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EFFICIENCY OF TETRAMERIC SHORT TANDEM REPEATS IN PRESTICE BLACK-PIED PIG FOR TRACEABILITY AND PARENTITY TESTING

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Abstract

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The main goal of the research was to evaluate suitability of 11 tetrameric STRs (Short Tandem Repeats) marker panel for the Prestice Black-Pied pig (PC) breed as the only genetic resource in the Czech Republic.The analysis was carried out in 522 breeding and slaughter PC individuals. We observed 94 alleles overall across whole 11 STR panel. The observed heterozygosity H_O was 0.677, the polymorphism information content *(PIC)* was in average 0.664 per locus. The probability of identity of two independent samples (*PI*) using all 11 STR loci was $4.037 \cdot 10^{-11}$ and the probability of identity related individuals *(PISibs)* was $8.315 \cdot 10^{-5}$. The power of exclusion for loci combinations when both parents are known *(P1)*, when only one of the parent is known (*P2*) and for two putative parents (*P3*) were 0.9996, 0.9899 and 0.9999. The efficiency of the 11 tetrameric STRs (Animaltype Pig kit) is higher in PC in comparison to commercial breeds and slaughter crossbred pigs. In genetic resource PC, the 11 STRs panel is usable for forensic purpose such parentity testing and traceability.

Keywords: tetrameric STRs, Prestice Black-pied pig, traceability, parentity

INTRODUCTION

DNA identification and parentity testing using molecular-genetic methods is applicated in protection and preservation endangered animal populations as well as in food safety management. The selection of the most informative genetic markers is crucial for these DNA analyses. The STR markers fulfil this requirement with their high informative value and variability in populations.

The STRs are applied for evaluation of genetic diversity within and between breeds by many authors over the world (e.g. Herrero-Medrano *et al.*, 2013; Zaman *et al.*, 2013; Li *et al.*, 2014; Vrtková, 2015). The STR analysis was used for research of genetic diversity of different pig's populations.

The STRs are used in pigs and also in other species for individual identification and parentage analysis (e.g. Nechtelberg *et al.*, 2001; Costa *et al.*, 2012; Wang *et al.*, 2015).

The usage of STR markers for traceability of the pig reveals Ipate *et al.* (2009). They present possibility of slaughter meat identification at abattoir based on the 10 STRs loci testing. The pig tissue samples were collected by ear tagging. The samples of meat were collected at abattoir and were compared with the saved samples of tissue. The authors proclaim the 10 STRs analysis results in 100% identification of meat products in examined pigs' individuals.

With the development of Single Nucleotide Polymorphism (SNP) arrays, the research started on SNP markers and their application to breed-specific testing for breed assignment purposes. Ramos *et al.* (2011) confirmed 193 SNPs as breed-specific based on the PorcineSNP60 beadchip. DNA pools were prepared for Duroc (D), Landrace (L), Large White (LW), Pietrain (PN) and Wild Boar. Wilkinson *et al.* (2012) posted the newly created 96-plex assay using selected markers from the PorcineSNP60 beadchip.

The assay enables powerful assignment of samples to breed origin and allows identify mislabelling and provides a highly effective tool for DNA analysis in food forensics.

The pig's genetic resource of the Czech Republic Prestice black-pied pig is small closed population. The breed was geneticly improved by other breeds, especially L, PN and some other breeds.

Admixture of L, LW and PN breeds in PC boars evaluated Vrtková (2015) by using 10 STRs dimeric markers. Differentiation between mentioned breeds was proved by cluster method. The PC breed was defined within four clusters.

Since 2013 the 11 STRs tetrameric markers panel (Animaltype Pig Amplification Kit) is used for determination of parentity and DNA identification of pigs all breeds and hybrids in accreditation laboratories in the Czech Republic (CZ). It was developed by Biotype Diagnostic Gmbh (Germany) specially for genotyping of breeding livestock samples for proof-of-origin in meat products and generally for quality management in food industry. The test kit is recommended for proof of origin according the EU-Directive and kinship testing in context with control of breeding. The Animaltype Pig amplification kit was developed and attested especially for parentity usage and kinship testing in breeds German Large White (GLW), German Landrace (GL) and Piétrain.

