International Scholars Journals

African Journal of Plant Breeding ISSN 2375-074X Vol. 2 (1), pp. 136-142, January, 2014. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Is HRM analysis suitable for SSR genotyping of a mapping population? A comparison of HRM and capillary electrophoresis for SSR genotyping in a mapping population

Bartosz Kozak[1], Renata Galek[1], Ewa Sawicka Sienkiewicz[1]

[1]Department of Genetics, Plant Breeding and Seed Production, Wroclaw University of Environmental and Life Sciences, 24a pl. Grunwaldzki, 50–363 Wroclaw, Poland.

Accepted 24 July, 2015

The aim of the study was to compare two methods for detection of polymorphism in a microsatellite locus, i.e. Capillary Electrophoresis (CE) - ABI and QIAxcel, and High Resolution Melting (HRM), in respect of correct and efficient genotyping of a narrow-leafed lupin mapping population. The difference in length of the generated products by three SSR primers, between the parental forms were: less than 10 bp, more than 10 bp, more than 20 bp. Both tested CE systems allowed to perform correct genotyping of the analyzed RILs and their parents. The HRM technique failed to correct genotype RILs and their parental forms with the markers generating polymorphic product difference in a length less than 10 bp. However, this technique enables correct and effective genotyping with the markers that generate polymorphic product difference in a length more than 10 pb. Of the two CE systems compared, QIAxcel was less time-and money-consuming.

Key words: SSR assay, capillary electrophoresis, ABI, QIAxcel, HRM, narrow-leafed lupin.

INTRODUCTION

Simple sequence repeats (SSR) markers, which are reproducible and co-dominant, represent one of the most frequently used systems in the molecular research of plants (Varshney et al. 2009). The high polymorphism of microsatellite sequences and their dispersal over the whole genome are responsible for the fact that analyses with the application of SSR markers yield reliable results when genetic diversity assessments are being attempted. Polymorphic SSR markers – if they have been found correlated with the genes that determine important agronomic traits – can also become a significant tool in selection activity, thus contributing to acceleration of the advancement in breeding of new cultivars. (Piquemal *et al*. 2005; Hearnden *et al*. 2007; Collard & Mackill 2008) SSR markers are particularly valuable due to their co-dominant character of inheritance. Nowadays, they are ranked among the most precise systems of markers used in defining the genetic variability of specimens within a population. Microsatellite sequences are built of long tandem replications of 1–6 nucleotides, located mainly in the non-coding parts of a genome (Wang *et al.* 1994). The SSR technique consists in identification of changes in the sequences of replications. Despite their numerous advantages, the SSR markers have also certain shortcomings, which lie behind their limited application in genetic investigations. The most significant of them is associated with the need to know the flanking sequence of the microsatellite in order to make it possible to elaborate specific starters as well as the difficulties involved with detection of the polymorphism of those loci which are characterized by insignificant difference in the number of replications of the microsatellite motif (Kalia et

Corresponding author. E-mail: renata.galek@up.wroc.pl

al. 2011). However, thanks to the dynamic development of the DNA sequencing techniques and rapid growth in different type of DNA sequence material deposited in data banks, searching for microsatellite sequences and developing specific starters get easier and easier from year to year (Bräutigam & Gowik 2010; Varshney *et al.* 2009). Consequently, SSR markers become more and more common in studies on further plant species, also those of less economic importance. The most frequently used systems of SSR markers separation are based on Capillary Electrophoresis (CE) (Wang et al. 2009). Recently many researchers have reported that High Resolution Malting (HRM) analysis can be a cost-effective alternative for CE in SSR genotyping (Mackay et *al.* 2008; Vossen *et al.* 2009).

Traditional agarose and polyacrylamide gel electrophoreses were widely used in the earliest microsatellite analyses. The media mentioned require simple devices and, what is more, the electrophoretic procedure can be carried out in most laboratories. It is laborintensive (e.g. gel preparation, staining and photographing), especially in the case of genomic linkage mapping, for which a abundant population and more molecular markers are needed. The major limitations of these two electrophoresis protocols are that they are not always amenable for accurately determining the sizes of alleles and recording data in an electronic format, thus making a downstream analysis problematic. The observed development of the techniques of DNA particles separation enables to solve the problems experienced when variability analyses with the use of the SSR technique are undertaken. Nowadays, microsatellite DNA analyses are most often performed with the application of automatic sequencers and fluorescently marked starters, which makes it possible to acquire precise results and have the process of data collecting automated. Yet, it is linked with higher costs of the analyses (Nowakowska 2006).

