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Genetics and identification of markers linked to multiflorous spikelet in hexaploid oat

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Abstract – *The formation of naked grains is directly associated with the formation of multiflorous spikelets in oats. The objectives of this study were to determine the genetics of multiflorous spikelet and to identify molecular markers linked to this character in hexaploid oat. Genetic analysis for multiflorous spikelet was performed in the F_5 and F_6 generations of two oat populations. DNA extracted from $F_{5:6}$ plants were assayed with 6,000 genome-wide single nucleotide polymorphism (SNP) markers using a genotyping platform developed for oat. Genetic analysis indicated the presence of a major gene controlling multiflorous spikelet in the UFRGS 01B7114-1-3 x UFRGS 006013-1 population. The SNP marker GMI_ES17_c5923_221 showed strong association with the multiflorous spikelet phenotype. These results suggest that the marker GMI_ES17_c5923_221 should be linked to a gene controlling multiflorous spikelet in the oat lines evaluated in this study.*

Key words: Avena sativa*, naked oat, molecular marker, quantitative trait loci (QTL), spikelet morphology.*

INTRODUCTION

Oat is an important cereal crop used for food and feed worldwide. Oat is adapted to a wide variety of environments; however, this crop grows mainly in temperate regions or in the cold seasons of subtropical regions such as southern Brazil (Locatelli et al. 2008). Cultivated oat, *Avena sativa* L., belongs to the Poaceae family. This species is autogamous, allohexaploid (2n = 6x = 42) and derived from the natural aggregation of the three ancestral diploid genomes AA, CC and DD (Rines et al. 2006).

In addition to cultivated hulled oat, the species *Avena sativa* subsp. *nudisativa* is also noteworthy. This species known as 'naked oat' differs from hulled oat in that the naked grain threshes free (Ougham et al. 1996). Another striking difference in naked oat is the presence of panicles with indeterminate multiflorous spikelets. Multiflorous spikelets usually have four to seven fertile florets per spikelet, although naked oat can produce up to 12 fertile florets per spikelet (Burrows 1986). In hulled oat, spikelets are normal and determinate with one to three fertile florets per spikelet; four florets are rarely observed in the same spikelet. Multiflorous spikelets are also found in other species of agricultural interest, including rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) (Chuck et al. 2007, Lee et al. 2007, Brown and Bregitzer 2011).

From a nutritional standpoint, oat cultivars producing naked grains have higher values of linoleic acid, starch and essential amino acids compared to hulled cultivars (Givens et al. 2003). Naked oat has great potential as a feed for monogastric animals, including pigs, birds and horses; once hulled oat is not suitable for these animals (Peltonen-Sainio et al. 2004). Naked oat may also be used in malting processes, which renders it attractive to the beer industry (Wilhelmson et al. 2001). However, the naked oat shows variable expressivity among different genotypes. This characteristic limits the growth of naked oat at the commercial scale. This variable expressivity results in the presence of hulled and hulless grains in the same panicle.

The formation of naked oat grains is directly associated with the formation of multiflorous spikelets. However, the mechanisms of genetic inheritance of these traits are not fully

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elucidated. Genetic studies conducted by Kibite and Taylor (1994) suggest that both "naked grain" and "multiflorous spikelet" characters have monogenic inheritance. Based on this model, the naked grain character is controlled by the *Naked1* (*N1*) gene. This gene acts on lemma and palea lignification. The multiflorous spikelet character is controlled by the *Multiflorous1* (*Mf1*) gene. The *N1* and *Mf1* genes show genetic linkage, with the allelic combination *N1_* (naked grains) being dominant over *n1n1* (hulled grains) and *Mf1_* (multiflorous spikelets) is partly dominant over *mf1mf1* (normal spikelets). Genetic studies involving Brazilian oat genotypes indicated that more than one gene is involved in controlling the formation of naked grain, besides the action of modifier genes (Cabral et al. 2000, Valentine et al. 2014).