Robino *et al.* (2008) proved forensic applications on a set of 55 Landrace x Large White crossbred pigs from Italy farms. Carrati *et al.* (2010) evaluated the use this panel for wild pigs. They described its suitability for individual identification wild pigs and possibility of forensic application too.

A new 13-plex of tetrameric markers and amelogenin marker for forensic identification of pigs was developed and tested by Lin *et al.* (2012). They specified a genetic variability in 341 pigs of 11 breeds from Taiwan. They verified their tetrameric STRs are appropriate for individual identifications, parentage testing, breed assignment, as well as phylogenetic studies and forensic usage.

The aim of our research was to confirm a scale of efficiency and reliability of the 11 tetrameric STRs panel (Animaltype Pig Kit) in the closed population of Prestice Black-Pied pig for parentage testing and traceability of meat products.

MATHERIALS AND METHODS

Sample Collection and DNA Extraction

We analysed 522 individuals of PC breed, thereof 2/3 breeding and 1/3 slaughter. DNA for analysis was isolated from blood, tissue or hair samples using Genomic DNA Mini Blood/Tissue Kit (Geneaid).

PCR Typing

The 11 tetrameric STRs (*387A12F, S0655, SBH1, SBH2, SBH4, SBH10, SBH13, SBH18, SBH19, SBH20,* and *SBH22*) included in Animaltype Pig kit (Biotype Diagnostic Gmbh) were analysed. Multiplex PCR amplification of STRs markers was carried out using the Animal Type Pig PCR amplification kit, following the manufacturer's recommendations.

STRs markers were separated by fragment analysis on genetic analyser ABI PRISM 310 (Applied Biosystems, USA). The fragment analysis was obtained GeneScan 3.7 and Genotyper 3.7 software.

Statistical Analysis

The software Genalex v. 6.5. (Peakall, Smouse, 2012) was used to calculate the number of alleles per locus (N_a), the number of effective alleles per locus (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), the fixation index (*F*), the deviations of Hardy Weinberg Equilibrium proportions (*HWE*), the probability of identity of two independent samples (*PI*), the probability among siblings (*PISibs*) and the power of exclusions when both parents are known, when only one of the parent is known and for two putative parents (*P1, P2, P3*). The polymorphism information content (*PIC*) was obtained across different loci using the Excel Microsatellite Toolkit v. 3.1.1. (Park, 2001).

RESULTS

Genetic Variability in Prestice Black-Pied Pig

The characterisation of population is important for parentity testing and traceability. The primary parameters of genetic variability of PC such as allele frequencies of the 11 STR loci, exact test of *HWE* and genetic variation such as allele number N_a, number of effective alleles N_e, observed and expected heterozygosity H_o and H_e, fixation index *F* and polymorphism information content *PIC* are summarized in Tab. I. We detected overall 94 alleles in 11 STR tetrameric loci in 522 PC pigs. Although average number of alleles per locus N_a was of 8.55, number of effective alleles N_e was in average of 3.92. Expected heterozygosity H_e 0.706 was higher than observed heterozygosity H_o 0.677. The H_e for individual markers varied between 0.841 (*SBH4*) and 0.525 (*SBH13*). The lowest H_o was found in loci *SBH13* (0.533), *SBH19* (0.542) and *S0655* (0.578), the highest H_o in locus *SBH4* (0.794). The negative fixation index was found in five loci. These negative values indicate the excess of heterozygotes. Higher *F* values were found for loci *SBH18* (0.263) and *SBH19* (0.115). *PIC* increased from 0.456 (*SBH22*) to 0.818 (*SBH18*).

We found 6 STR loci significantly deviated from Hardy-Weinberg equilibrium ($p < 0.001$).

Identity and Parentity Testing

Probability of identity determination and power of exclusion were various for different loci. Decrease effectiveness is shown in Tab. II.

The lowest predicative value and least suitable and effective were found markers *SBH22, SBH13, SBH19* and *S0655* (with increasing effective level).

