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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Parameters of variability and diversity of tetrameric STRs for practical use in Zlotnicka White and Zlotnicka Spotted pigs*

Irena Vrtková[1], Andrzej Filistowicz[2], Janusz T. Buczyński[3], Štěpán Vrtek[4], Lenka Falková[1]**

[1] Mendel University in Brno, Department of Morphology, Physiology and Animal Genetics, Zemedelská 1, 613 00 Brno, Czech Republic

[2] Wroclaw University of Environmental and Life Sciences, Institute of Animal Breeding, Chelmonskiego 38c, 51-630 Wroclaw, Poland

[3] Poznan University of Life Sciences, Faculty of Animal Breeding and Biology, Wojska Polskiego 28, 60-637 Poznan

[4] Masaryk University, Department of Mathematics and Statistics, Msaryk University, Kotlarska 2, 611 37 Brno, Czech Republic

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As a goal of this paper, the assessment of genetic variability of Zlotnicka White (ZW) and Zlotnicka Spotted (ZS), was chosen in order to verify the appropriateness of 11 tetrameric Short Tandem Repeats (STRs) panel for use in genetic resources of pigs. Analyses were carried out in sets of 91 ZW and 250 ZS pigs. Seventy-one alleles in ZW and 85 alleles in ZS were detected at all 11 STRs loci. An average number of alleles at locus (MNA) was 6.455 in ZW and 7.727 in ZS. An average number of effective alleles (MNe) was 3.532 in ZW and 3.431 in ZS. Observed heterozygosity Ho was 0.659 in ZW and 0.637 in ZS. On average, polymorphism information content (PIC) reached 0.639 and 0.619 per locus in ZW and ZS. The probability of identity of two independent samples PI using all 11 STRs loci in ZW amounted to 3.118×10^{-10} and to 5.921×10^{-10} in ZS while the probability of identity related individuals PISibs was 1.331×10^{-4} and 1.749×10^{-4} in ZW and ZS. The power of exclusion for loci combinations when both parents are known, when only one of the parent is known and for

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*Corresponding author: irenav@mendelu.cz

two putative parents P1, P2 and P3 were in ZW versus ZS 0.99903 v. 0.99887, 0.97998 v. 0.97654 and 0.99999 v. 0.99998, respectively. Identified estimates of stated parameters illustrate suitability of tetrameric STRs for practical application in the management of genetic resources, verification of parentage and traceability in ZW and ZS. Based on the results, we recommend the panel of tetrameric STRs loci as suitable for parentage, traceability and differentiation of subpopulations in genetic pig resources of similar history. H_e.

KEYWORDS: diversity / pigs / tetrameric STRs / Zlotnicka White / Zlotnicka Spotted

Small closed pig populations, which are bred as genetic resources, require specific management for their sustainability. Different molecular genetic markers are helpful for deciding on the breeding procedure. Predominantly, Short Tandem Repeats (microsatellite sequences) are used. However, different loci and different numbers of STRs are used in different countries.

Majority of the reports regard the Spanish genetic resource – the Iberian pig [Martínez *et al.* 2000, 2012, Toro *et al.* 2002, Fabuel *et al*. 2004, Alves *et al.* 2006, Gama *et al.* 2013].

Scarce knowledge about microsatellites has been made available regarding other genetic resources and local breeds [Berthouly-Salazar *et al.* 2012, Guastella *et al.* 2010, Herrero-Medrano *et al.* 2012, 2013]. Microsatellite data are also used for different studies in commercial breeds [Wilkinson *et al.* 2011, Li *et al.* 2014, Szmatola *et al.* 2016].

The use of tetrameric STRs from the commercial panel at genetic resources has not been met in the available literature. Vrtková [2015] and Vrtková *et al.* [2016] published results of the first large analysis with the panel of 11 tetrameric microsatellites in pig genetic resources in Central Europe. Prestice Black-Pied Pig breed, the genetic resource in CZ, was the object of the study. Tetrameric STRs proved to be very handy. In order to acknowledge that, observations of other genetic resources were necessary to be made. We chose Polish Zlotnicka pigs because of the similarities in the development and the fact that it is kept as a closed population [Szulc, Buczynski 2012].