Recently, various genes with key roles in regulating the architecture, identity and development of floral organs were identified (Ciaffi et al. 2011). The *AGAMOUS* gene family stands out among them. These genes participate in the determinacy of reproductive floral organs, including stamens, carpels and ovules. *AGAMOUS* genes also participate in defining the number of floral organs by controlling the floral meristem determinacy (Dreni and Kater 2014). These genes have been identified in a large number of monocot species, including barley, wheat, rice and maize, and are associated with the number of florets per spikelet (Reinheimer and Kellogg 2009). Genes of the *AGAMOUS* family have not yet been identified or associated with the expression of multiflorous spikelets in oat. Thus, the identification and characterization of *AGAMOUS* genes and other genes involved in floral meristem determinacy in future studies will be key to a complete understanding of the genetic factors controlling the expression of multiflorous spikelet in oat. The objectives of this study were to determine the genetics of multiflorous spikelet and to identify molecular markers linked to this character in hexaploid oat.

MATERIAL AND METHODS

Genetic populations

For the present study, two oat populations were developed through the following crosses: 'UFRGS 01B7114-1-3 x UFRGS 006013-1' and 'URS Taura x UFRGS 017004-2'. The genealogy and the type of spikelet (normal or multiflorous) for each parent is shown in Table 1. The parents UFRGS 01B7114-1-3, UFRGS 006013-1, URS Taura and UFRGS 017004-2 were developed by the Oat-Breeding Program of the Universidade Federal do Rio Grande do Sul (UFRGS). Both populations of oat lines were derived by single-seed descent to the F_5 generation.

Phenotypic screening for multiflorous spikelet

Oat lines and parents from both populations were analyzed for multiflorous spikelet. The experiments were conducted at the Agronomy Experimental Station of the UFRGS, which is located in Eldorado do Sul, RS (Lat 30° 05' 27'' S, Long 51° 40' 18'' W) at 46 m above sea level. The soil is classified as Typical Red Dystrophic Argisol (EMBRAPA 1999). The climate is classified as Cfa (subtropical humic) according to the Köppen-Geiger classification. Parental lines and recombinant lines from each population were evaluated in 2012 (F_5 generation) and 2013 (F_6 generation). A total of 144 and 191 lines were evaluated for multiflorous spikelet in the populations UFRGS 01B7114-1-3 x UFRGS 006013-1 and URS Taura x UFRGS 017004-2, respectively. Each parent and line was sown in double rows (2.0-m long) in the field, with a spacing of 0.20 m between rows and 0.40 m between plots.

At the harvest maturity stage, the lines were visually analyzed in the field and classified according to the following spikelet types: i) normal spikelet, where all plants within the same line expressed panicles with 100% normal spikelets, or ii) multiflorous spikelet, where all plants within the same line expressed panicles with 100% multiflorous spikelets. Panicles with normal spikelets showed a determinate growth pattern, with two to three fertile florets per spikelet and grains adhered to well-lignified lemma and palea. Conversely, multiflorous spikelets showed an indeterminate growth pattern, with four or more fertile florets per spikelet and grains coated with soft glumes that consisted of poorly lignified lemma and palea.

Table 1. Genealogy and characterization of multiflorous spikelet for the parental lines used in the development of the oat genetic populations

Genetic population	Parental line[†]	Genealogy	Type of spikelet[§]
1	UFRGS 01B7114-1-3	Pc68/5*Starter / UFRGS 10	Normal
	UFRGS 006013-1	Cocker 492/Starter-1 // UFRGS 8	Multiflorous
2	URS Taura	UFRGS 970216-2 / UFRGS 970461	Normal
	UFRGS 017004-2	Cocker 492/Starter-1 // UFRGS 8	Multiflorous

[†] Parental lines developed by the Oat-Breeding Program of the Universidade Federal do Rio Grande do Sul (UFRGS);
[§] Normal spikelet = one to three fertile florets per spikelet; multiflorous spikelet = four or more fertile florets per spikelet.