I: *Allele distribution and genetic variation of 11 STR markers*

Alleles	387A12F	S0655	SBH1	SBH2	SBH4	SBH10	SBH13	SBH18	SBH19	SBH20	SBH22	Mean
5		0.516										
6				0.008								
7		0.002										
9	0.111	0.025					0.177	0.001				
10		0.029	0.077						0.072			
11		0.115					0.002	0.295	0.367			
12		0.312	0.112									
12.1	0.014											
13	0.034	0.001	0.296				0.067	0.014				
14			0.348				0.045	0.114	0.491			
14.1	0.347											
15	0.355		0.154				0.660	0.098	0.069			
16			0.013				0.049	0.163				
17								0.112				
18	0.064										0.009	
19								0.101		0.030	0.061	
20				0.002						0.001	0.007	
21	0.070			0.095								
22	0.005							0.057				
23				0.145				0.044			0.583	
23.3											0.340	
24								0.001			0.001	
25				0.008								
26				0.394						0.006		
27				0.013								
28				0.112								
29				0.001								
30				0.046								
31				0.005						0.101		
32				0.028						0.016		
33				0.142								
34				0.001		0.219				0.257		
36						0.024						
37						0.245				0.005		
38										0.131		
39										0.448		
40						0.001				0.004		
41						0.064						
42						0.101				0.001		
46						0.099						
48						0.003						
49						0.210						
53						0.034						
55.1					0.045							
56					0.006							
57					0.105							
58					0.298							
59					0.073							
60					0.006							

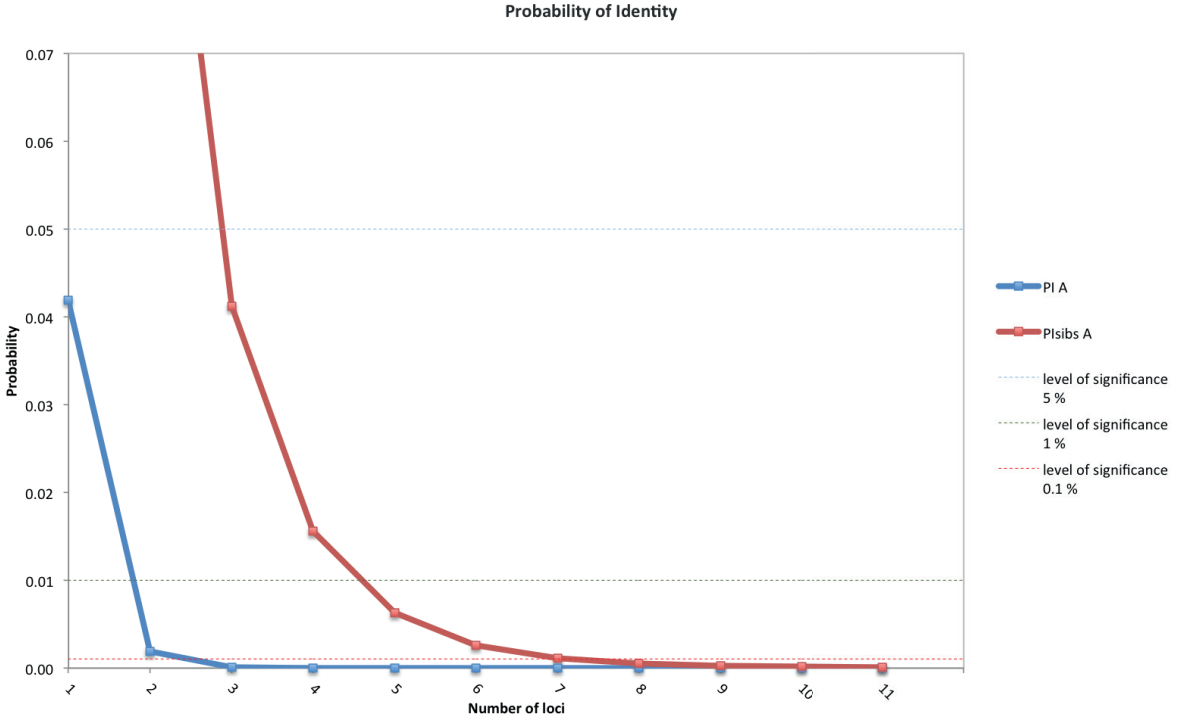
Alleles	387A12F	S0655	SBH1	SBH2	SBH4	SBH10	SBH13	SBH18	SBH19	SBH20	SBH22	Mean
62					0.035							
63					0.064							
64					0.099							
65.1					0.115							
66.1					0.155							
Na	8	7	6	14	11	10	6	11	4	11	6	8.545
Ne	3.714	2.648	3.987	4.519	6.276	5.620	2.104	6.090	2.589	3.384	2.178	3.919
H_O	0.757	0.578	0.784	0.763	0.794	0.764	0.533	0.616	0.542	0.711	0.603	0.677
H_e	0.731	0.622	0.749	0.779	0.841	0.822	0.525	0.836	0.614	0.705	0.541	0.706
HWE	ns	ns	ns	s	s	s	ns	s	s	ns	s	
F	–0.035	0.071	–0.047	0.020	0.056	0.071	–0.015	0.263	0.117	–0.009	–0.115	0.034
PIC	0.689	0.560	0.709	0.755	0.824	0.799	0.489	0.818	0.541	0.662	0.456	0.664