The fact that a detailed microsatellite structure has not been studied yet in the Polish genetic resources of Zlotnicka White and Zlotnicka Spotted, was another reason to work with Polish Zlotnicka. Kurył *et al.* [1997] described only the genetic structure of immunogenic, biochemical and some single nucleotide polymorphism (SNP) markers.

The goal of our study was the estimation of parameters important for breeding management and the demonstration of suitability of tetrameric STRs for the Polish genetic resource of Zlotnicka pigs.

Material and methods

Sample collection and DNA extraction

A total of 341 boars and sows of Zlotnicka pigs were analysed, which included 91 Zlotnicka White and 250 of Zlotnicka Spotted pigs. DNA for the analysis was isolated from hair samples, using Genomic DNA Mini Tissue Kit (Geneaid).

Microsatellite, PCR amplification

Microsatellites were analysed by the Animaltype Pig PCR Amplification Kit (Biotype Diagnostic Gmbh), which allows for specification of 12 tetrameric microsatellites (*SBH2, SBH18, SBH4, S0655, SBH23, SBH20, SBH1, SBH10, SBH13, 387A12F, SBH22, SBH19*), including the sex specific marker for Amelogenin *(SBH23)*, which was not used for the variability assessment. Multiplex PCR amplification of microsatellite markers was carried out using the Animaltype Pig PCR amplification kit, following the manufacturer's recommendations. The Animaltype Pig kit is a PCR test specifically developed for the genotyping of breeding livestock samples for proof-of-origin in meat products and generally for quality management in the food industry. The test kit is recommended for the following applications: Proof of origin according the EU-Directive, Kinship testing in the context of breeding control, Status of inbreeding for herd book populations.

Microsatellites markers were separated by fragment analysis on genetic analyser ABI PRISM 310 (Applied Biosystems, Foster City, USA). The fragment analysis was carried out using the GeneScan 3.7 and Genotyper 3.7 software.

Statistical analysis

The Genalex v. 6.5. software (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 from 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].

Genetic differentiation and population subdivision were tested with the algorithm of Pritchard *et al.* [2000], implemented in the Structure v. 2.2 software. Individual animals were assigned to two or more subpopulations based on their microsatellite genotypes. The Structure is able to determine for each pig the proportion of genes originating from the "K" potential clusters. The Structure algorithm based on the Markov chain Monte Carlo method was used to define the natural algorithm of the probability that a given genotype belongs to the assumed K clusters. The run length was set to burn-in period of 10^5 interactions followed by 10^5 interactions suggested by authors. The program was tested for a range of possible cluster numbers (K) from 2 to 5.

Results and disscusion

Genetic variability of Zlotnicka pigs

The primary parameters of genetic variability of Zlotnicka pigs such as allele frequencies of the 11 STRs loci, exact test of HWE and genetic variation such as allele

number (Na), number of effective alleles (Ne), observed and expected heterozygosity (Ho and He), fixation index (F) and polymorphism information content (PIC) are summarized in Table 1 and 2.

Table 1. Genetic variability at microsatellite loci in Zlotnicka White

Locus	N_a	N_e	H_o	H_e	PIC	F	HWE
387A12F	11.000	4.951	0.711	0.802	0.777	0.109	ns
S0655	4.000	2.492	0.596	0.602	0.526	0.005	ns
SBH1	5.000	1.899	0.495	0.476	0.434	-0.044	ns
SBH2	6.000	2.658	0.648	0.627	0.566	-0.039	ns
SBH4	8.000	4.690	0.753	0.791	0.754	0.043	s
SBH10	8.000	2.747	0.584	0.640	0.606	0.081	ns
SBH13	5.000	2.597	0.733	0.618	0.538	-0.193	ns
SBH18	7.000	3.813	0.429	0.743	0.697	0.419	s
SBH19	4.000	3.369	0.636	0.707	0.649	0.095	ns
SBH20	10.000	6.722	0.901	0.856	0.833	-0.059	ns
SBH22	3.000	2.912	0.767	0.660	0.642	-0.168	ns
Mean	6.455	3.532	0.659	0.684	0.638	0.023	-