Genetics of multiflorous spikelet

The number of genes controlling multiflorous spikelet was determined using the F_5 and F_6 generations of the oat populations UFRGS 01B7114-1-3 x UFRGS 006013-1 and URS Taura x UFRGS 017004-2. A genetic hypothesis was tested for each population based on the observed results. The observed frequencies were compared to the expected frequencies using the Chi-square test (χ^2). The hypothesis of a single major gene controlling multiflorous spikelet was tested for the UFRGS 01B7114-1-3 x UFRGS 006013-1 population. The hypothesis of two genes controlling multiflorous spikelet was tested for the URS Taura x UFRGS 017004-2 population. In the evaluated populations, only lines expressing the normal spikelet phenotype or the multiflorous spikelet phenotype were included in the tests of the genetic models for one or two genes. Ratios of one normal spikelet line:one multiflorous spikelet line and three normal spikelet line:one multiflorous spikelet line were expected in the UFRGS 01B7114-1-3 x UFRGS 006013-1 and URS Taura x UFRGS 017004-2 genetic populations, respectively. The calculated χ^2 value was compared to the critical χ^2 value. One degree of freedom and a 5% significance level were considered.

DNA extraction and analysis of SNP markers

The population derived from the cross UFRGS 01B7114-1-3 x UFRGS 006013-1 was genotyped in this study. DNA extracted from $F_{5:6}$ plants were assayed with 6,000 genome-wide single nucleotide polymorphism (SNP) markers using a genotyping platform developed for oat. A total of 94 lines in the F_6 generation and the respective parents UFRGS 01B7114-1-3 and UFRGS 006013-1 were included in this analysis. Plant tissues from seedlings from each line and the parents were collected seven days after planting. Tissues were frozen in liquid nitrogen and ground. DNA extraction was conducted using the modified cetyltrimethylammonium bromide (CTAB) method. Sodium chloride (NaCl) and polyvinylpyrrolidone (PVP) were used to remove polysaccharides and polyphenols, respectively, as reported by Lodhi et al. (1994). A 20 µL DNA aliquot at a concentration of 50 ng μL^{-1} for each line was sent to the Biosciences Research Laboratory, United States Department of Agriculture (USDA) – Agricultural Research Service (ARS) located in Fargo, North Dakota (ND), USA. The GoldenGate genotyping platform from Illumina (www.illumina.com) was used for the analysis of SNP markers, according to the specifications reported by Tinker et al. (2014).

Linkage mapping

A genetic linkage map for the UFRGS 01B7114-1-3 x UFRGS 006013-1 population was designed using the program JoinMap 4.0 (Kyazma, the Netherlands). Linkage groups were formed using a logarithm of odds (LOD) score of six and a maximum frequency of recombination of 40%. The maximum likelihood-mapping algorithm was used to generate the linkage groups. The Kosambi mapping function was used to convert recombination frequencies into centimorgans (cM). SNP markers with the expected segregation pattern and polymorphic between parents were used to design the genetic linkage map.

Identification of SNP markers linked to multiflorous spikelet

The single gene mapping method was used to identify SNP markers linked to multiflorous spikelet in the UFRGS 01B7114-1-3 x UFRGS 006013-1 mapping population. Phenotypic data collected in 2012 (F_5 generation) and 2013 (F_6 generation) were converted to the JoinMap's mapping code system and included in the genetic mapping. In 2012, the morphological marker was termed Mf1a (Mf = Multiflorous); in 2013, this marker was termed Mf1b. Lines expressing the normal spikelet phenotype were labeled with the mapping code "a" (same as the parent UFRGS 01B7114-1-3). Lines expressing the multiflorous spikelet phenotype were labeled with the mapping code "b" (same as the parent UFRGS 006013-1). DNA sequences containing SNP molecular markers associated with multiflorous spikelet were aligned to sequences available in the open-access sequence database GenBank (http://www.ncbi.nlm.nih.gov/genbank/), using the Basic Local Alignment Search Tool (BLAST). The SNP markers associated with multiflorous spikelet were also compared to the consensus map of oat developed by Oliver et al. (2013) and Tinker et al. (2014) in order to identify the chromosomal location.