Na – number of alleles, *Ne* – number of effective alleles, H_O – observed heterozygosity, H_e – expected heterozygosity, *HWE* (ns = not significant, s = significant p – value lower than 0.1% level), *F* – fixation index, *PIC* – polymorphism information content

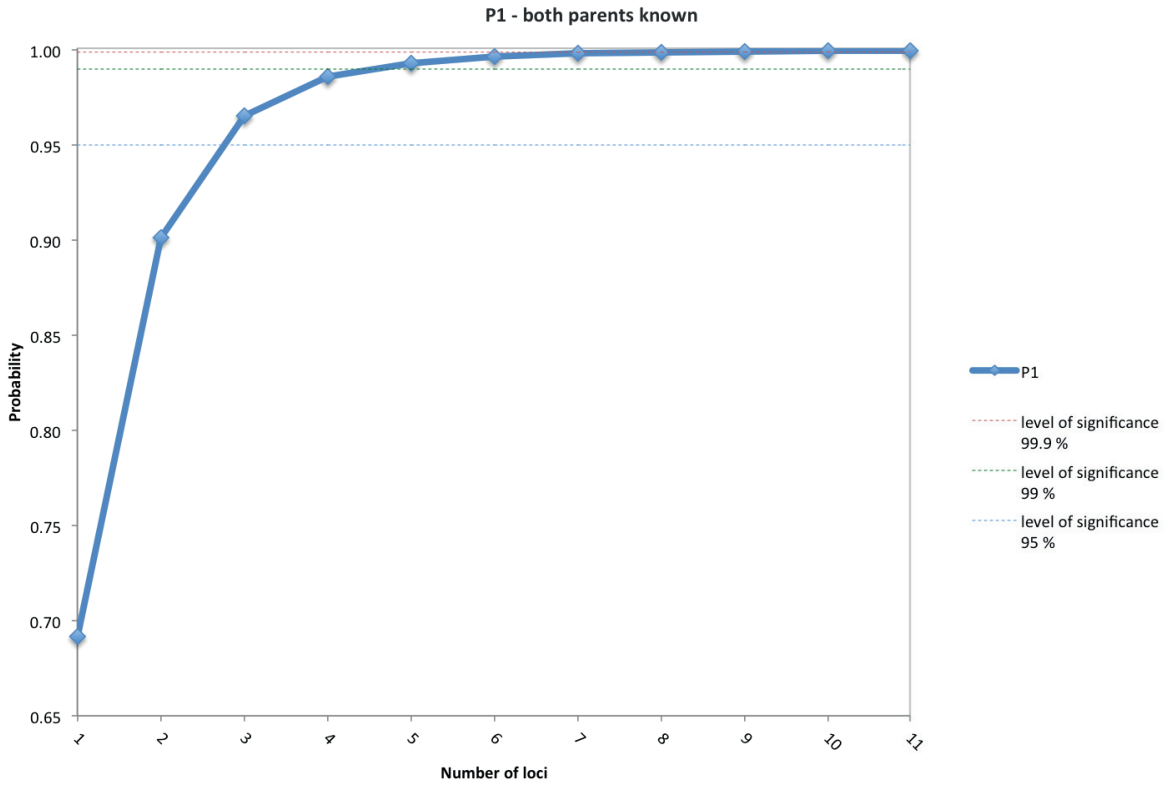
II: *Probability of identity and probability of exclusion by locus*