Recently, the molecular research makes more and more frequent use of the HRM technique, which involves melting of double-stranded DNA (dsDNA). Polymorphism in dsDNA is monitored by fluorescence variations and displayed as melting curves for the intercalating fluorescent dye can only bind to dsDNA (Mao *et al.* 2007). HRM has many advantages when compared with other genotyping techniques for it does not require any specialist equipment. Moreover, it allows to define not only differences in length but also in the sequences of the examined product (possibility to detect point mutations of single nucleotide polymorphism – SNP) (Reed & Wittwer 2004). This method is fast and cheap, too. It has been developed to be applied for identification of point mutations in the genetic research on humans (Margraf *et al.* 2006), but subsequently it found a quick use in genotyping also in plants. In the last years numerous reports have appeared on the application of this technique in analysis of plant genetic diversity with the use of SSR markers (Croxford *et al.* 2008; Knopkiewicz *et al.* 2014; Ganopoulos *et al.* 2011; Yu *et al.* 2013). These abundant reports indicate that the HRM technique can successfully be employed as a cheaper alternative to capillary systems, though the obtained results do not always agree with those acquired with the application of the latter methods. Also, there are non-numerous reports on the use of the HRM technique for analysis of the SSR markers polymorphism in mapping populations.

The aim of the study was a comparison of three methods of microsatellite array analyses: two devices – ABI 310 GeneticAnalyzer and QIAxcel System – both based on capillary electrophoresis, and the HRM system. Their cost and time absorption, accuracy of the obtained results and possibility of automation of the data analysis process have been evaluated.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

For analysis 10 randomly chosen RIL form *L. angustifolius* mapping population and their parental forms, cv Emir and breeding line LAE-1, were used.

Genomic DNA was extracted from young leaves using the CTAB method (Doyle & Doyle 1990) with minor modification.

SSR Markers amplification, ABI, QIAxcel and HRM genotyping

Preliminary analysis of 24 SSR primers pars (Gao *et al.* 2011) on parental form of mapping population allow to select 3 pairs with large, medium and small difference in generated makers between ‘Emir’ and LAE-1 (Tab1).

Amplification conditions for two capillary methods and sample separations - ABI 310 GeneticAnalyzer and the QIAxcel System Advance

Amplification of DNA was performed in a 15 µl reaction mixture using a 2xPCR mixture that contained Taq polymerase 0.1U/ul, dNTP mix 2mM, MgCl2 4mM (AA Biotechnology, Poland), 0.280 µM each primer in the case of QIAxcel and 45 ng of DNA using a Biometra thermal cycler for 35 cycles. In a case of ABI separation lower primers concentration were used: LaSSR_002 - 0.210 µM, LaSSR_003 – 0.140 µM, LaSSR_008 – 0.140 µM. Primers were also libed with fluorescent dye: for LaSSR_002 i LaSSR_003 JOE and for LaSSR_008 FAM (Sigma Aldrich)

After initial denaturation for 5 min at 95°C, each cycle was composed of 30-sec denaturation at 95°C, 30-sec annealing at 55–60°C depending on the primer (Table 1), 25-sec extension at 72°C with a final extension for 7 min at 72°C at the end of 35 cycles.

Sample separation - QIAxcel

The amplified products were analyzed with the application of the QIAxcel (Qiagen, Germany) capillary electrophoresis system with the QIAxcel DNA screening

Table 1. Characteristic of used SSR primers.

SSR Marker ID	Expected product size	Fwd primer Tm	Fwd primer sequence	Rev primer Tm	Rev primer sequence
LaSSR_002	215	60	TGATATCATGTGAGGCAACTACG	60	CTTCTCAATGCTGATTCAAGTACC
LaSSR_003	296	55	AAGTAGTATGATCAACGGAGAGG	55	CGAGTGAGAGAGAAGTGTTACC
LaSSR_008	291	60	TAAGCCGGAATGACTTCACC	60	TATGATCAGTTCACAGGCTTCG

Table 2. The length (in bp) of three SSR primers for tested L.angustifolius chosen ten recombinant inbred lines (RILs) and their parental forms.

