTC GAT ATT CAA AA 3'	5' GTG AGT TGA GGC GCA TGA TA 3'	60	(GA)32	215
WMC657 (4B)	5` CGG GCT GCG GGG GTA T 3'	5' CGG TTG GGT CAT TTG TCT CA 3'	61	-	119
WMC664 (3A)	5' GGG CCA ACA AAT CCA AT 3'	5' TCT ACT TCC TTC ATC CAC TCC 3'	61	-	157
WMC625 (3A, 3B)	5` CAC AGA CCT CAA CCT CTT CTT 3'	5' AGT ACT GTT CAC AGC AGA CGA 3'	61	-	113
WMC413 (4B)	5`CAC TGG AAA CAT CTC TTC AAC T 3`	5' ACA GGA AAG GAT GAT GTT CTC T 3'	51	(GT)9	162
GWM257 (2B)	5'AGA GTG CAT GGT GGG ACG 3'	5' CCA AGA CGA TGC TGA AGT CA 3'	60	(GT)30	-
WMC765 (5D)	5` GGG ATC AGA CTG GGA CTG GAG 3'	5' GGG TTG GCT TGG CAG AGA A 3'	61	-	167
CFD26 (5D)	5' TCA AGA TCG TGC CAA ATC AA 3'	5' ACT CCA AGC TGA GCA CGT TT 3'	60	(GAGAA)2(GA)37	271
WMC245 (2D, 2B)	5` GCT CAG ATC ATC CAC CAA CTT C 3'	5' AGA TGC TCT GGG AGA GTC CTT A 3'	61	-	129,150
GWM494 (4A)	5` ATT GAA CAG GAA GAC ATC AGGG 3'	5' TTC CTG GAG CTG TCT GGC 3'	-	(CA)13	-

mostly from England and France; 30 genotypes (Q4), from Croatia; and 40 genotypes (Q6), from the USA.

The distribution could be explained partly by geographical origin and partly by pedigree data.

Table 3. Basic parameters of genetic diversity in wheat (*Triticum aestivum*).

SSR markers	Chromosome	Allelic Nº/ locus	Most frequent allele	PIC	Gene diversity	Observed heterozygosity	Allele size (bp)
WMC28	5B	10	0.356	0.706	0.746	0	177–203, null
CFD65	1A	10	0.271	0.739	0.777	0.004	180–202, null
GWM292	5D	14	0.342	0.777	0.802	0	196–222, null
WMC167	2D	13	0.649	0.516	0.545	0.028	141–191
GWM271	5B	7	0.528	0.626	0.663	0.004	142–166, null
CFA2257	7A	6	0.570	0.426	0.525	0.021	123–141
WMC83	7A	9	0.289	0.799	0.823	0	151–165, null
GWM148	2B	11	0.269	0.785	0.812	0.011	140–168, null
PSP3200	6D	7	0.391	0.701	0.742	0.018	159–177, null
GWM181	3B	19	0.232	0.827	0.846	0.176	117–157, null
CFA2086	2A	24	0.190	0.896	0.905	0.004	207–275, null
WMC170	2A	10	0.366	0.701	0.741	0.218	182–226, null
GWM11	1B	11	0.300	0.802	0.825	0	184-206, null
WMC44	1B	21	0.379	0.801	0.816	0.014	204–266, null
CFA2114	6A	12	0.236	0.837	0.855	0	199–221, null
BARC101	2B	10	0.535	0.619	0.655	0.011	102–130, null
WMC216	1D	12	0.794	0.354	0.364	0.004	106–132, null
GWM294	2A	19	0.229	0.858	0.871	0.004	65–111
WMC166	7B	4	0.634	0.513	0.553	0	306–310, null
WMC18	2D	14	0.284	0.830	0.847	0.004	218–248, null
CFD71	4A	16	0.180	0.876	0.888	0.007	169–207, null
GWM157	2D	7	0.826	0.297	0.309	0.014	77–107
WMC144	2D	6	0.582	0.425	0.522	0	137–157
WMC407	2A	11	0.379	0.738	0.769	0.004	121–153
WMC333-1A	1A	3	0.512	0.400	0.517	0	157–161
WMC333-6A	6A	6	0.466	0.552	0.626	0	161–183
GWM160	4A	8	0.248	0.785	0.813	0.004	166–186, null
WMC154	2B	15	0.220	0.818	0.840	0.004	117–161
GWM219	6B	15	0.166	0.875	0.888	0	163–195, null
GWM368	4B	19	0.387	0.766	0.789	0	202–258, null
Total/average		349/11.5	0.394	0.688	0.722	0.020	