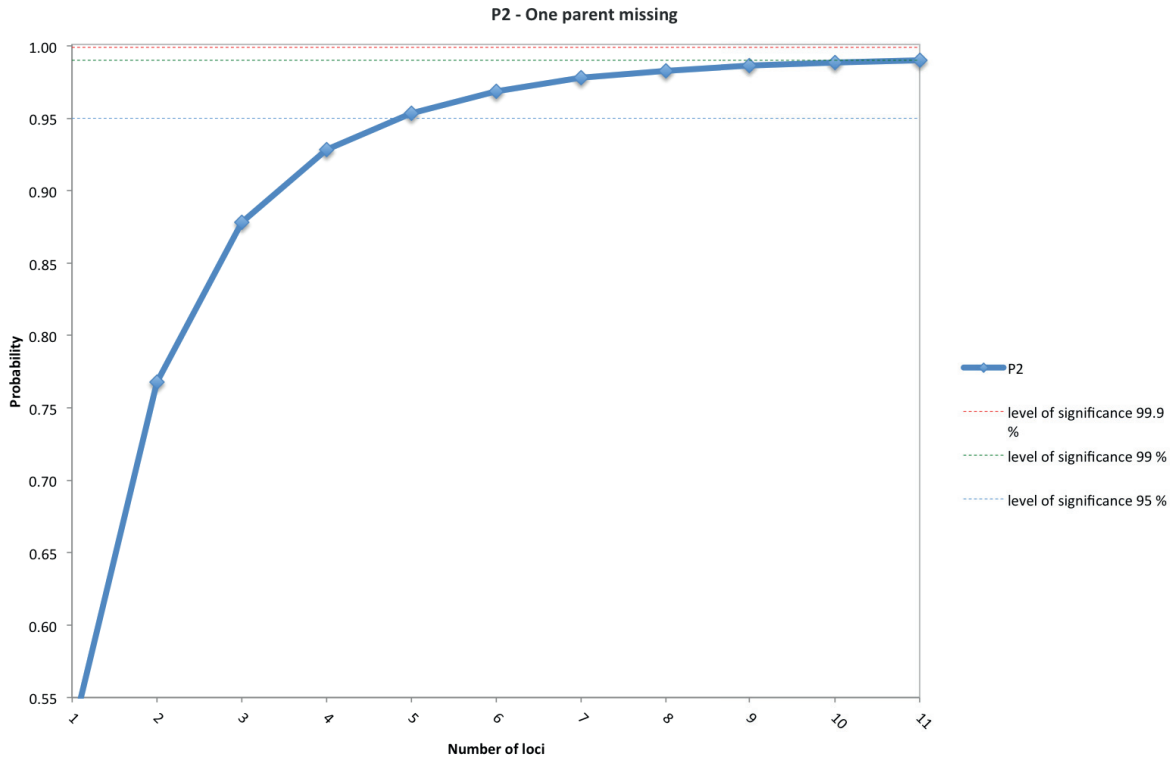
	Short Tandem Repeats										
Probability	**SBH4**	**SBH18**	**SBH10**	**SBH2**	**SBH1**	**387A12F**	**SBH20**	**S0655**	**SBH19**	**SBH13**	**SBH22**
PI	0.0419	0.0451	0.0552	0.0728	0.1028	0.1144	0.1296	0.2050	0.2220	0.2616	0.2929
PISibs	0.3401	0.3434	0.3528	0.3788	0.4011	0.4132	0.4301	0.4901	0.4986	0.5531	0.5528
P1	0.6919	0.6804	0.6479	0.5965	0.5250	0.5058	0.4748	0.3619	0.3405	0.3138	0.2669
P2	0.5249	0.5110	0.4746	0.4151	0.3473	0.3306	0.2985	0.2092	0.1991	0.1517	0.1488
P3	0.8667	0.8564	0.8256	0.7918	0.7099	0.6950	0.6646	0.5305	0.5002	0.4923	0.4045