RESULTS AND DISCUSSION

The formation of multiflorous spikelet was analyzed in two oat populations, which were derived from the following crosses: UFRGS 01B71114-1-3 x UFRGS 006013-1 and URS Taura x UFRGS 017004-2. For the UFRGS 01B71114-1-3 x UFRGS 006013-1 population evaluated in 2012, 54 lines expressed the multiflorous spikelet phenotype; 63 lines expressed the normal spikelet phenotype. From the observed results, the genetic hypothesis of a single gene controlling multiflorous spikelet was tested. The calculated value of the Chi-square test was 0.70 ($p = 0.40$). Thus, the hypothesis of a major gene controlling multiflorous spikelet in oat could not be rejected (Table 2). In 2013, the frequency

of lines with multiflorous and normal spikelets was 55 and 73, respectively. The calculated value of the Chi-squared test was 2.5 (p = 0.11). This finding also indicated the hypothesis of a major gene controlling the character could not be rejected (Table 2).

Genetic studies aiming to determine the number of genes controlling multiflorous spikelet in oats began over a century ago. Several studies demonstrated the character controlled by a single gene with a strong effect on the phenotype (Gaines 1917, Caporn 1918, Love and McRosstie 1919, Boland and Lawes 1973, Cabral et al. 2000). Therefore, the results of the genetic analysis for the UFRGS 01B71114-1-3 x UFRGS 006013-1 population corroborate the results reported in previous studies.

For the URS Taura x UFRGS 017004-2 population evaluated in 2012, 48 lines expressed the multiflorous spikelet phenotype; 112 lines expressed the normal spikelet phenotype. This frequency indicates that the single gene model, as previously described for the UFRGS 01B71114-1-3 x UFRGS 006013-1 population, did not fit the results observed in this population. Thus, the hypothesis of two genes controlling the multiflorous spikelet character was tested. The calculated value of the Chi-squared test was 2.1 (p = 0.14), indicating the hypothesis of two genes could not be rejected in this population (Table 2). In 2013, 122 of the 172 lines analyzed expressed the normal spikelet phenotype; 50 lines expressed the multiflorous spikelet phenotype. The calculated value of the Chi-squared test was 1.5 (p = 0.22), which fit the genetic hypothesis of two genes.

The genetic analysis of multiflorous spikelet performed for the UFRGS 01B71114-1-3 x UFRGS 006013-1 and URS Taura x UFRGS 017004-2 populations demonstrated that the number of genes controlling this character diverged between these populations. The origin of the second gene in the URS Taura x UFRGS 017004-2 population is not known based on the genealogy of the parental lines (Table 1). In previous studies, several authors suggested the action of modifier genes in addition to a primary gene. These modifier genes act to form a wide array of phenotypes observed in different genotypes of naked oat (Jenkins and Hanson 1976, Cabral et al. 2000, Valentini et al. 2014). However, we cannot consider the action of a modifier gene in this study. With the action of "modifier gene", the expression of the "modified gene" would be reduced or suppressed, contributing to a deviation from the 3:1 (normal:multiflorous spikelet) frequency observed here. Therefore, further studies are needed to validate the presence of both genes and determine their roles in controlling multiflorous spikelet in the URS Taura x UFRGS 017004-2 population.

Based on the results of the genetic analysis performed for the UFRGS 01B71114-1-3 x UFRGS 006013-1 population, the lines from this population were subjected to a genotypic analysis using SNP markers. Of the total of 631 SNP markers identified as polymorphic between the parental lines, 502 markers were grouped into 42 linkage groups. The remaining markers showed no linkage to any group or were not considered in the analysis due to distortion of Mendelian segregation.

