doi: 10.17221/153/2014-CJGPB

A Rapid and Cost-effective Procedure for Delineation and Utilization of Genomic Microsatellites for Paralleled Genotyping in *Vicia faba*

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Abstract

Aleksić J.M., Banović B., Miljuš-Đukić J., Jovanović Ž., Mikić A., Ćupina B., Zlatković B., Anđelković S., Spanu I., Jelić M., Maksimović V. (2015): A rapid and cost-effective procedure for delineation and utilization of genomic microsatellites for paralleled genotyping in *Vicia faba*. Czech J. Genet. Plant Breed., 51: 36–39.

Although more than 400 microsatellite loci are currently available for *Vicia faba* L. (faba bean), an important food and feed grain crop legume, they have not yet been used for comprehensive molecular characterization of this crop. We report a three-step procedure for rapid and cost-effective delineation and utilization of informative genomic nuclear SSRs for paralleled genotyping in faba bean suitable also for other species: (*i*) pre-selection of loci generating PCR products of expected lengths which are potentially polymorphic (achieved by PCR amplification in bulked samples); (*ii*) exclusion of loci burdened with persistent null alleles and multilocus amplification products (based on PCR amplification of pre-selected loci in individual genotypes), and (*iii*) multiplexing. We demonstrate also that genomic SSRs are promising molecular tools for molecular characterization of faba bean required also for crop improvement.

Keywords: faba bean; genomic SSRs; multiplexing; Type-it kit

Faba bean (*Vicia faba* L.), an important grain crop legume used mainly for food and feed (Duc 1997; Duc *et al.* 2010), has been studied genetically to date by rather diverse molecular markers (reviewed in Duc *et al.* 2015). To date, more than 400 nuclear microsatellites (simple sequence repeats – SSRs), which are preferred molecular tools for genotyping in plant and animal species because they are hypervariable, multiallelic, co-dominant, highly reproducible and amenable to automation and high throughput genotyping (Ellegren 2004; Varshney *et al.* 2005), have been developed for faba bean (reviewed in Duc *et al.* 2015), but have not yet been used for large-scale molecular characterization of this crop. How-

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ever, utilization of genomic SSRs, which are found usually within non-coding and anonymous regions of the nuclear genome (Morgante *et al.* 2002; Ellegren 2004), may be rather challenging in faba bean, characterized by exceptionally large (c. 13 Gb) and repetitive nuclear genome (above 85%) (Young *et al.* 2003) with a high number of retrotransposon copies (Pearce *et al.* 1996), because they may generate multilocus amplification products especially if found within retrotransposons as observed in pea (Smýkal *et al.* 2009).

We report a rapid and cost-effective procedure for delineation and utilization of informative and reliable genomic SSRs, which are presumably more polymorphic than EST-SSRs (expressed sequence tagged SSRs, Ellegren 2004; Varshney *et al.* 2005), for paralleled genotyping in faba bean. Ten out of 73 genomic SSRs reported by Zeid *et al.* (2009), selected based on repetition motifs (di-nucleotides), structure (perfect and interrupted SSRs) and polymorphism information content values (≥ 0.53, Zeid *et al.* 2009), were used (Table 1). The plant material comprised 37 individuals from three faba bean traditional landraces from Serbia (nine individuals from Bački Petrovac 2 – abbreviation BAP2; 13 individuals from Pepeljevac – PPL, and 15 from Zalogovac – ZLG), which are spring, major seed size faba beans. The seeds, acquired from local farmers in autumn 2012, were sown and grown to seedlings. Approximately 30 mg of silica-gel dried young leaves per individual, homogenized with TissueLyser II (Qiagen, Valencia, USA), was used for DNA extraction following the procedure of Aleksić *et al.* (2012).

SSR loci were validated via a three-step procedure: (a) pre-selection of loci, applied for testing whether SSRs loci would generate amplicons of expected lengths and display polymorphism in our source material; (b) discrimination of loci, required for assessing whether pre-selected loci harbour null alleles, and to distinguish between single-copy loci and those generating multilocus amplification products; and (c) multiplexing via three approaches (post-PCR multiplexing, also called multi-pooling; multiplexing via simultaneous PCR amplification of two or more loci in a single PCR reaction, hereafter referred to as standard multiplexing; and multiplexing using Type-it Microsatellite PCR kit (Qiagen, GmbH, Hilden, Germany).