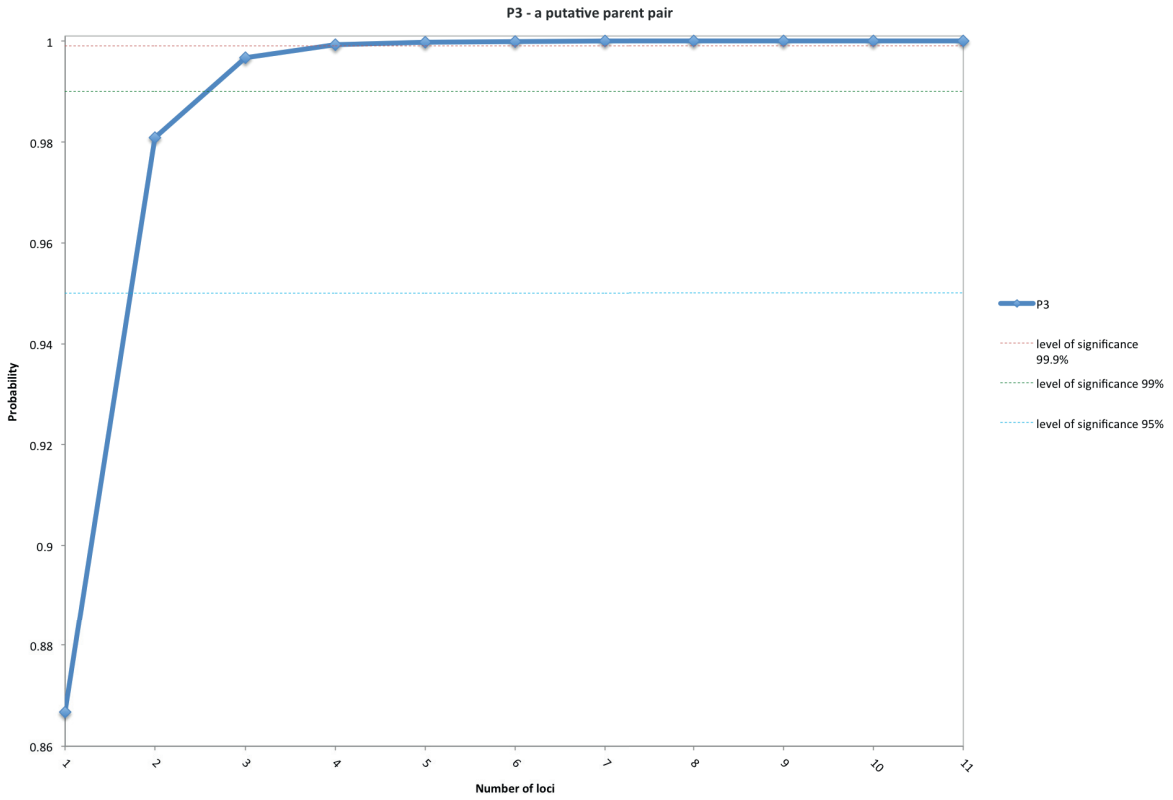
PI – probability of identity of independent samples, PISibs – probability of identity among siblings, *P1* – probability of exclusion when both parents are known and one parent is wrongly identified, *P2* – probability of exclusion when only one parent is known, *P3* – probability of exclusion for two putative parents (excluding a putative parent pair), both parents are wrongly identified



1: *The probability of identity in PC population for increasing combinations of the 11 loci*
PI–probability of identity of two independent samples, *PISibs*–probability among siblings


P1 - both parents known
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
Probability
1
2
3
4
5
6
7
8
9
10
11
Number of loci
P1
level of significance 99.9 %
level of significance 99 %
level of significance 95 %


P2 - One parent missing
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
Probability
1
2
3
4
5
6
7
8
9
10
11
Number of loci
P2
level of significance 99.9 %
level of significance 99 %
level of significance 95 %



2: *Exclusion probabilities in PC population for increasing combinations of the 11 loci*
P1 – probability of exclusion when both parents are known, *P2* – probability of exclusion when only one parent is known, *P3* – probability of exclusion for two putative parents (excluding both parents), Significant level = 99.9%

The most appropriate markers for identity and parentity testing are *SBH2, SBH10, SBH18* and *SBH4* (increasingly). That means, the probability of identical genotypes (*PI*) in reference population PC was 4.2% in locus *SBH4* against locus *SBH22* with probability agreement 29.3%.