The number of molecular markers in each linkage group ranged from two in linkage groups 35 to 42 to 61 in linkage group 1 (Figure 1). The genetic distance of each linkage group ranged from 0 cM (co-segregating markers; in linkage groups 35, 38 and 39) to 188.5 cM (in linkage group 2; Figure 1). The complete genetic linkage map for the UFRGS 01B71114-1-3 x UFRGS 006013-1 mapping

Table 2. Observed and expected frequencies for the multiflorous spikelet phenotype in two oat populations evaluated in field tests during the growing seasons of 2012 and 2013

	UFRGS 01B7114-1-3 x UFRGS 006013-1							
Phenotype	**2012 (F_5)**				**2013 (F_6)**			
	F_{obs} †	F_{exp} §	χ^2	p*	F_{obs}	F_{exp}	χ^2	p
Multiflorous	54	58,5	0,35		55	64	1,27	
Normal	63	58,5	0,35		73	64	1,27	
Total	117	117	0,70	**0,40**	128	128	2,5	**0,11**
	URS Taura x UFRGS 017004-2							
Phenotype	**2012 (F_5)**				**2013 (F_6)**			
	F_{obs}	F_{exp}	χ^2	p	F_{obs}	F_{exp}	χ^2	p
Multiflorous	48	40	1,60		50	43	1,14	
Normal	112	120	0,53		122	129	0,38	
Total	160	160	2,1	**0,14**	172	172	1,5	**0,22**

† F_{obs} = observed frequency of oat lines for the multiflorous spikelet phenotype;
§ F_{exp} = expected frequency of oat lines for the multiflorous spikelet phenotype, according to the genetic hypothesis of one gene (UFRGS 01B7114-1-3 x UFRGS 006013-1) and two genes (URS Taura x UFRGS 017004-2);
* p = probability of the calculated chi-square value, considering one degree of freedom.

