COBISS: 1.08
Agris category code: L10

MICROSATELLITE MULTIPLEX METHOD FOR POTENTIAL USE IN BLACK SLAVONIAN PIG BREEDING

Polona MARGETA [1], Vladimir MARGETA [2], Kristina GVOZDANOVIĆ [3], Dalida GALOVIĆ [4], Ivona DJURKIN KUŠEC [5], Goran KUŠEC [6]

ABSTRACT

Previous studies on Black Slavonian pig breed were focused on the main coat color MC1R locus and on development of the method for Black Slavonian cross-breeds identification, based on coat color genotype. Microsatellites have been used for population studies in pigs for last 25 years and are convenient markers for evaluating the genetic diversity of pig breeds, for parental analysis and identification of pigs as well as traceability of different pig breeds meat in meat products. We developed a PCR multiplex, containing 9 microsatellites in a single reaction. The method was tested on 50 Black Slavonian pig samples (30 homozygous and 20 heterozygous for MC1R locus). For the 9 microsatellite loci analyzed, 79 alleles were detected. The number of detected alleles was high for all loci, with the number of alleles per locus ranging from 5 for S0026, Sw24 and Swr1941 to 22 alleles for S0005. Our results demonstrate that described MS multiplex method may provide a reasonable amount of genetic information to be suitable for use in Black slavonian pig breeding programs in the future.

Key words: pigs, breeds, Black Slavonian pigs, genetics, microsatellites, multiplex PCR

1 INTRODUCTION

Black Slavonian pig is Croatian autochthonous pig breed, established at the end of the 19th century near Osijek in Slavonia by crossing a locally raised Mangalitsa pigs with Berkshire, Poland China and Large black pig breeds. It is black, resistant and convenient for keeping in extensive (pastures, woods) and half-extensive conditions (pens with some free space). It is also of great meat quality, convenient for producing traditional meat products like kulen (dry cured sausage), ham, bacon and other sausages (Karolyi *et al.*, 2007).

In the second half of the 20th century this pig breed was raised mostly in extensive conditions by local farmers, which provided opportunity for uncontrolled crossing of this pig breed with commercial pig breeds (Large White, Yorkshire, Pietrain, Duroc). Phenotypic distinguishing between purebred and F1 crossbred pigs is not possible because of the dominant black color of Black Slavonian pig. Previous study of the *Extension* locus (*melanocortin receptor 1*) in Black Slavonian pigs revealed presence of the E^{D1} allele (Margeta *et al.*, 2009). Because the Black Slavonian pig breed is the only one with E^{D1} genotype raised in Croatia, the genotyping of the *MC1R* gene was used to determine purity of the Black Slavonian pigs (Margeta *et al.*, 2013). The advantages of this method are that it is fast, simple and low-cost. On the other hand, method is based only on one gene and could lead to excessive elimination of pigs from breeding program.

Microsatellites have been intensely used for population studies in commercial as well as rare pig breeds in the last 25 years (Giuffra *et al.*, 2000; SanCristobal *et al.*,

1 J.J.Strossmayer University of Osijek, Kralja Petra Svačića 1d, Osijek, Croatia, e-mail: polona.frajman@gmail.com
2 Same address as 1, e-mail: vmargeta@pfos.hr
3 Same address as 1, e-mail: kgvozdanovic@pfos.hr
4 Same address as 1, e-mail: dgalovic@pfos.hr
5 Same address as 1, e-mail: idurkin@pfos.hr
6 Same address as 1, e-mail: gkusec@pfos.hr

2006; Gama *et al.*, 2013). Microsatellites have been proposed as convenient markers for evaluating the genetic diversity of domestic animals because of their abundant, even distribution in the genome, high level of polymorphism and ease of genotyping. The International Society of Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO) have recommended a set of 30 microsatellite loci (ISAG/FAO, 2011) for evaluating the genetic diversity of pigs. Microsatellites are also powerful tool for parentage analysis in domestic pigs and wild boars (Costa *et al.*, 2012; Nechtelberger *et al.*, 2001). Microsatellites have higher variability and consequently increased power for parentage assignment when compared to the same number of bi-allelic markers such as SNPs (Schlotterer, 2004). For the same reason they are used for traceability of different pig breeds meat in meat products (Jae-Don Oh *et al.*, 2014).