PIC, polymorphic information content.

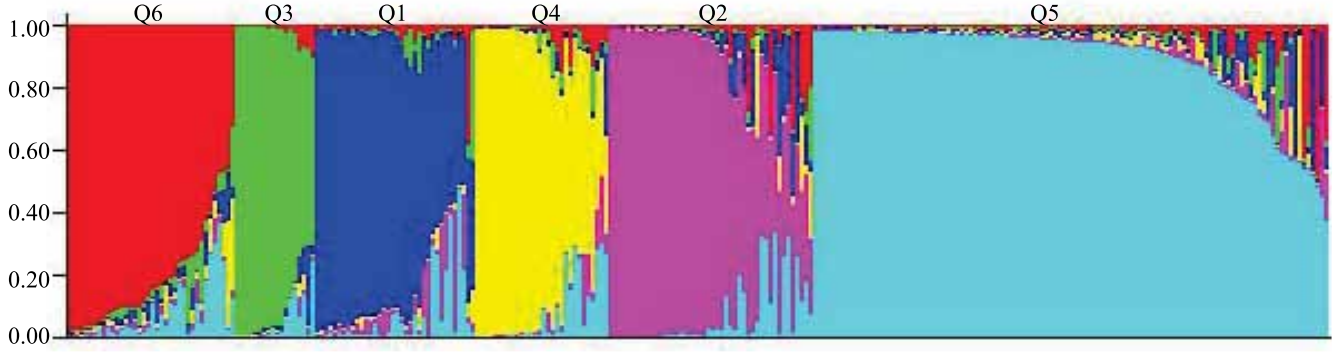

Figure 1. Population structure of 283 wheat (*Triticum aestivum*) genotypes estimated using the model-based Bayesian algorithm implemented in the Structure software (Pritchard et al., 2000) performed with 30 microsatellite loci. Q1 to Q6, genotype clusters on the Q matrix.

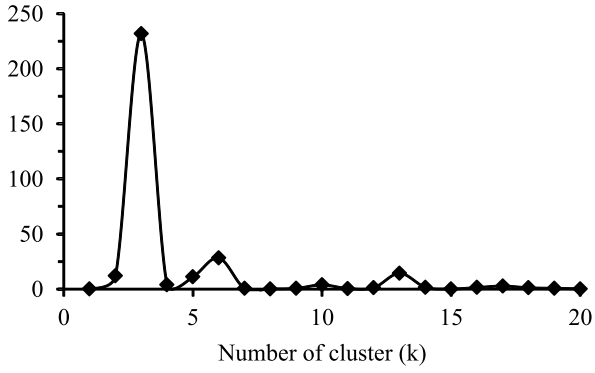

Figure 2. Correction of number of clusters (ΔK) according to Evanno et al. (2005) for the different Bayesian clustering analyses implemented by the Structure software (Pritchard et al., 2000).