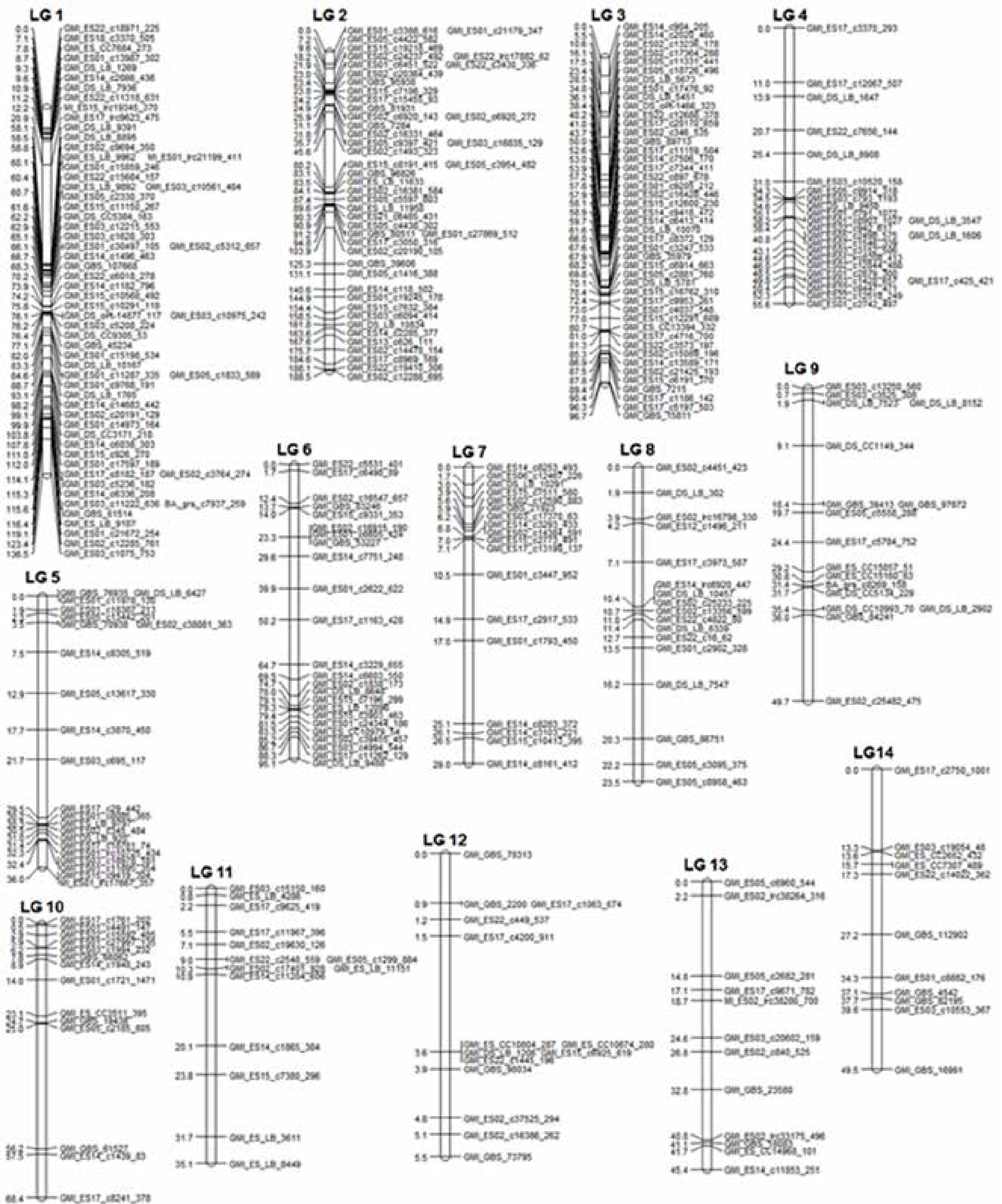
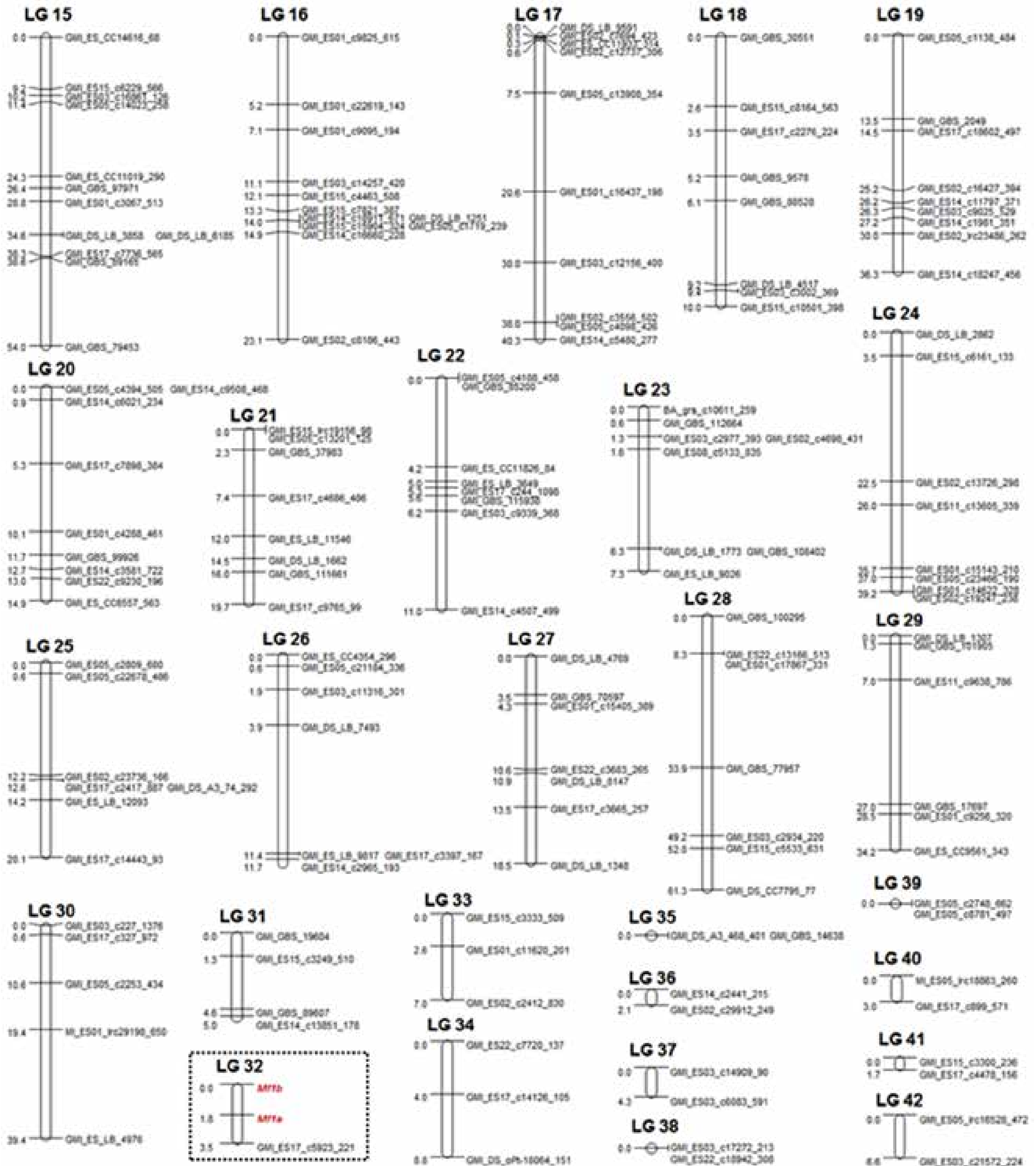