		SSR primers								
		LaSSR_002			LaSSR_003			LaSSR_008		
	Genotypes	System of product separation								
		ABI310	QIAxcel	HRM	ABI310	QIAxcel	HRM	ABI310	QIAxcel	HRM
No		pz	pz	°C	pz	pz	°C	pz	pz	°C
1	EL/02/2/16-4	216	210	75,4	294	288	81,9	268	262	74,8
2	EL/02/2/20-1	219	211	75,4	297	292	81,8	267	264	74,8
3	EL/02/2/21	181	178	75,1	294	288	82,0	268	262	74,8
4	EL/02/2/22-8	181	177	75,2	294	289	81,9	258	251	75,2
5	EL/02/2/32	180	178	75,2	294	289	82,0	258	251	75,2
6	EL/02/2/39	216	209	75,5	297	291	81,8	259	251	75,2
7	EL/02/2/40-5	219	210	75,3	297	292	81,7	259	250	75,2
8	EL/02/2/41	216	211	75,5	293	288	81,7	268	264	74,8
9	EL/02/2/42	216	211	75,4	293	288	81,8	268	263	74,8
10	EL/02/1/1	180	181	75,1	297	296	81,9	259	255	75,2
11	EMIR	181	177	75,1	297	291	81,8	268	261	74,7
12	LAE-1	216	210	75,4	293	289	82,0	258	251	75,2

Kit (Qiagen, Germany) and the AM420 method. The QX Alignment Marker 15 bp/3 kbp (Qiagen, Germany) and QX DNA Size Marker 100 bp–2.5 kbp (Qiagen, Germany) were used in the analysis (Figure 2).

Sample separation - ABI 310

One µl of PCR product for tree tested primers pairs were taken and mix with 12 µl of foramide and 0,5 µl ROX500. Samples were heated to 95°C and then chilled on ice. Separation was performed on device ABI 310. Separation parameters were as follow: module GS STR POP4 (1mL) F, temp 60°C, inject samples at 15.0 KV for 10s, separation at 15.0 KV for 30'

Amplification and HRM analysis

The HRM curve was acquired and analyzed on Ilumina Eco® real-time PCR system.

Amplification of DNA was performed in a 10 µl reaction mixture containing 5 µl MIX (Bioline – 2xHRM Mix) and final concentrate of each pairs starters 0,4 µM, and 30 ng genomic DNA.

The HRM reaction procedure and melting analysis were performed as follows: a 3-min initial denaturation followed by 45 cycles of denaturation at 95°C for 15 s, annealing temperatures at 55-60 °C (Tab.1) for 15 s and extension at 72°C for 15 s.

The amplification cycles were immediately followed by the high-resolution melting steps: 95°C for 1 min, cooling to 55°C for 15s, and then the temperature was raised to 95°C with 25 fluorescent acquisitions per degree Celsius at this step.