The probability of identical genotypes between siblings (*PISibs*) in monitored population is 34% in *SBH4* locus versus 55.3% in *SBH22*. These results correlate with number of effective alleles N_e 6.3 (*SBH4*) and 2.2 (*SBH22*) and observed heterozygosity H_o 0.79 and 0.60. Parentity testing of *P1* – we have genotypes of both parents and one of them is incorrect – the effectiveness each of the first 6 loci in Tab. II is higher than 50% (from 69% in *SBH4* to 50% in *387A12F*). The effectiveness of these loci for *P2* (we know just one parent genotype and the parent is incorrect) varies from 53% to 33%. In case of two putative parents (both are wrongly identified) is effectiveness from 87% to 70%. The effectiveness can be compared with *PIC* values in Tab. I (range from 0.456 in *SBH22* to 0.824 *SBH4*).

The STR markers panel from Tab. II is used for controlling of all breeding pigs in CZ. The efficiency in our genetic resource PC involving breeding and slaughter individuals are shown in Figs. 1 and 2. The markers are sorted by probability value from the strongest to lowest see Tab. II.

The probability of identity usable for traceability of the PC products, *PI* and *PISibs* using all 11 loci were $4.037 \cdot 10^{-11}$ and $8.315 \cdot 10^{-5}$ respectively.

Exclusion probabilities for loci combinations *P1, P2* and *P3* were 0.999635, 0.989994 and 0.999998. Combined power of exclusion (*CEP*) was 0.999999996.

Fig. 2 (*P1*) states that eight STR loci with the highest value (*SBH4, SBH18, SBH10, SBH2, SBH1, 387A12F, SBH20* and *S0655*) are sufficient for parentity testing in PC.

With a knowledge of breed management of PC in the Czech Republic and other available results (even those not included here), the verification (that slaughter PC meat does not come from the breed given by vendor) is right with a probability $P2^n$ where *P2* is value for loci combinations (98.99%) and n is a number of boars in given breed.

DISCUSSION

Comparison of Alleles Occurrence in PC and Other Breeds

Biotype Diagnostic Gmbh performed huge study regarding presence of alleles in GLW, GL and PN breeds. They recognized 165 alleles overall, our result is 94 alleles in PC. It is very interesting to judge the occurrence of alleles in breeds which were a part of unregulated breeding PC during 20th

century. Our research showed that PC breed has got alleles, which did not appear in breeds above, in 7 loci *(387A12F, SBH2, SBH4, SBH10, SBH18, SBH20* and *SBH22).*

Our results in allele distribution in 11 STRs can be compared partly with Robino *et al.* (2008) and their set of 55 slaughter hybrids L x LW originated from several farms of area of Piedmont region. They detected overall 100 different alleles in F1 hybrids, we determined 94 alleles in PC set. More important is the occurrence of alleles in each locus. According to breeding improvement of PC by breed L, we expected minimal difference between hybrids and PC. However, at least one different allele was detected at each one of 11 STR loci. It is caused by little set of hybrids, probably.

The allele distribution in 11 STR loci described Caratti *et al.* (2010) in 412 individuals of commercial pig breeds. They presented 170 alleles overall. PC breed has got in three loci (*SBH2, SBH4* and *SBH18*) at least one allele which doesn't occure in commercial breeds. The history of origin the Prestice Black-Pied pig breed is quite complicated so we expected the occurrence of these alleles in other regional breeds such as British Saddleback, Angler Sattelschwein and Nero Siciliano (Megens *et al.*, 2008) some of which probably participated on creation of PC. It would be interesting to find out whether these alleles appear in these breeds. Unfortunately, no information were found at available literature.