Figure 1. A framework version of the 'UFRGS 01B7114-1-3' x 'UFRGS 006013-1' linkage map derived from single nucleotide polymorphism (SNP) markers.



population encompassed a total genetic distance of 1,397.5 cM (Figure 1). These results are in agreement with the results of molecular mapping previously performed in hexaploid oats (O'Donoughue et al. 1995, Wight et al. 2003, Zhu and Kaeppler 2003, Tinker et al. 2009). The expected number of linkage groups is 21, corresponding to the number of chromosomes in each haploid cell (*n*) of hexaploid oat. In all of the above studies, the number of linkage groups assessed was greater than 21. The development of a consensus map and a physically anchored genetic map in oat has been

hampered by the genome size and complexity, the scarcity of molecular markers and a lack of aneuploid oat stocks (Oliver et al. 2013). The first physically anchored chromosomal map of oat was recently developed using various modern genotyping strategies. This map contains 21 linkage groups, and most of the identified and mapped molecular markers are annotated to their corresponding chromosomes in the hexaploid oat genome (Oliver et al. 2013, Tinker et al. 2014). Therefore, the genetic linkage map for the UFRGS 01B71114-1-3 x UFRGS 006013-1 mapping population covered approximately 80% of the oat genome.

The linkage map for the UFRGS 01B71114-1-3 x UFRGS 006013-1 population was used to identify SNP markers associated with multiflorous spikelet in hexaploid oats. Based on the single gene mapping strategy used in the present study, only the SNP marker GMI_ES17_c5923_221 showed association with the multiflorous spikelet phenotype. The SNP marker GMI_ES17_c5923_221 and the markers for spikelet morphology (normal or multiflorous) 'Mf1a' and 'Mf1b' were mapped to linkage group 32 of the genetic linkage map designed for the UFRGS 01B71114-1-3 x UFRGS 006013-1 mapping population (Figure 1). In 2012 (F_5 generation), the SNP marker GMI_ES17_c5923_221 was mapped at a genetic distance of 1.8 cM from the morphological marker of the multiflorous spikelet trait *Mf1a*. In 2013 (F_6 generation), the genetic distance between the markers GMI_ES17_c5923_221 and *Mf1b* was 3.5 cM (Figure 1). The greater genetic distance between the SNP and morphological marker estimated in 2013 compared to 2012 may be directly associated with a higher frequency of recombination. In 2013 (F_6 generation), one additional cycle of meiosis occurred when compared to the year of 2012 (F_5 generation). Therefore, the genetic distance of 3.5 cM between the SNP marker GMI_ES17_c5923_221 and the morphological marker (Mf1) must represent a more accurate measurement of the physical distance between these markers on the oat chromosome.