RESULTS

Genotyping

Using three pairs of LaSSR starters, it was possible to

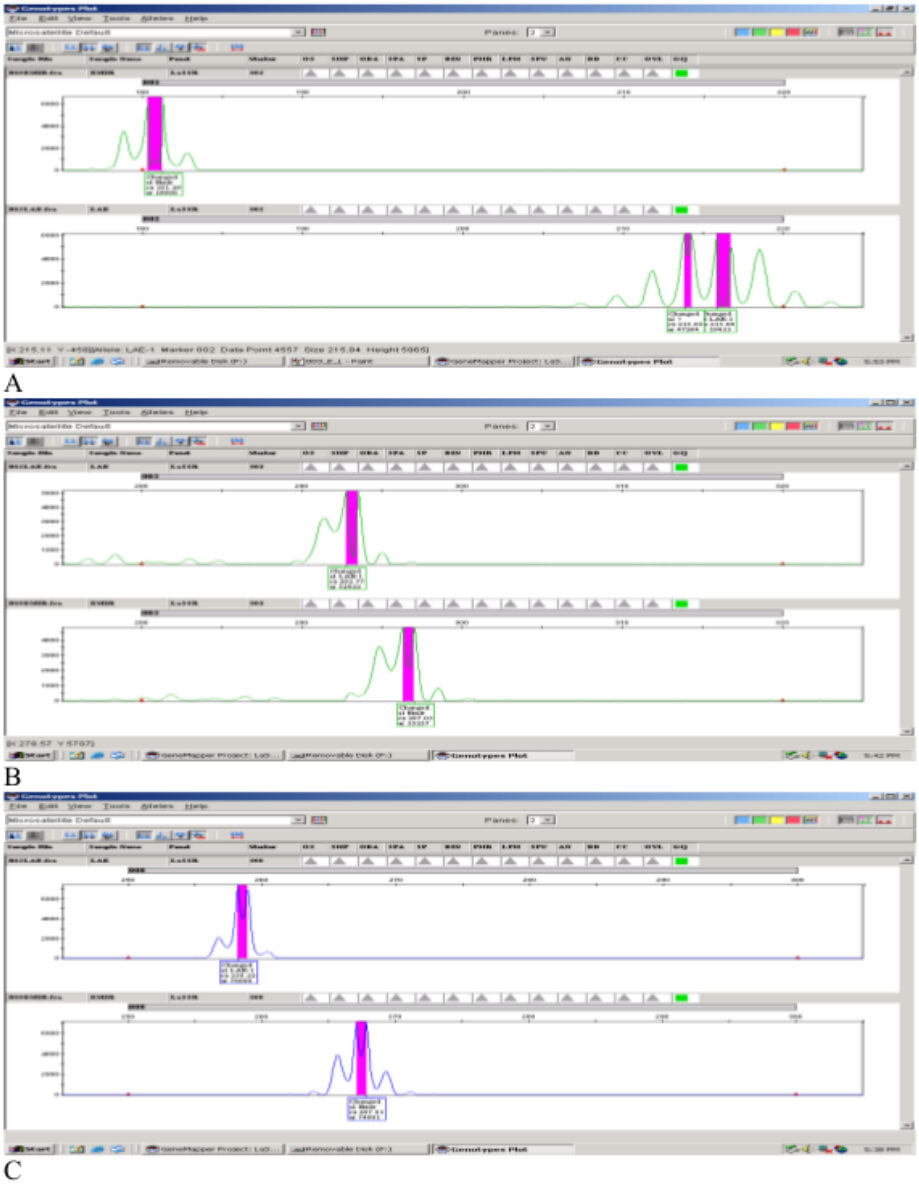

Figure 1. SSR markers genotyping using ABI 310 for parental forms (‘Emir’ – upper and LAE-1 – lower), A - LaSSR_002, B- LaSSR_003, C - LaSSR_008.

obtain polymorphic products of amplification for the genotypes of parental lines ’Emir’ and LAE-1 (Table 2) (Figure 1, Figure 2, Figure 3). The amplification products obtained approximated in size to the desired product (Table 1). In the analyzed recombinant inbred lines, a product characteristic of form ‘Emir’, i.e. allele of the “Emir” type, was amplified and in the case of form LAE-1 – allele of the ”LAE-1” type (Table 2).

When the ABI 310 system was applied for separation of the amplification products after using LaSSR_002 for the initial forms – ‘Emir’ and LAE-1 – of the mapping population, peaks 181 and 216 were obtained respectively (Table 2) (Figure1). When the QIAxcel system was used, the size of the product for ‘Emir’ was smaller by o 4 bp (177 bp), and for LAE-1 it was 6 bp (210 bp) (Table 2). In the case of analyses performed with the HRM technique, the result for parental forms ‘Emir’ and LAE-1 was respectively 75.1°C and 75.4°C (Figure 3).

With the application of the ABI 310 device for separation of amplification products after the use of LaSSR_003 for initial forms – ‘Emir’ and LAE-1 – of the mapping population, products of 297 bp and 293 bp were obtained respectively (Table 2). When comparing the obtained size of the products after amplification for the inbred lines (Table 2), it is clearly visible that four lines are character-

