J. Bányai, P. Szűcs, I. Karsai, K. Mészáros, Cs. Kuti, L. Láng, Z. Bedő

Agricultural Research Institute of the Hungarian Academy of Sciences,
Martonvásár 2462, Hungary; Corresponding author: bjudit@mail.mgki.hu

SUMMARY

A total of 96 winter wheat (*Triticum aestivum* L.) cultivars registered in Hungary were analysed using 15 wheat microsatellite markers located on different chromosome arms. Analyses revealed 91 SSR alleles with sizes ranging from 123–239 base pairs. The total number of alleles per locus ranged from 2 (Gwm664 and Gwm415) to 11 (Gwm219) with an average number of 6.1. The polymorphic information content (PIC) values ranged from 0.06 to 0.85 with an average number of 0.60 for all markers. Several markers included allele sizes characteristic of a single or a small number of cultivars. At most 9 SSR markers were required to distinguish the 96 cultivars, so the simple sequence repeats could serve as a relatively cheap, rapid method for identifying winter wheat cultivars.
Key words: marker, microsatellite, rare alleles, unique alleles

INTRODUCTION

One important application of molecular markers in plant system involves improvement in the efficiency of conventional plant breeding by carrying out indirect selection through molecular markers linked to the traits of interest – both simple and quantitative trait loci (QTL) – because these markers are not influenced by the environment and can be scored at all stages of plant growth (Gupta *et al.* 1999). DNA markers can also be used for testing origin, pedigree and homogeneity. In addition, molecular markers are able to reveal differences between the DNA sequences of different varieties, making them suitable for variety identification. PCR-based genotyping is favoured over other techniques because it is fast, does not require radioactive labelling and can be carried out with very small amounts of genomic DNA (Lima *et al.* 2003, Nagy 1999, Szűcs *et al.* 2000).

Microsatellites are direct tandem repeated sequences of DNA with a repeat size ranging from one to six base pairs. They are locus-specific, usually very polymorphic due to the high level of variation in the number of repeats, hyper-variable and co-dominant, so their information content is very high (Gianfranceschi *et al.* 1998, Gupta *et al.* 2002, Matus and Hayes 2002, Plaschke *et al.* 1995, Rampling *et al.* 2001, Roussel *et al.* 2004, Tixier *et al.* 1997). As markers are defined for use in a breeding programme, the focus changes to a smaller set of primer pairs that will be used to screen large numbers of DNA samples either from the analysis of progeny from a cross or the routine checking of cultivar identity in the industry (Rampling *et al.* 2001). Even though the genome of bread wheat is hexaploid and extremely large, the microsatellite allelic patterns generated through PCR are capable of individualizing cultivars (Lima *et al.* 2003).

In many countries variety characterisation based on the field evaluation of morphological and physiological descriptors is used for the protection of plant breeders' rights and for registration in national catalogues. New approaches relying on the use of biochemical and molecular characteristics are currently being considered, in particular regarding their use for DUS (Distinctness, Uniformity, Stability) testing and for the

assessment of essential derivation (Teriaca *et al.* 2004). Manifesto *et al.* (2001) concluded that molecular markers are a useful complement to morphological and physiological characterization of cultivars because they are plentiful, independent of tissue or environmental effects, and allow cultivar identification early in plant development.

The present work demonstrates the application of wheat microsatellites for cultivar identification, and the estimation of the usefulness of microsatellites for investigations in genetically closely related material.

MATERIAL AND METHODS

Plant material. Analyses were made on 96 cultivated winter wheat varieties, of which 61 originated from Hungary, 21 from Western Europe, 11 from Middle Europe and 3 from Eastern Europe (Table 1).