The Utilization for Parentity and Traceability Testing of Sold PC Meat

Let's consider the values of combined power of exclusinon (*CEP*) for 11 STR panel described by Biotype Diagnostic for clear Germany breeds GLW – *CEP* 0.9987, GL – *CEP* 0.9997 and PN – *CEP* 0.9998 (Anonym, 2007). Next, we consider values of hybrids of F1 generation *CEP* 0.99993 (Robino *et al.*, 2008) as the first step in hybridisation programmes. Then we continue with slaughter pigs produced in hybridisation programmes in Piedmont region in Italy – *CEP* 0.9991 (Carrati *et al.*, 2010).

As *CEP* = 0.9999 set by us in PC is highest, we can consider this panel as most effective in parentage testing in PC. This resulted in its suitability for forensic traceability in PC whose need is expected in the Czech Republic. Carrati *et al.* (2010) determined *CEP* 0.9998 in wild pigs which is lower than at genetic resource of PC, too. From the point of view of food safety management, the value of probability of identity of two related samples is highly informative.

In our examined population of PC, we recognized the probability of identity among siblings 0.008% in 11 STRs. This cumulative calculation, presented on Fig. 1, will be considered in case of reducing of loci which are discussted below. For comparison, Robino *et al.* (2008) described in F1 LW x L siblings random match probability 0.0009%.

The PC meat is delivered to special shops, farm markets and specific restaurants. Because of marketing, vendors feature the place of origin of the meat (farm or slaughterhouse). Using a breed management and knowledges gained by our laboratory so far, the probability that the meat does not come from the farm it is featured from is possible to calculate as 0.9899^n where n is number of boars used on given farm.

The PC is breed in the Czech Republic in the organic farms where could a higher number of boars and sows live together. Assignment of piglets to their parents is a need occasionally. The 11 STRs panel is suitable for this usage due to *P1, P2* and *P3* values (99.96%, 98.99% and 99.99%).

Economy – Efficiency of Tetrameric Loci

There are 8 loci fulfilling sufficient reliability which is set as 99.99% for parentage testing at PC (based on value of *P1* presented at results). That means 3 loci less then are used in the Czech Republic so far. Therefore, the laboratory costs could be reduced by a quarter.

Another helpful possibility how to use testing only 8 authenticated STRs without raising contemporary laboratory costs (cost of the whole Animaltype Pig kit) would be finding di or tetra STR where anyone of alleles appears with a huge frequency at current population of PC. Or, it is possible to replace some STRs by those ones which gives shorter fragments during PCR reaction. Shorter fragments are better for amplification at less quality samples of DNA as treated meat, meat treated by heat etc. where DNA could be degraded.

Another opportunity contributing to sustainability of farms of PC in the Czech Republic can be seen in inclusion of particular SNP or haplotype (associated, for instance, with the quality of PC meat, content of intramuscular fat, boar taint etc.) to compulsory parentage testing at boars. Diagnosed genotypes of SNP would serve as a basis for market assisted selection or mating schemes.

Breeding of PC due to the quality of its meat is suitable for expansion according to us. Our study shows the need and benefits of molecular genetic research for sustainable breeding of PC in the Czech Republic.

CONCLUSION

The efficiency of 11 tetrameric STRs (*387A12F, S0655, SBH1, SBH2, SBH4, SBH10, SBH13, SBH18, SBH19, SBH20,* and *SBH22*) included in Animaltype Pig kit (Biotype Diagnostic Gmbh) in Prestice Black-Pied pig was verified in 522 individuals of PC from which 2/3 were breeding and 1/3 slaughter. According to allele frequencies for each loci and specified probability of exclusion *P1* (99.96%), we concluded that 8 STR loci out of those given above are enough for parentity testing in PC.

The occurrence of uncommon alleles, which were not detected in commercial breeds, participates on high effectivity at PC.
The 11 STRs panel efficiency for traceability of meat, in conditions of production of PC pigs in CZ, with the use of parameters (the probability of identity of independent samples *PI* ($4.037 \cdot 10^{-11}$), the probability of identity of related individuals *PISibs* ($8.315 \cdot 10^{-5}$) and combined power of exclusion CEP (0.99999999596) is sufficient for forensic use.
This work contents design of food safety management of PC meat products.

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