For the pre-selection step, equal quantities of DNAs (100 ng) from all individuals comprising each out of three landraces were pooled, and such a bulk DNA was used as a template for PCR amplification of individual SSRs. Pre-selected SSRs were used for the discrimination step, i.e. for PCR amplification

Table 1. Characteristics of ten genomic microsatellites tested in faba bean and results of their PCR amplification in bulked samples from three traditional landraces from Serbia

No.	Locus	Data taken from Zeid *et al.* (2009)		PIC	Number of alleles and size range per locus in three traditional landraces from Serbia					
		repeat motif and length	expected size (bp)		BAP2 (9)		ZLG (13)		PPL (15)	
					alleles	range (bp)	alleles	range (bp)	alleles	range (bp)
1	VfG1	(AG)15	224	0.70	2	205–231	6	203–241	7	203–233
2	VfG3	(AG)7 + 14	159	0.62	2	149–153	4	151–167	9	181–219
3	VfG10	(AG)5	219	0.53	6	217–249	8	219–287	14	217–251
4	VfG13	(AG)10	191	0.57	3	186–190	6	181–209	7	181–207
5	VfG19	(AG)9	174	0.72	5	173–201	10	175–211	6	165–203
6	VfG27	(AG)17	206	0.54	6	193–224	11	181–227	13	181–225
7	VfG28	(AG)19	220	0.65	2	205–266	4	196–228	5	196–238
8	VfG41	(AG)7 + 10	217	0.67	5	193–217	7	189–217	9	189–221
9	VfG69	(AG)9	195	0.61	4	189–221	6	191–221	7	191–221
10	VfG87	(AG)10	242	0.61	8	244–266	9	234–262	5	232–260

PIC – polymorphism information content; BAP2, ZLG and PPL – traditional landraces Bački Petrovac 2, Zalogovac and Pepeljevac, respectively; numbers in brackets represent numbers of individuals from each landrace used for assembling bulk DNAs

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in all nine individuals from a single landrace, BAP2. In these two steps, PCR reactions were carried out following Zeid *et al.* (2009) with minor modifications. They were performed in 25 µl volumes containing: 100 ng template DNA; 1 × *Taq* Buffer with $(NH_4)_2SO_4$ (Fermentas, Vilnius, Lithuania); 2.5mM $MgCl_2$ (Fermentas, Vilnius, Lithuania); 0.2mM dNTPs (Fermentas, Vilnius, Lithuania); 0.1µM of each F and R (reverse) primer; 0.80% BSA (Bovine Serum Albumin, Promega, St. Louis, USA); and 0.025 U of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). PCR profiles were as follows: denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 s; annealing at 60°C for 1 min; extension at 72°C for 1 min.; and final extension of 10 min at 72°C.

Loci passing a discrimination step were used for multiplexing. For multi-pooling, amplicons obtained in a discrimination step were pooled, while for each PCR assay of the standard multiplexing, equal amounts of primers (0.1µM of each primer) for amplification of two or more loci were used in a single reaction. PCR reactions and cycling profiles were the same as those used in previous steps. Multiplexing with Type-it kit was performed following manufacturer's instructions except that PCR assays were carried out in 6 µl volumes containing 1 µl of template DNA (50 ng) and 5 µl of Type-it ready-to-use PCR mix.

In all PCR assays, fluorescently labelled forward (F) primers (Dye Set DS-33, Applied Biosystems, USA) from each primer pair were used, and PCR products were separated commercially via capillary electrophoresis by Macrogen Europe, Amsterdam, The Netherlands (http://dna.macrogen.com/eng/) using 96-capillary 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Inc., Foster City, USA). Sizing of fragments was performed manually with GeneMapper ver. 4.0 (Applied Biosystems, Foster City, USA).