Because of its great quality, the Black Slavonian pig meat is indispensable in the production of traditional meat products. Last year, the process for protection of Black Slavonian pig meat at the base of origin (PDO-Protected Designation of Origin) was initiated.

Present work follows the idea to develop a microsatellite based method, which could be potentially used for pedigree analysis, identification of Black Slavonian pigs and also for traceability of Black Slavonian pig meat in meat products.

2 MATERIALS AND METHODS

2.1 MICROSATELLITE MARKERS

Nine microsatellite markers from the ISAG/FAO recommendation list were selected based on their size and annealing temperature. The markers used, chromosome location, and expected sizes of fragments are summarized in Table 1. Primers were labeled with fluorescent markers (6-FAM, HEX and ATTO550) to distinguish between fragments of similar size, and microsatellite markers were grouped in a single multiplex reaction, according to PCR conditions and expected fragment sizes (Table 1).

2.2 SINGLE LOCUS PCR AMPLIFICATION

A PCR program to amplify all loci individually was designed. Reaction mixture included 50 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each primer and 1 U of *Taq* DNA polymerase (*ThermoFisher scientific*) per 20 µL reaction volume. Amplification was performed in the thermal cycler (*Eppendorf*) as follows: initial activation step 7 min at 95 °C, followed by 35 cycles of denaturation (45 s at 95 °C), annealing (50 s at 55 ± 3 °C) and extension (20 s at 72 °C). Cycling program ended with final extension 7 min at 72 °C. Results of PCR optimization were compared on 3 % agarose gel.

Table 1: *Microsatellite markers with corresponding chromosome location, indication of dye used for labeling (F- 6FAM, H- HEX, A-ATTO550), annealing temperature and allele range*

Nr.	Name	Chr.	Primer sequence (5' -> 3') F,A,H- indicate Dye labelled primer	Annealing temperature	Allele range
1.	S0026	16	**F**-AACCTTCCCTTCCCAATCAC CACAGACTGCTTTTTACTCC	55 °C	87–105
2.	S0155	1	**F**-TGTTCTCTGTTTCTCCTCTGTTTG AAAGTGGAAAGAGTCAATGGCTAT	55 °C	142–162
3.	S0005	5	**F**-TCCTTCCCTCCTGGTAACTA GCACTTCCTGATTCTGGGTA	55 °C	203–267
4.	Sw2410	8	**A**-ATTTGCCCCCAAGGTATTTC CAGGGTGTGGAGGGTAGAAG	50 °C	90–131
5.	Sw830	10	**A**-AAGTACCATGGAGAGGGAAATG ACATGGTTCCAAAGACCTGTG	50 °C	168–203
6.	S0355	15	**A**-TCTGGCTCCTACACTCCTTCTTGATG TTGGGTGGGTGCTGAAAAATAGGA	50 °C	244–271
7.	Sw24	17	**H**-CTTTGGGTGGAGTGTGTGC ATCCAAATGCTGCAAGCG	55 °C	95–124
8.	Sw632	7	**H**-TGGGTTGAAAGATTTCCCAA GGAGTCAGTACTTTGGCTTGA	55 °C	148–178
9.	Swr1941	13	**H**-AGAAAGCAATTTGATTTGCATAATC ACAAGGACCTACTGTATAGCACAGG	55 °C	202–224