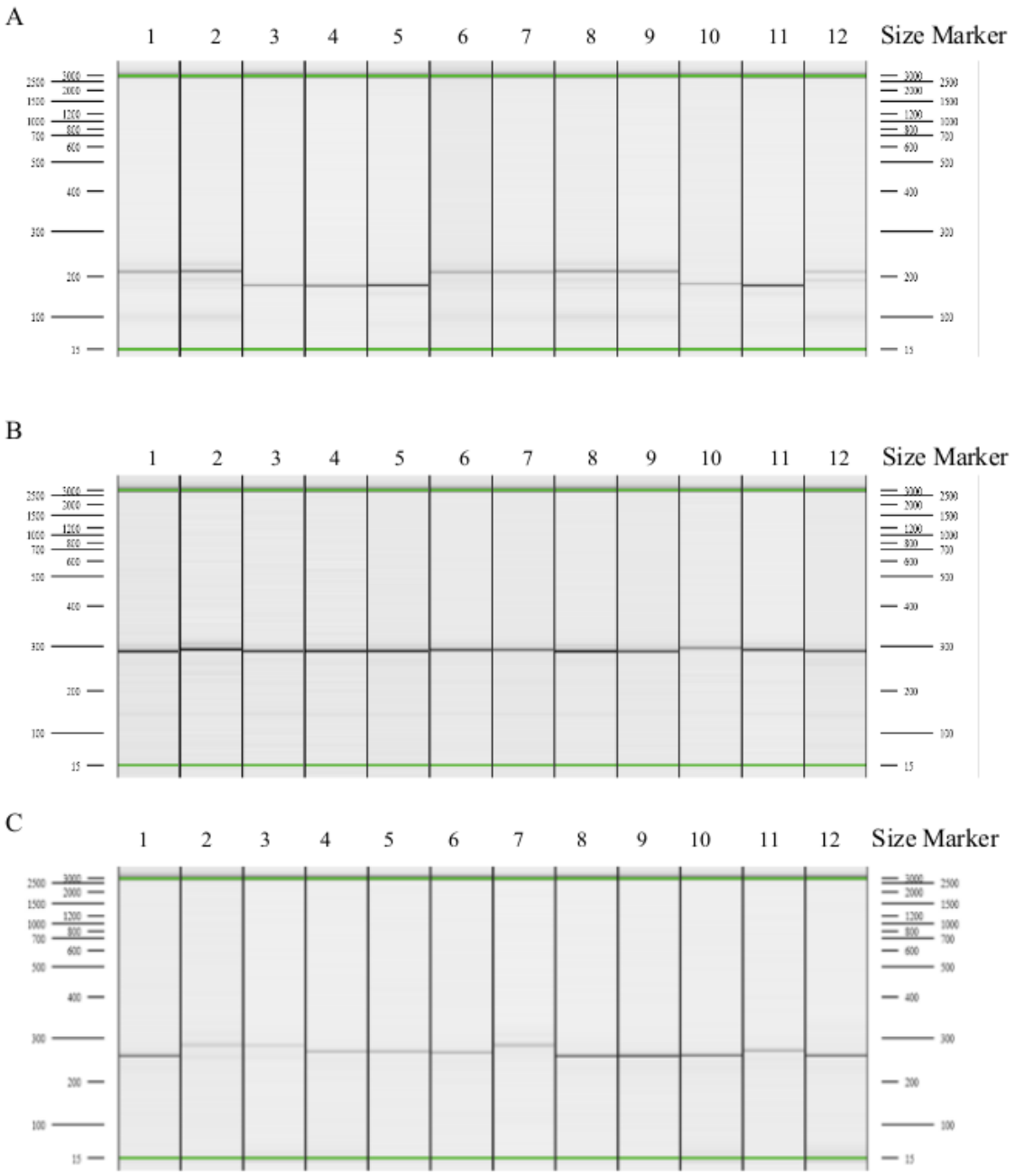

Figure 2. SSR markers genotyping using QIAxcel for parental forms ('Emir' – 11 and LAE-1 – 12), A - LaSSR_002, B- LaSSR_003, C - LaSSR_008.

ized by products typical of 'Emir', and six – of LAE-1. An analogous relation occurred with separation by the QIAxcel system, although with this technique the size of the product for 'Emir' was larger by 6 bp (291 bp), and that for LAE-1 – by 4 bp (289 bp). HRM analysis revealed nearly identical temperatures for the initial forms and for 90% of the analyzed inbred lines (Table 2).

When the ABI 310 device was used for separation of the products of amplification after application of LaSSR_008 for initial forms – 'Emir' and LAE-1 – of the mapping population, products of 268 bp and 258 bp were obtained respectively (Table 2). A comparison of the obtained size of the products after amplification for inbred lines (Table 2) clearly shows that five of the ten analyzed lines were