Likewise, strict distribution according to origin is difficult because of the use of breeding and elite lines through and by different breeding centers. Even the distribution of genotypes originating from the same regions points to a similar selective pressure in wheat breeding during domestication and the subsequent breeding process (Laido et al., 2013). Moreover, internal genetic structure using PCoA separated the largest subpopulation (Q5) and group (Q2) mostly consistent with grouping by the Structure software (Figure 3). In addition, the groups from Croatia (Q4) and from the USA (Q3) took a particular position in the coordinate system, whereas the remaining two clusters (Q1 and Q6) showed dispersed distribution in the coordinate system. However, certain overlapping

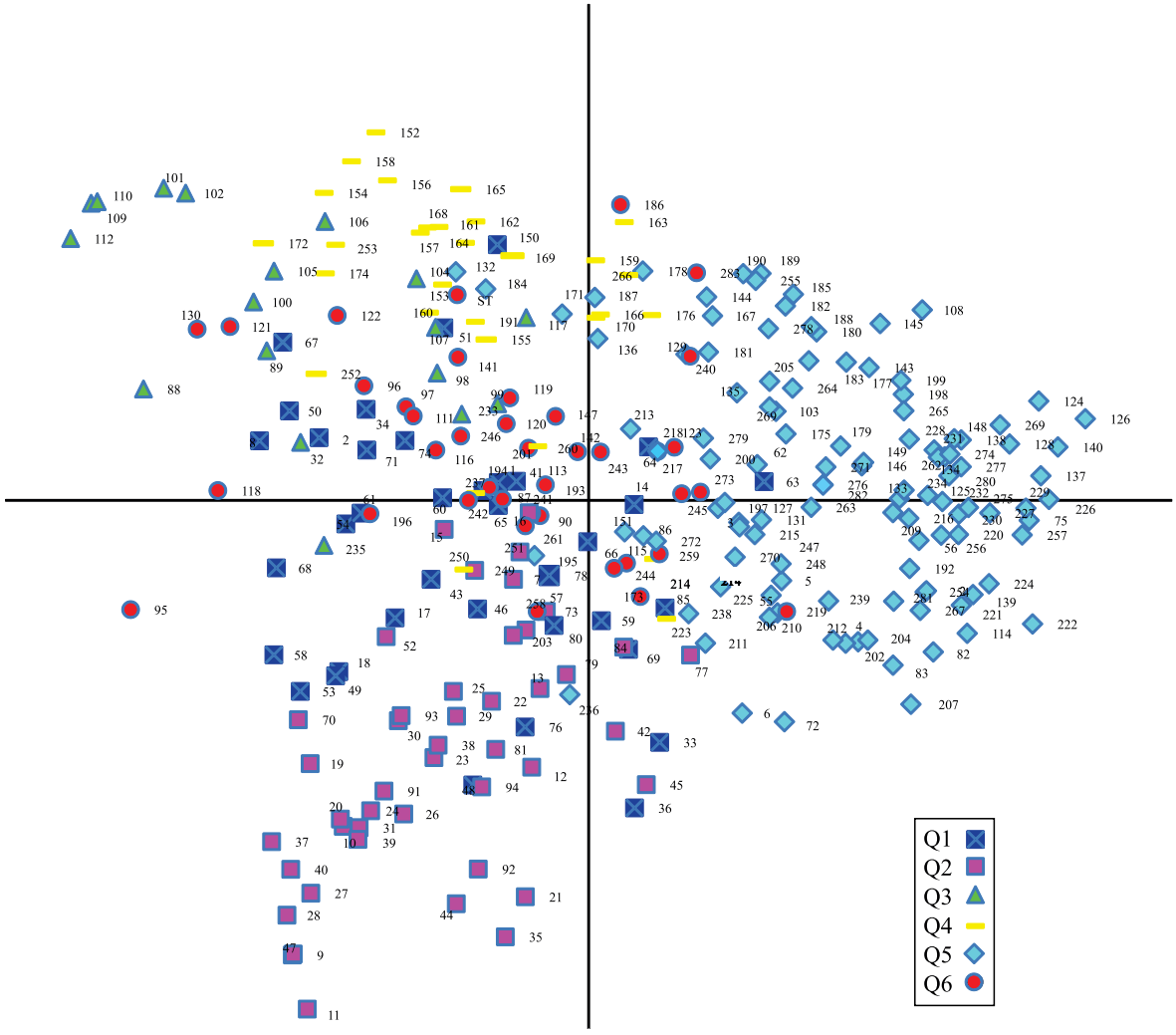

Figure 3. Principal coordinate analysis of the 283 wheat (*Triticum aestivum*) varieties. Each mark represents a sample obtained by the Structure software (Pritchard et al., 2000). Q1 to Q6, genotype clusters on the Q matrix.

within some subpopulations could be a result of the frequent use of certain varieties as parents, as well as of the inclusion of a great number of genotypes into the analysis. Population structure determined by model-based clustering in the Structure software was the most appropriate tool for determining genetic structure and a key component for further association studies (Yu et al., 2006).

The total number of detected marker-trait associations in the five evaluation years was of 192 using the GLM method, but decreased to 76 for all analyzed traits and years using the MLM approach (Table 4). The advantage of the MLM approach is the detection of more real loci associated with agronomic traits, without false positive associations (Zhang et al., 2013). Neumann et al. (2011) suggested the usefulness of both models because a great number of associations could be neglected using only the MLM, resulting in many MTAs that might not be recognized as potential loci. This statement is in accordance with Yu et al. (2009), who proposed that new loci detected by GLM are also useful and should be additionally validated to avoid false-positive associations. Furthermore, the differences detected by these two models could be trait-dependent (Neumann et al., 2011).