2.3 MULTIPLEX PCR AMPLIFICATION

A multiplex PCR reaction was performed with 2x QIAGEN Multiplex PCR Master Mix following the manufacturer instructions. Primers were dissolved in TE buffer (10 mMTris·Cl, 1 mM EDTA, pH 8.0) to obtain 100 µM stock solution. For easy and reproducible handling of nine primer-pairs used in multiplex PCR a primer mix containing all primers at equimolar concentrations was prepared by mixing 5 µL of each primer stock solution and adding TE buffer to the final volume of 250 µL. The multiplex PCR reaction mix was prepared containing 10 µL of 2x QIAGEN Multiplex PCR Master Mix,10x primer mix, 2 µM each primer, 2 µL of 5x Q-Solution (optional), 50–100 ng of template DNA and RNase-free water to the final volume of 20 µL. Microsatellite cycling protocol began with initial activation step 15 min at 95 °C, followed by 35 cycles of denaturation (30 s at 94 °C), annealing (90 s at 57 °C) and extension (60 s at 72 °C). Cycling program ended with a final extension for 30 min at 60 °C.

2.4 MICROSATELLITE ANALYSIS

Microsatellite multiplex PCR products were sent to Macrogen (*Macrogen Inc., Netherlands*), where they were analyzed using GeneScan350 ROX internal standard size marker on ABI3730XL capillary gene analyzer. Data processing of *.fsa* files was performed with the Peak Scanner (Applyed Biosystems) and the Peak Studio (Fodor Lab UNCC 2012) software. Markers and method parameters have been empirically optimized for specific peak detection and precise sizing of amplified products.

Analyzes were performed on 50 unrelated Black Slavonian pigs, 30 of them were homozygous for the *MC1R* locus (black coat color genotype) while 20 were heterozygous. Basic information like total number of alleles per marker, allele frequencies, observed and expected heterozygosity (Nei, 1973), F_{IS} were obtained with Genepop version 4.2 online software (Raymond and Rousset, 1995; Rousset, 2008). Microsatellite format conversions were conducted using MS toolkit software.

3 RESULTS AND CONCLUSION

A quality microsatellite assay requires specific PCR amplification of microsatellite regions, automated specific peak detection and precise sizing of amplified fragments. Hereby we present optimized conditions for the amplification of nine microsatellite loci in one multiplexed PCR reaction. We also show the results of two different programs for detection of specific peaks of amplified products and precise sizing of microsatellite fragments in order to achieve reproducible results.

3.1 SINGLE LOCUS PCR AMPLIFICATION

PCRs for all microsatellite primer pairs were first optimized in single locus amplifications with annealing temperature ranging from 52 ° to 58 **°C**. Checking the products on the agarose gel revealed that microsatellites were all well amplified when annealing temperature was 57 **°C**.

3.2 MULTIPLEX PCR AMPLIFICATION

Based on results of the single locus PCR, we decided to optimize multiplex PCR reaction containing all nine primer pairs using gradient annealing temperature between 55 ° and 58 **°C** and with two different reaction mixes, one without and one containing Q-solution. Again, best results with clearly visible bands on agarose gel and with low background were obtained with annealing temperature 57 **°C** and with primer mix containing Q-solution. Although predicted annealing temperatures of 18 primers ranged from 50 to 55 **°C**, with optimization of the annealing temperature and ingredients of the reaction mix we were able to combine them into a single multiplex PCR. During optimization process it was also noticed that quality and quantity (between 50 and 100 ng per 20 **µL**) of template DNA play important role in obtaining optimal results.

3.3 MICROSATELLITE ANALYSIS

After analyzis on ABI3730XL capillary gene analyzer the .fsa data were processed using the Peak Scanner (Applyed Biosystems) and the Peak Studio (Fodor Lab UNCC 2012) software (Fig. 1).

For the 9 microsatellite loci analyzed, 79 alleles were detected in 50 individuals of the Black Slavonian pig breed (30 homozygous and 20 heterozygous for *MC1R* locus; Table 2).