Table 1. List of wheat cultivars analysed and their origin

Cultivar	Origin	Cultivar	Origin	Cultivar	Origin	Cultivar	Origin
ABONY	H	GK-CIPÓ	H	GK-TÜNDÉR	H	MV-MAGDALÉNA	H
ADRIANA	HR	GK-CSONGRÁD	H	GK-VÉKA	H	MV-MAGVAS	H
ALEX	RO	GK-ÉLET	H	GK-VERECKE	H	MV-MAMBO	H
ALFÖLD	H	GK-FAVORIT	H	GK-ZUGOLY	H	MV-MARISKA	H
ÁTRIUM	A	GK-GARABOLY	H	GUARNI	F	MV-MARSALL	H
BALADA	CZ	GK-GÓBÉ	H	HAJDUSÁG	H	MV-MARTINA	H
BORA	CZ	GK-HATTYÚ	H	HP-ÁRKUS	H	MV-MEZŐFÖLD	H
BŐSÉG	F	GK-HÉJA	H	HUNOR	H	MV-PÁLMA	H
BOSZANOVA	CZ	GK-HOLLÓ	H	JAREBICA	YU	MV-PALOTÁS	H
BREA	CZ	GK-JÁSZSÁG	H	YUBILEINAYA-50	UA	MV-SUBA	H
BRUTUS	A	GK-KALÁSZ	H	KG-KUNHALOM	H	MV-SÜVEGES	H
BUZOGÁNY	F	GK-MALMOS	H	KG-MAGOR	H	MV-SZIGMA	H
CAPO	A	GK-MARCAL	H	KOMPOLTI-3	H	MV-TAMARA	H
CARLO	A	GK-MISKA	H	LINDA	A	MV-VERBUNKOS	H
COMPLET	D	GK-MURA	H	LUDWIG	A	MV-VILMA	H
DEA	A	GK-ÖTHALOM	H	LUPUS	A	NIAGARA	CZ
EUREKA	F	GK-PETUR	H	MAXIMUS	D	ORPIC	F
FATIMA-2	RO-H	GK-PINKA	H	MF-KAZAL	FR	ORVANTIS	F
FLORI-2	F	GK-RÁBA	H	MV-CSÁRDÁS	H	POBEDA	YU
FURORE	A	GK-RUBINTOS	H	MV-DALMA	H	RENESANSA	YU
GASPARD	F	GK-SÁRA	H	MV-EMESE	H	RUSIJA	YU
GK-ATI	H	GK-SAS	H	MV-EMMA	H	SATURNUS	A
GK-BAGOLY	H	GK-SMARAGD	H	MV-KÖDMÖN	H	UKRAINKA	UA
GK-CINEGE	H	GK-SZIVÁRVÁNY	H	MV-KUCSMA	H	ZLATKA	YU

SSR allele identification. DNA was isolated from 100 mg ten-day-old leaf tissue of two individuals per cultivar with the Dneasy Plant Mini Kit (Viogene, France). Gwm (also known as Wms) primers were previously developed and mapped by Röder *et al.* (1998). The primers used in the present SSR analyses are listed in Table 2. PCR reactions were performed in a volume of 15 µl reaction mixture containing 400 nM each of forward and reverse primers, respectively, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer (Promega, WI), 0.5 U Taq polymerase (Promega, WI) and 50-100 ng of template DNA. The following two-step PCR program was run in a Perkin-Elmer GeneAmp 9700 thermocycler (Applied Biosystems, CA). After 2 min at 94°C, 35 cycles were performed with 20 s at 94°C, 1 min at either 63, 65 or

66°C (depending on the individual microsatellite), and a final extension step of 10 min at 72°C, followed by a 4°C holding step. To separate the amplification products, sequencing poliacrylamide gel electrophoresis was carried out on a FB-SEQ-3545 gel system (Fisher Biotech, Australia). Samples were run for 2 hours at constant 75 W. For silver staining the 6% polyacrylamide gel was placed in 10% acetic acid for 20 min with gentle shaking. The gel was washed with water three times, each for 2 min, and stained in a solution containing 0.1% silver nitrate and 0.15% formaldehyde for 30 min. After a quick rinse with water the patterns were revealed by adding the developer (3% sodium carbonate, 0.15% formaldehyde, 0.1% sodium thiosulphate). The reaction was stopped in 10% acetic acid and the gel was washed with water three times and dried at room temperature.

Data analysis. The total number of alleles and the number of rare and unique alleles were observed. An allele was considered to be rare if its frequency was lower than 0.05. Unique alleles are characteristic of a single variety. The polymorphic information content (PIC) value was calculated for each locus (Anderson *et al.* 1993):

$$PIC = 1 - \sum_{i=1}^{n} p^2 i$$

where p_i is the frequency of the *i*th allele.

Using the database created using the Breeder Program Package (Láng *et al.* 2001) the number of primers was reduced iteratively to obtain the optimum marker combination that was necessary and sufficient to distinguish the varieties.

RESULTS AND DISCUSSION

The SSR allele size range, the total number of alleles, the number of rare alleles and unique alleles, the polymorphic information content (PIC) values are presented for each of the 15 microsatellite locus in Table 2. The majority of the varieties were homozygous at all the SSR loci, but 11 varieties exhibited heterozygosity at six loci (Gwm044, Gwm011, Gwm149, Gwm190, Gwm219, Gwm312). The highest level of heterozygosity (4.4%) was found at Gwm312, four cultivars (Brutus, Gk-Garaboly, Gk-Verecke, Mv-Kucsma) were heterozygous. One cultivar (Pobeda) was heterozygous with respect to two markers (Gwm044, Gwm219). If amplification products were missing from the gel, the reaction was repeated. In several cases a product was still not obtained, but this could not necessarily be interpreted as a null allele. The occurrence of null/missing alleles was observed in 10 of the 15 markers. The total number of alleles per locus ranged from 2 (Gwm664 and Gwm415) to 11 (Gwm219) with an average number of 6.1 in the 123-239 bp size range. The number of rare alleles varied, with none at two loci (Gwm005, Gwm415) but 5 at Gwm219 and Gwm312.