It is important to highlight that only the stable associations detected in more than three evaluation years, at 1% probability, using the GLM and MLM approaches, were reported (Table 5). Four closely located markers (*wmc*18, *wmc*167, *wmc*144, and *gwm*157) on chromosome 2D were significant for the detection of QTLs for number of spikelets per spike, number of sterile spikelets per spike, and grain number per spike. This observation agrees with the results of high partial correlations obtained for these traits (Table 6). Besides being a carrier of three key genes for height reduction (*Rht*8), photoperiod (*Ppd*1), and yellow rust (*Yr*16), which are essential for adaptation, chromosome 2D contained most markers associated with the agronomically important traits.

The proximity region of the *Ppd*-1 gene, near *gwm*484, was responsible for the expression of many yield components and spike morphology, showing its high value for wheat improvement (Dodig et al., 2012). On the integrated genetic map of this chromosome created with scaffolds and markers in *Aegilops tauschii*, Jia et al. (2013) identified 33 QTLs or genes. One of them was the QTL for test weight near marker *wmc*167, which was significant for spike-related traits in the present study. Marker *wmc*144 showed the highest effect on phenotypic variation of spikelets per spike with mean value of 40.7%. QTLs for grain number per spike and spike length were found in association with marker *gwm*294, derived by Yao et al. (2009), located on the long arms of chromosome 2A.

Table 4. Total number of marker-trait associations ($p \leq 0.01$) detected with the general linear model (GLM) and the mixed linear model (MLM) methods in three evaluation years.

Traits	Significant GLM associations	Significant MLM associations	Unique GLM	Unique MLM
Spikelets per spike	42	22	34	0
SSS[(1)]	46	18	30	0
Grains per spike	30	16	15	1
Spike length	29	7	28	0
Spike weight	12	4	17	0
GWS[(2)]	12	4	12	0
Spike index	12	3	24	0
Peduncle length	9	2	7	1
Total	192	76	167	2

[(1)]SSS, sterile spikelets per spike. [(2)]GWS, grain weight per spike.

Table 5. Markers associated ($p \leq 0.01$) with spike-related traits in more than three evaluation years using the mixed linear model method, and the mean value of phenotypic variation (%).