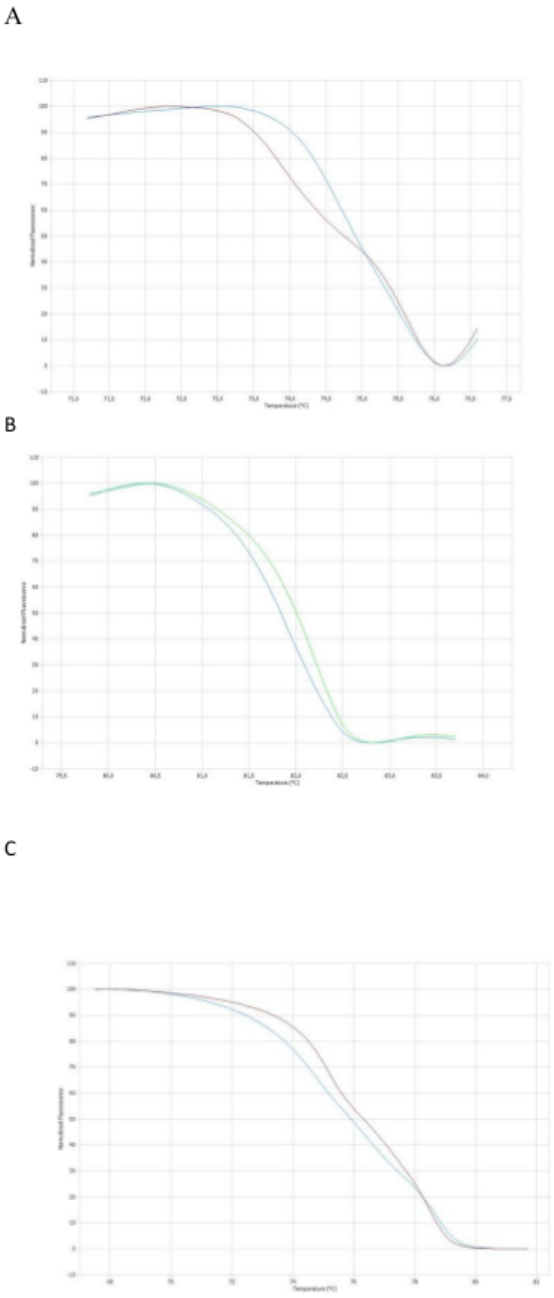

Figure 3. SSR markers genotyping using HRM for parental forms (‘Emir’ and LAE-1), A - LaSSR_002, B- LaSSR_003, C - LaSSR_008.

characterized by products of the number of bp typical of ‘Emir’, and the remaining – of LAE-1. An analogous interdependence was found in the case of separation by QIAxcel and HRM (Table 2).

An analysis of the results obtained for the LaSSR_002 and LaSSR_008 starters with the employment of three methods of genotyping revealed existence of polymorphism between parental forms cv ‘Emir’ and LAE-1 (Table 2). Out of the ten lines analyzed, six were characterized by an allele of the ”LAE-1” type, and four of the ”Emir” type for the LaSSR_002 starter. In the case of LaSSR_008, five lines had an allele of the ”Emir” type while the remaining an allele of the ”LAE-1” type at all the three separation methods applied, which has been confirmed by a statistical analysis (Table 2).

With the LaSSR_003 starter – for which the difference in the size of products in parental forms was the smallest of the three starters under analysis – statistically significant

differences between the group of the ”LAE-1” allele (6 lines) and that of the ”Emir” allele (4 lines) were obtained for two methods of the capillary separation system (ABI 310, QIAxcel). In the case of the third method employed, the differences between the allele types were found insignificant (Table 2).

Consumptiveness of work and money

The time required for carrying out the genotyping of 12 object with three SSR markers (understood as the time indispensable for preparation of samples, performing the PCR and an analysis of products, and for data collecting) reached 3 hours for the HRM analyses, 3.5 hours for the analyses with the application of the QIAxcel device, and 10 hours for the analyses using the ABI 310 device. The costs of the analyses with the compared systems amounted to 4.5 PLN (ca €4) per sample in the case of the HRM analyses, 5 PLN (ca €4.15) per sample for the QIAxcel system and 20 zł (ca €5) per sample for the ABI 310 device. The costs were assessed at the assumption of 200 samples needed for analysis with each marker.

DISCUSSION

The HRM technique did not properly differentiate the studied lines in the case of the pair of LaSSR003 starters, although this pair differentiated the parental forms. The HRM results are reliable for loci with a larger difference in the size of generated products (LaSSR002 and LaSSR008). On account of this, with the HRM system being used, one can assume occurrence of mistakes in genotyping, which can make correct construction of a linkage map impossible. For this reason, the system – in spite of having many advantages, such as low costs of analyses and low consumptiveness of labour – should not be applied to genotyping of a mapping population.