PIC values ranged from 0.061 to 0.846 with an average number of 0.598 for all markers. The lowest PIC value was found for microsatellite marker Gwm664 (0.061), but this was associated with a rare allele, a fragment of 154 bp being obtained for three varieties, compared with 156 bp for all the other varieties. This marker is thus ideal for distinguishing these three varieties (Dea, Mv-Mariska, Orvantis).

Nine microsatellite markers (Gwm044, Gwm135, Gwm149, Gwm194, Gwm219, Gwm257, Gwm261, Gwm312, Gwm566) revealed allele size unique for a single cultivar -six of them in two cultivars each- giving a total of 15 unique alleles characteristic of a single variety. Using the database created with the Breeder Program Package, the number of primers was reduced iteratively to obtain the optimum marker combination that was necessary and sufficient to distinguish the 96 varieties.

Table 2. Chromosome localisation, primer annealing temperature (T_a), allele size range, total number of alleles, number of rare alleles, rare allele frequency (%), number of unique alleles and polymorphic information content (PIC) values for 15 microsatellite loci

SSR loci	Chromosome localisation	T_a (°C)	Allele size range (bp)	No. of alleles	No. of rare alleles	Rare alleles (%)	No. of unique alleles	PIC
Gwm005	3A	68.0	165-180	4	0	0	0	0.632
Gwm011	1B	62.0	190-208	8	3	38	0	0.804
Gwm044	7D	68.0	160-184	10	4	40	2	0.804
Gwm135	1A	64.0	138-198	6	2	33	2	0.724
Gwm149	4B	63.5	154-170	7	4	57	1	0.489
Gwm190	5D	65.0	200-216	6	3	50	0	0.535
Gwm194	4D	63.0	131-140	6	2	33	2	0.684
Gwm219	6B	67.5	166-202	11	5	45	2	0.846
Gwm257	2B	65.0	194-198	3	1	33	1	0.511
Gwm261	2D	66.0	164-196	6	2	33	2	0.475
Gwm312	2A	65.0	191-239	10	5	50	2	0.751
Gwm415	5A	65.5	134-136	2	0	0	0	0.465
Gwm566	3B	65.5	123-135	7	2	29	1	0.757
Gwm610	4A	64.0	170-174	3	1	33	0	0.443
Gwm664	3D	65.0	154-156	2	1	50	0	0.061

The allele combinations of eight markers (Gwm011, Gwm044, Gwm149, Gwm190, Gwm194, Gwm219, Gwm261, Gwm312) were able to distinguish all but two of the varieties (Gk-Jászság, Gk-Marcal). Five further Gwm primer pairs were found (Gwm135, Gwm257, Gwm415, Gwm566, Gwm610), any one of which could be added to these eight markers to allow all 96 varieties to be distinguished. The Gwm610 microsatellite marker could be used to distinguish between Mv-Pálma and its sister line Mv-Vilma (Figure 1), while Gwm044 was suitable for distinguishing between the sister lines Gk-Héja and Gk-Holló.
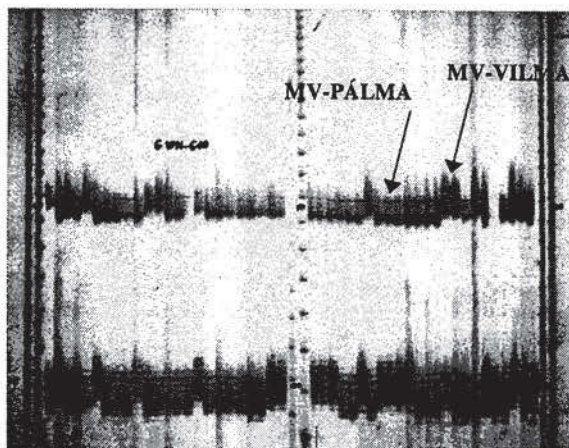

Figure 1. The sister lines Mv-Pálma and Mv-Vilma can be distinguishing using the marker Gwm610

Microsatellite markers produce high level of polymorphism, so SSR markers are extremely useful for variety identification. In the present experiment a set of 15 SSR primer pairs was selected, optimised and used to analyse 96 European winter wheat cultivars. Using 9 SSR primer pairs, all the cultivars could be distinguished from one another. It proved possible to distinguish even between sister lines, despite the very slight difference in their genomic composition. Röder *et al.* (2002) distinguished 502 European wheat varieties using 19 microsatellite markers, but varieties originating from the same parental lines gave identical patterns. It is clear that microsatellite markers represent a powerful tool for wheat cultivar discrimination. There is a growing demand from wheat breeders for a public, constantly updated international database containing the microsatellite allele sizes of all known varieties (Röder *et al.* 2002). The microsatellite allele size data obtained in the present work could form part of such a database.