All ten genomic SSRs tested in faba bean passed preselection as they all generated amplicons, and displayed multiple amplification products indicative of their potential multiallelic nature. Otherwise, they would be discarded at this stage. However, based on outcomes of the discrimination step, four loci were discarded: VfG1 and VfG28, due to the occurrence of null alleles (amplicons were obtained in only three and four individuals, respectively), and VfG10 and VfG87, due to the occurrence of multilocus amplification products (up to four amplicons of expected lengths per locus). The number of alleles at the remaining six loci, combined for paralleled genotyping in nine individuals of BAP2 as follows – the first set of loci comprised VfG27 (yellow), VfG41 (green) and VfG69 (blue), the second set of loci comprised VfG3 (yellow), VfG13 (green) and VfG19 (blue), ranged from two alleles per locus (VfG3) to six alleles per locus (VfG27) in BAP2.

However, during both pre-selection and discrimination steps, abundant non-specific amplicons of lengths lower than expected were observed at almost all loci, and in some cases during the pre-selection, it was unclear whether they represent non-specific products or alleles. The discrimination step additionally revealed that the heights of these non-specific peaks in electrophoregrams may be much higher than the heights of peaks of expected lengths. These peaks are generally undesired because they may hamper allele sizing on an automated DNA fragment analyzer due to the inability of genotyping software to fully correct for background fluorescence resulting from the partial overlap of emission spectra for fluorescent dyes (Hayden *et al.* 2008). This was particularly pronounced in electrophoregrams obtained upon multi-pooling and standard multiplexing, which were rather difficult to read because non-specific amplicons of one locus were in the range of amplicons of expected length of another locus. Furthermore, the heights of peaks of expected lengths were generally below a threshold of 500 relative fluorescence units (RFU), and had a distorted appearance in some cases. Although it was possible to perform accurate allele sizing at certain loci in certain individuals, these findings altogether reveal that all six loci, which passed pre-selection and discrimination step, should be discarded.

Surprisingly, multiplexing with Type-it kit was successful. Non-specific shorter amplicons were generally missing, and the heights of peaks of expected lengths were much above the threshold of 500 RFU. This was most likely due to the specific features of components of this kit which is specifically developed for multiplex PCR-based analyses of microsatellite and minisatellite loci and is able to ensure high product yields for all amplicons in a multiplex experiment and to overcome the problem of uneven product yield, large differences in the intensity of multiple fluorescent signals, overrepresentation of stutter and n–1 peaks, and allelic drop-outs. To date, multiplexing of SSRs with Type-it kit was reported in numerous studies (e.g. Lepais & Bacles 2011; Postolache *et al.* 2014), while in faba bean, this kit in addition enabled the usage of loci that would most likely be discarded. However, due to the potential inconsistent shifts in lengths of fragments obtained during standard and multiplex assays (the lengths of amplicons obtained by multiplexing with Type-it kit were shifted towards longer lengths in a systematic manner, i.e. plus two to three bases depending on the locus, while at locus VfG19,

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shifts in lengths were more pronounced at lower than at higher lengths, data not shown), some additional tests may be required for harmonizing allele sizing when combining these two approaches.

Based on 174 PCR and capillary electrophoresis assays each (30, 90 and 54 assays of both kinds for pre-selection, discrimination and multiplexing, respectively), we found that 60% of tested genomic SSRs reported by Zeid *et al.* (2009) may be used for genotyping in faba bean via standard PCR protocols but with a limited success due to the abundant non-specific amplicons that hamper allele scoring, which, on the other hand, preclude their utility for paralleled genotyping via multi-pooling and standard multiplexing. Given the large and repetitive faba bean nuclear genome (Young *et al.* 2003) with a high number of retrotransposon copies (Pearce *et al.* 1996), these findings question the utility of genomic SSRs for genotyping in this crop. Nonetheless, even these loci may be used for paralleled genotyping with Type-it kit and furthermore, they may turn out to be rather informative. Along with recently reported SNP loci in faba bean (Cottage *et al*. 2012), these markers are promising molecular tools for molecular characterization of faba bean required for crop improvement. Our procedure, developed for genomic SSRs in faba bean, may be used for rapid delineation and utilization of other informative microsatellites (genomic and/or EST-SSRs) in this crop, and is suitable also for other markers and species as well.

Acknowledgements. This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Research Grant No. 173005, and represents a contribution towards the project of bilateral collaboration between the Republic of Serbia and the Republic of France "Pavle Savić" FABAGRALE for the year 2012-2013, No. 680-00-132/2012-09/15.

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Received for publication July 22, 2014
Accepted after corrections January 13, 2015

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