Capillary electrophoresis in combination with the application of fluorescently labelled primers provides high detection sensitivity to amplified DNA fragments (Ramachandran *et al.* 2003; Shi *et al.* 2003). However, the cost of the device, fluorescently labelled primers and sample preparation makes the use of most capillary sequencers uneconomical for routine microsatellite analyses. In comparison, the QIAxcel system is a relatively inexpensive instrument that uses disposable micro-channel cartridges containing a sieving-gel matrix with ethidium bromide (EtBr) dye to generate both gel images and allele sizes (Amirkhanian & Liu 2002; Liu *et al.* 1995). Most small- to mid-sized laboratories can afford this device for SSR assays. The QIAxcel does not require special primer labelling and provides comparable resolution as other capillary sequencers in one-tenth of running time (for 96 PCR samples). In our research, both systems allowed to correctly genotype the studied objects (Table 2). The obtained non-identical data on the size of separated amplification fragments may stem from the employed method of estimation of the size of the fragments analyzed by these two capillary systems.

The outcome of our study clearly indicates high time-consumptiveness of the ABI 310 system. It mostly results from the fact that this particular system uses a single capillary, i.e. each sample is analyzed separately, which is followed by an analysis of further samples consecutively. If more modern systems, such as ABI 3100 or ABI 3730 – the former with 16 and the latter even as many as 96 capillaries – were used, the time needed for analyzing 12 samples might be shortened to about 2.5 hours, which means a comparable time span as in the case of HRM analysis or one with the use of the QIAxcel device. When the number of samples to be analyzed by the QIAxcel device increases to 96, the time span will expand to about 12 hours as 12 samples can simultaneously be analyzed (the tool is equipped with 12 capillaries). For the ABI 310 system, the time indispensable for analysis of 96 samples will extend to 64 hours. However, when more modern devices, equipped with a larger number of capillaries (ABI 3100, ABI 3730), are used, this time can be reduced even up to 2–3 hours. Since such systems employ fluorescently stained starters, it is possible to separate 3 or even a greater number of SSR loci at the same time. Taking into consideration the time required and the cost of analyses, the QIAxcel system can be regarded as the most optimum for application in genotyping of a mapping populations with the use of SSR markers.

CONCLUSION

When an abundant mapping population is being genotyped, it is important that there is a possibility to automate the activities. Also, the costs involved in the analyses and the reliability of the obtained data are of equal significance. The HRM technique proved to require the least labour whereas genotyping with the use of the ABI system was found the most work-consuming. Taking into account the obtained results, the capillary methods effectively differentiate the parents and progeny, while the HRM technique is useful for genotyping loci of large or medium difference in size (up to about 10 bp). The costs of HRM analysis and those based on the QIAxcel device are approximately the same, whereas the analyses with the application of the ABI 310 device are about four times more expensive. The obtained results allowed to choose the QIAxcel capillary electrophoresis system as the most optimum method for genotyping a mapping population with SSR markers.

REFERENCE

Amirkhanian VD, Liu MS (2002). Low-Cost and High-throughput Multi-Channel Capillarybased Electrophoresis

(MCCE) System for DNA Analysis. Biomedical Nanotechnology Architectures and Applications, Bornhop DJ, Dunn DA, Mariella RP, Murphy Jr CJ, Nicolau DV, Nie S, Palmer M, Raghavachari R (Eds). Proc. SPIE 4626: 238-246.

Bräutigam A, Gowik U (2010): What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. Plant Biology, 12(6): 831-841

Collard BC, Mackill DJ (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philosophical Transactions of the Royal Society B: Biological Sciences, 363(1491): 557-572, doi:10.1098/rstb.2007.2170

Croxford AE, Rogers T, Caligari PD, Wilkinson MJ (2008): Highresolution melt analysis to identify and map sequence-tagged site anchor points onto linkage maps: a white lupin (*Lupinus albus*) map as an exemplar. New Phytology, 180: 594–607

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus, 12: 13-15

Ganopoulos I, Argiriou A, Tsaftaris A (2011): Microsatellite high resolution melting (SSR-HRM) analysis for authenticity testing of protected designation of origin (PDO) sweet cherry products. Food Control, 22(3): 532-541, doi:10.1016/j.foodcont.2010.09.040