The level of polymorphism was high for all loci, with the number of alleles per locus ranging from 5 for S0026, Sw24 and Swr1941 to 22 alleles for S0005. Some alleles at 6 loci can only be found in *MC1R* heterozygotes, probably as a result of crossing with other pig breeds. In order to clarify the origin and thus the usefulness of these alleles for genetic studies in the future, the study was extended to other pig breeds, which are currently raised in

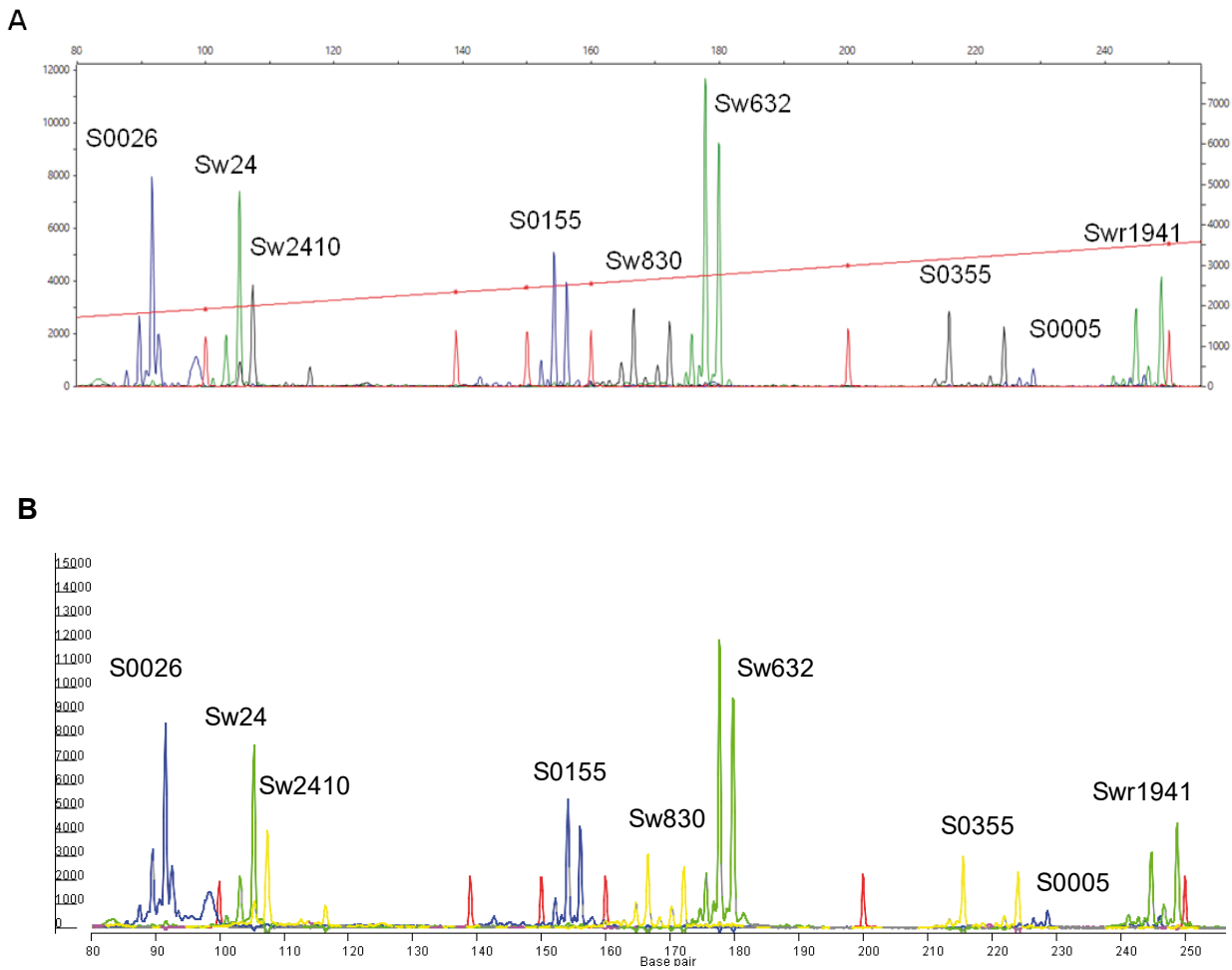

Figure 1: *Electropherograms of group I markers. The x-axis represents DNA fragment size in base pairs, and the y-axis represents fluorescence units. (A) Complete spectrum of group I markers labeled with specific fluorescent tags. Sw2410, Sw830 and S0355 labeled with Atto550, Sw24, Sw632 and Swr1941 labeled with Hex, and S0026, S0155 and S0005 labeled with 6-Fam. Internal size standard is 350Rox. Results are comparable when analyzed with Peak Scanner (Applyed Biosystems; A) and Peak Studio (Fodor Lab UNCC 2012; B) software. Similarly labeled fragments are distally spaced in size by assay design and nonspecific signals are kept at a minimum.*

Table 2: *Number of allels (A), heterozygosity (HExp, HObs) and inbreeding coefficient (FIS) for 9 loci of MC1R homozygous and MC1R heterozygous Black Slavonian pigs*

MS locus	Black Slavonian pig*MC1R* homozygotes (n = 30)					Black Slavonian pig*MC1R* heterozygotes (n = 20)				
	A	H_{Exp}	H_{Obs}	$F_{IS(W\&C)}$	$F_{IS(R\&H)}$	A	H_{Exp}	H_{Obs}	$F_{IS(W\&C)}$	$F_{IS(R\&H)}$
S0026	5	0.6514	0.57	0.132	0.109	5	0.6962	0.65	0.068	0.057
S0155	7	0.7384	0.67	0.099	0.037	9	0.8615	0.80	0.073	0.017
S0005	21	0.9119	0.87	0.050	0.034	22	0.8974	0.85	0.054	0.027
Sw24	5	0.4588	0.47	−0.018	0.042	5	0.3885	0.40	−0.031	−0.051
Sw632	5	0.5961	0.7	−0.178	−0.122	6	0.5026	0.50	0.005	−0.002