The molecular marker GMI_ES17_c5923_221 showed no genetic linkage to any other SNP markers among the linkage groups formed for the UFRGS 01B71114-1-3 x UFRGS 006013-1 mapping population. In addition, this marker was not associated with any linkage groups in the physically anchored map designed by Oliver et al. (2013) or the consensus map designed by Tinker et al. (2014). The reason for the lack of genetic linkage between the GMI_ES17_c5923_221 marker and other markers identified thus far is still unknown.

The nucleotide sequence of the DNA segment in which the GMI_ES17_c5923_221 marker was identified in the parental lines UFRGS 01B71114-1-3 and UFRGS 006013-1 is shown in Figure 2. The DNA segment consists of 120 nucleotides, and the genetic polymorphism is characterized by a point mutation involving the substitution of one thymine (T) in the parental line UFRGS 01B71114-1-3 (normal spikelet) with a cytosine (C) in the parental line UFRGS 006013-1 (multiflorous spikelet) (Figure 2a). Sequence alignments demonstrated that the mutation occurred at position 61 (Figure 2b). This base-pair substitution [T/C], albeit still genetically uncharacterized, may have an evolutionary contribution to the phenotypic divergence of spikelet development in hexaploid oats.

The nucleotide sequence of the SNP marker GMI_ES17_c5923_221 was also compared with sequences available

Figure 2. Identification of the SNP marker GMI_ES17_c5923_221 in hexaploid oat. **a)** The nucleotide sequence of the parental lines demonstrates that the SNP marker GMI_ES17_c5923_221 involves the replacement of a pyrimidine with other pyrimidine [T/C]. Such base-pair substitutions are called transitions. **b)** The sequence alignment indicates that the base-pair substitution occurred at position 61 between the parental lines UFRGS 01B71114-3 e UFRGS 006013-1.

in the open-access sequence database GenBank (http://www.ncbi.nlm.nih.gov). The comparison was carried out using the Basic Local Alignment Search Tool (BLAST). The oat sequence showed similarity to only one wheat (*Triticum aestivum* L.) sequence with the identification HG670306 (Choulet et al. 2014). Oat sequence showed a molecular identity of 92% with the wheat sequence. In wheat, the sequence is located onto a region of chromosome 3B, with approximately 774 megabases, 5,326 protein-coding genes and 1,938 pseudogenes; transposable elements make up 85% of the chromosome sequence (Choulet et al. 2014).

The molecular marker GMI_ES17_c5923_221 could not be located on the genetic linkage map developed for the UFRGS 01B71114-1-3 x UFRGS 006013-1 mapping population. The high association between this marker and the multiflorous spikelet phenotype in this population, as determined in the present study, indicates that this marker may be linked to the major gene involved in controlling the character. The validation of this marker in other populations and germplasm of hexaploid oats is key to its use as diagnostic marker in the differentiation between genotypes with normal spikelet or multiflorous spikelet phenotypes. The GMI_ES17_c5923_221 marker may also be of use in molecular-marker assisted selection, as the multiflorous spikelet trait is highly correlated with the naked trait. This would increase the efficiency of breeding programs in selecting stable naked oat genotypes, eliminating the environmental effect on the expression of the naked trait in oat.

Thus, the present study reports the first dataset containing SNP markers associated with the multiflorous spikelet in hexaploid oat. The results assessed may help in the efficient development of naked oat genotypes in genetic breeding programs. These data also form a foundation for future studies seeking to elucidate the genetic and molecular mechanisms involved in the control of multiflorous/naked traits in hexaploid oat.

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