Gao LL, Hane JK, Kamphuis LG, Foley R, Shi BJ, Atkins CA, Singh KB (2011). Development of genomic resources for the narrow-leafed lupin (*Lupinus angustifolius*): construction of a bacterial artificial chromosome (BAC) library and BAC-end sequencing. BMC Genomics, 12: 521 doi:10.1186/1471-2164-12-521

Hearnden PR, Eckermann PJ, McMichael GL, Hayden MJ, Eglinton JK, Chalmers KJ (2007). A genetic map of 1,000 SSR and DArT markers in a wide barley cross. Theoretical and Applied Genetics, 115(3): 383-391, doi:10.1007/s00122-007-0572-7

Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011). Microsatellite markers: an overview of the recent progress in plants. Euphytica, 177(3): 309-334, doi:10.1007/s10681-010-0286-9

Knopkiewicz M, Gawłowska M, Święcicki W (2014). The Application of High Resolution Melting in the Analysis of Simple Sequence Repeat and Single Nucleotide Polymorphism Markers in a Pea (*Pisum sativum* L.) Population. Czech J. Genet. Plant Breed. 50(2): 151-156

Liu MS, Zang J, Evangelista RA, Rampal S, Chen FTA (1995). Doublestranded DNA analysis by capillary electrophoresis with laser induced fluorescence using ethidium bromide as an intercalator. BioTechniques, 18: 316-323.

Mackay JF, Wright CD, Bonfiglioli RG (2008). A new approach to varietal identification in plants by microsatellite high resolution melting analysis: application to the verification of grapevine and olive cultivars. Plant Methods, 4: 8–17, doi:10.1186/1746-4811-4-8

Mao F, Leung WY, Xin X (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. BMC Biotechnology, 7: 76–91, doi:10.1186/1472-6750-7-76

Margraf RL, Mao R, Highsmith WE, Holtegaard LM, Wittwer CT (2006). Mutation scanning of the RET protooncogene using highresolution melting analysis. Clinical Chemistry, 52: 138–141

Nowakowska JA (2006). Zastosowanie markerów DNA (RAPD, SSR, PCR-RFLP I STS) w genetyce drzew leśnych, entomologii, fitopatologii i łowiectwie. Leśne Prace Badawcze, 1: 73–101.

Piquemal J, Cinquin E, Couton F, Rondeau C, Seignoret E, Perret D, Blanchard P (2005). Construction of an oilseed rape (*Brassica napus* L.) genetic map with SSR markers. Theoretical and Applied Genetics, 111(8): 1514-1523, doi:10.1007/s00122-005-0080-6

Ramachandran A, Zhang M, Goad D, Olah G, Malayer JR, El Rassi Z (2003). Capillary electrophoresis and fluorescence studies on molecular beacon-based variable length oligonucleotide target discrimination. Electrophoresis, 24(1-2): 70-77, doi:10.1002/elps.200390033

Reed GH, Wittwer CT (2004): Sensitivity and specificity of singlenucleotide polymorphism scanning by high-resolution melting analysis. Clinical Chemistry, 50: 1748–1754

Shi L, Khandurina J, Ronai Z, Li BY, Kwan WK, Wang X, Guttman A (2003). Micropreparative capillary gel electrophoresis of DNA: Rapid expressed sequence tag library construction. Electrophoresis, 24: 86–92, doi:10.1002/elps.200390035

Varshney RK, Nayak SN, May GD, Jackson SA (2009). Next-generation sequencing technologies and their implications for crop genetics and breeding. Trends in biotechnology, 27(9): 522-530, doi:10.1016/j.tibtech.2009.05.006

Vossen RH, Aten E, Roos A, Den Dunnen JT (2009): High-resolution melting analysis (HRMA): more than just sequence variant screening. Human Mutation, 30: 860–866, doi:10.1002/humu.21019

Wang X, Rinehart TA, Wadl PA, Spiers JM, Hadziabdic D, Windham MT, Trigiano RN (2009). A new electrophoresis technique to separate microsatellite alleles. Afr. J. Biotechnol. 8(11): 2432-2436

Wang Z, Weber JL, Zhong G, Tanksley SD (1994): Survey of plant short tandem DNA repeats. Theoretical and Applied Genetics, 88(1): 1-6

Yu RH, Shan XH, Wang S, Li XH, Jiang Y, Tan H, Li YD (2013). A screening method for detecting simple sequence repeat (SSR) polymorphism of *Zea mays* using highresolution melting-curve analysis. Afr. J. Biotechnol. 10(73): 16443-16447