Traits/ markers	GWM157 2D	WMC144 2D	WMC167 2D	WMC18 2D	GWM11 1B	BARC101 2B	GWM294 2A	CFA2086 2A	WMC333-2 6A
Spikelets per spike	12.2	40.7	26.6	13.7	ns	ns	ns	ns	ns
Sterile spikelets per spike	9.7	8.6	22.2	14.6	10.7	ns	ns	ns	ns
Grains per spike	7.6	ns	11	8.5	ns	8.2	9.8	ns	ns
Spike index	ns	ns	ns	ns	ns	ns	ns	14.3	ns
Peduncle length[(1)]	ns	ns	ns	ns	ns	ns	12	11.1	ns
Spike length	7.6	ns	ns	ns	ns	ns	11	14.6	4.7

[(1)]Measured only in three years, and significant marker-trait associations in more than two years. [ns]Nonsignificant.

Table 6. Partial correlations of significant phenotypic traits with mean values for each genotype and coefficient of variation (CV) for each trait.

Traits	Spikelets per spike	Sterile spikelets per spike	Grains per spike	Spike length	Spike index	Spike weight	Grain weight per spike	Peduncle length	CV (%)
Spikelets per spike	1	-	-	-	-	-	-	-	33.26
Sterile spikelets per spike	0.796**	1	-	-	-	-	-	-	80.96
Grains per spike	0.667**	0.396**	1	-	-	-	-	-	22.53
Spike length	0.444**	0.410**	0.432**	1	-	-	-	-	17.40
Spike index	-0.330**	-0.340**	-0.159**	-0.319**	1	-	-	-	6.60
Spike weight	0.409**	0.181**	0.742**	0.399**	0.115**	1	-	-	20.94
Grain weight per spike	0.256**	ns	0.629**	0.269**	0.389**	0.953**	1	-	22.00
Peduncle length	0.155**	0.203**	ns	0.278**	0.085*	0.076*	0.076*	1	21.12

In the present study, this marker showed similar effects on the phenotypic variation of these traits (13 and 5%, respectively) (Table 5). Also, two markers (*gwm*294 and *cfa*2086) on chromosome 2A were associated with peduncle length apart from the previously detected QTL for this trait on chromosome 6A (Neumann et al., 2011). This trait has attracted great interest in recent studies due to its importance in avoiding ear diseases. Grain number per spike is one of the most important yield components of wheat (Ma et al., 2007), which was associated with the largest number of markers evaluated, i.e., five (Table 5). The specific marker for grain number was *barc*101 (2BL), which has not been previously associated with this trait, indicating the presence of a new QTL. The presence of QTLs near marker *gwm*11 for a large number of agronomic and adaptive traits has been proven by Wang et al. (2009), whereas, in the present study, the only association of this marker was found with sterile spikelets per spike. Only a limited number of QTL studies for sterile spikelet number per spike have been documented (Ma et al., 2007). The coefficient of variation for sterile spikelets per spike obtained by descriptive statistics was extremely high (Table 6), probably due to the selection of a relatively small number of varieties with branched architecture of wheat spikes. Grain weight per spike and spike weight were the only traits with absence of stable associations in more evaluation years. Using the collection of genotypes with a high level of polymorphism for association analysis and finding stable QTLs over a course of multiple years could be useful for the breeding process (Maccaferri et al., 2008). A potential new flowering-time gene on chromosome 6D (*psp*3200) was detected in similar material from the same core collection under contrasting water regimes (Dodig et al., 2012). However, this region has not shown importance for spike characteristics considering field conditions. The unique association between marker *wmc*333 on chromosome 6A and spike length detected in the present study could indicate the presence of new potential QTL with minor effect.

Conclusions

1. The evaluated collection of wheat (*Triticum aestivum*) genotypes shows genetic diversity, and population structure is an important tool for association analysis.

2. A significant number of associations is stable for six spike-related traits.

3. The statistical models evaluated increase the accuracy and power of the association analysis.

4. The new chromosome regions identified as responsible for spike-related traits are useful for wheat breeding programs.

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