Swr1941	4	0.3961	0.43	−0.096	−0.051	5	0.2731	0.30	−0.101	−0.064
Sw2410	9	0.7040	0.73	−0.042	−0.033	10	0.7885	0.85	−0.080	−0.051
Sw830	8	0.6429	0.57	0.120	0.040	8	0.7218	0.70	0.031	0.002
S0355	8	0.7441	0.73	0.015	−0.023	9	0.6269	0.70	−0.120	−0.096

Croatia (PIC, Topigs, Yorkshire, Landras, Pietrain, Duroc); the data are still being processed and are not shown in this work.

The selected microsatellites represent high level of informativeness and the expected heterozygosity values (Table 2) obtained at these loci are comparable with those reported in other MS studies in different pig breeds. This confirms the reliability of this set of markers and its power of resolution for genetic analyses. Genetic makers for identification of a certain breed need to have sufficient genetic diversity and power. Previous studies have indicated that for successful individual identification, H_{Ex} values need to be higher than 0.6. Our results show that H_{Ex} values are little lower for two MS markers (Sw24 and Swr1941), while for the other seven MS markers they are high enough, ranging even to 0.91 for S0005 MS marker.

These results demonstrated that 7 out of 9 MS markers tested may provide a reasonable amount of genetic diversity and information to be suitable for the use in breed identification, not only in live animals, but also for the identification of meat origin in meat products. Another possible implementation of described MS multiplex set is also for parentage testing and pedigree analyses of Black Slavonian pigs. Certanly, further analyses including other pig breeds and testing of MS multiplex on Black Slavonian pig families are needed to show a sutability of described MS multiplex method for use in Black slavonian pig breeding in the future.

4 ACKNOWLEDGMENT

This work has been fully supported by Croatian Science Foundation under the project number 3396.