ACKNOWLEDGEMENTS

REFERENCES

Anderson, J. A, G. A. Churchill, J. E. Autrique, S. D. Tanksley, M. E. Sorrells (1993). Optimising parental selection for genetic linkage maps. Genome 36: 181-186.

Gianfranceschi L, N. Seglias, R. Tarchini, M. Komjanc, C. Gessler (1998). Simple sequence repeats for the genetic analysis of apple. Theor. Appl. Genet. 96: 1069-1076.

Gupta, P. K, R. K. Varshney, P. C. Sharma, B. Ramesh (1999). Molecular markers and their applications in wheat breeding. Plant Breeding 118: 369-390.

Gupta, P. K, H. S. Balyan, K. J. Edwards, P. Isaac, V. Korzun, M. Röder, M.-F. Gautier, P. Joudrier, A. R. Schlatter, J. Dubcovsky, R. C. De la Pena, M. Khairallah, G. Penner, M. J. Hayden, P. Sharp, B. Keller, R. C. C. Wang, J. P. Hardouin, P. Jack, P. Leroy (2002). Genetic mapping of 66 new microsatellite (SSR) loci in bread wheat. Theor. Appl. Genet. 105: 413-422.

Láng, L, Cs. Kuti, Z. Bedő (2001). Computerised data management system for cereal breeding. Euphytica 119: 235-240.

Lima V. L, H. A. Seki, F. D. Rumjanek (2003). Microsatellite polymorphism in wheat from Brazilian cultivars; inter- and intra-varietal studies. Genetics and Molecular Biology, 26: 349-353.

Manifesto M. M, A. R. Schlatter, H. E. Hopp, E. Y. Suárez, J. Dubcovsky (2001). Quantitative evaluation of genetic diversity in wheat germplasm using molecular markers. Crop Sci. 41: 682-690.

Nagy, I. (1999). Improved PCR-based techniques for polymorphism investigations. Növénytermelés 48: 421-434.

Matus, I. A, P. M. Hayes (2002). Genetic diversity in three groups of barley germplasm assessed by simple sequence repeats. Genome 45: 1095-1106.

Plaschke J, M. W. Ganal, M. S. Röder (1995). Detection of genetic diversity in closely related bread wheat using microsatellite markers. Theor. Appl. Genet. 91: 1001-1007.

Rampling L. R, N. Harker, M. R. Shariflou, M. K. Morell (2001). Detection and analysis systems for microsatellite markers in wheat. Aust. J. Agric. Res. 52: 1131-1141.

Roussel V, J. Koenig, M. Beckert, F. Balfourier (2004). Molecular diversity in French bread wheat accessions related to temporal trends and breeding programmes. Theor. Appl. Genet. 108: 920-930.

Röder M. S, V. Korzun, K. Wendehake, J. Plaschke, M. H. Tixier, P. Leroy, M. W. Ganal (1998). A microsatellite map of wheat. Genetics 149: 2007-2023.

Röder M. S, K. Wendehake, V. Korzun, G. Bredemeijer, D. Laborie, L. Bertrand, P. Isaac, S. Rendell, J. Jackson, R. J. Cooke, B. Vosman, M. W. Ganal (2002). Construction and analysis of a microsatellite-based database of European wheat varieties. Theor. Appl. Genet. 106: 67-73.

Szűcs P., A. Juhász, I. Karsai, L. Láng, O. Veisz, Z. Bedő (2000). Use of molecular markers for studying genetic diversity in durum wheat (*Triticum durum* Desf.). J. Genet. Breed. 54: 25-33.

Teriaca M. S, C. Milanesi, M. Maccaferri, E. Noli, S. Conti (2004). Distinctness among lines of durum wheat based on morphological and molecular characteristics. Proceedings of the XLVIII Italian Society of Agricultural Genetics. 15-18. September.

Tixier M. H, P. Sourdille, M. Röder, P. Leroy, M. Bernard (1997). Detection of wheat microsatellites using a non radioactive silver-nitrate staining method. J. Genet. Breed. 51: 175-177.