5 REFERENCES

Costa, V., Pérez-González, J., Santos, P., Fernández-Llario, P., Carranza, J., Zsolnai, A., Anton, I., Buzgó, J., Varga, G., Monteiro, N., Beja-Pereira, A. (2012). Microsatellite markers for identification and parentage analysis in the European wild boar (Sus scrofa). *BMC Research Notes*, *5*, 479.

Gama, L.T., Martínez, A.M., Carolino, I., Landi, V., Delgado, J.V., Vicente, A.A., Vega-Pla, J.L., Cortés, O., Sousa, C.O.; BIOPIG Consortium, (2013). Genetic structure, relationships and admixture with wild relatives in native pig breeds from Iberia and its islands. *Genetics Selection Evolution*, *45*, 18.

Giuffra, E., Kijas, J.M., Amarger, V., Carlborg, O., Jeon, J.T., Andersson, L., (2000). The origin of the domestic pig: independent domestication and subsequent introgression. *Genetics*, *4*, 1785–1791.

ISAG/FAO. (2011). Molecular genetic characterization of animal genetic resources. Retrieved from http://www.fao.org/docrep/014/i2413e/i2413e00.pdf

Jae-Don, O., Ki-Duk, S., Joo-Hee, S., Duk-Kyung, K., Sung-Hoon, K., Kang-Seok, S., Hyun-Tae, L., Jae-Bong, L., Hwa-Chun, P., Youn-Chul, R., Min-Soo, K., Seoae, C., Eui-Soo, K., Ho-Sung, C.,Hong-Sik, K., Hak-Kyo, L., (2014). Genetic Traceability of Black Pig Meats Using Microsatellite Marker *Asian-Australasian Journal of Animal Sciences*, *27*(7), 926–931.

Karolyi, D., Salajpal, K., Kiš, G., Đikić, M., Jurić, I., (2007). Influence of finishing diet on fatty acid profile of longissimus muscle of Black Slavonian pigs. *Poljoprivreda*, *13*(1), 176–179.

Margeta, P., Margeta, V., Budimir, K. (2013). How black is really Black Slavonian pig? *Acta argiculturae Slovenica*, *Supplement 4*, 25–28.

Margeta, V., Kralik, G., Dovč, P., Jakšić, D., Margeta, P. (2009). A simple DNA based method for determination of pure Black Slavonian pigs. Proceedings of the 17th International Symposium Animal Science Days, Padova, 15–18 Sept. *Italian Journal of Animal Science*, *8*(3), 92–94.

Nechtelberger, D., Kaltwasser, C., Stur, I., Meyer, J.N., Brem, G., Mueller, M., Mueller, S. (2001). DNA microsatellite analysis for parentage control in Austrian pigs. *Animal Biotechnology*, *12*(2), 141–144.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA*,*70*(12), 3321–3323.

Raymond, M., and Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, *86*, 248–249.

Rousset, F., (2008). Genepop'007: a complete reimplementation of the Gene pop software for Windows and Linux. *Molecular Ecology Resources*, *8*, 103–106.

SanCristobal, M., Chevalet, C., Haley, C.S., Joosten, R., Rattink, A.P., Harlizius, B., Groenen, M.A., Amigues, Y., Boscher, M.Y., Russell, G., Law, A., Davoli, R., Russo, V., Désautés, C., Alderson, L., Fimland, E., Bagga, M., Delgado, J.V., Vega-Pla, J.L., Martinez, A.M., Ramos, M., Glodek, P., Meyer, J.N., Gandini, G.C., Matassino, D., Plastow, G.S., Siggens, K.W., Laval, G., Archibald, A.L., Milan, D., Hammond, K., Cardellino, R. (2006). Genetic diversity within and between European pig breeds using microsatellite markers. *Animal Genetics*, *37*(3),189–198.

Schlotterer, C., 2004. The evolution of molecular markers – just a matter of fashion? *Nature Reviews Genetics*, *5*, 63–69.