N_a – number of alleles, N_e – 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

Table 2. Genetic variability at microsatellite loci at Zlotnicka Spotted

Locus	N_a	N_e	H_o	H_e	PIC	F	HWE
387A12F	7.000	1.451	0.300	0.311	0.301	0.035	ns
S0655	10.000	4.229	0.753	0.765	0.724	0.014	s
SBH1	8.000	5.996	0.864	0.835	0.811	-0.037	ns
SBH2	10.000	5.264	0.876	0.812	0.783	-0.081	ns
SBH4	6.000	2.276	0.528	0.562	0.520	0.057	s
SBH10	9.000	3.609	0.688	0.725	0.682	0.048	ns
SBH13	7.000	3.632	0.750	0.726	0.680	-0.035	ns
SBH18	9.000	2.675	0.569	0.627	0.554	0.092	ns
SBH19	5.000	3.983	0.713	0.750	0.705	0.049	ns
SBH20	8.000	2.353	0.586	0.576	0.523	-0.020	ns
SBH22	6.000	2.274	0.380	0.561	0.525	0.322	s
Mean	7.727	3.431	0.637	0.659	0.619	0.040	-

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

We detected overall 71 alleles in 11 STR tetrameric loci in the ZW pigs. Although the average number of alleles per locus was 6.46, the number of effective alleles had an average of 3.53. Number of alleles per locus was in the range from 3 (*SBH22*) to 11 (*387A12F*). The expected heterozygosity (0.684) was close to the observed one at 0.659. The H_e for the individual markers varied between 0.856 (*SBH20*) and 0.476 (*SBH1*). The lowest Ho was found at the *SBH18* (0.429) and *SBH1* (0.495) loci whereas the highest H_o was at locus *SBH20* (0.901). A negative fixation index was found at five loci. These negative values indicate an excess of heterozygotes. Highest F value (0.419) was found for the *SBH18* locus. PIC was increasing from 0.434 (*SBH1*) to 0.833 (*SBH20*). We found 2 STRs loci significantly deviating from Hardy-Weinberg equilibrium ($p<0.001$).

In the ZS pigs, we detected overall 85 alleles. Average number of alleles per locus was 7.73, the number of effective alleles averaged to 3.43. The number of alleles per locus ranged from 5 (*SBH19*) to 10 (*S0655, SBH2*). H_e was 0.659, H_o was 0.637. The H_e for individual markers varied between 0.311 (*387A12F*) and 0.835 (*SBH1*). The lowest H_o was found in loci *387A12F* (0.300) and *SBH22* (0.380), the highest H_o in locus *SBH2* (0.876) and *SBH1* (0.864). The negative fixation index was found at four loci. Higher F values were recorded for locus SBH*22* (0.322); PIC increased from 0.301 (*387A12F*) to 0.811 (*SBH1*).

There were 3 STRs loci significantly deviating from Hardy-Weinberg equilibrium ($p<0.001$). As presented in Tables 1 and 2, there is a difference among populations of the Zlotnicka pigs. The difference is in the number of alleles found at each locus, in the number of effective alleles, expected and observed heterozygosity and in PIC. For N_a, H_o, H_e, and PIC the differences are as follows at particular loci (ZW vs. ZS) – N_a: *S0655* (4 vs. 10), H_o: 3*87A12F* (0.711 vs. 0.300), *SBH1* (0.495 vs. 0.864), H_e: *387A12F* (0.802 vs. 0.311), *SBH1* (0.476 vs. 0.835), PIC *387A12F* (0.777 vs. 0.301), *SBH1* (0.434 vs. 0.811). The difference in the number of effective alleles is of special interest. At *SBH20*, N_a differs by two (10 vs. 8), whereas N_e differs by more than 4 (6.72 vs. 2.35).

The *SBH4* locus significantly deviated from HWE, in both populations. Negative fixation index, which was identified in ZS, was found at same loci in ZW, as well.

The average number of alleles in ZS is by 1.272 higher than in ZW. However, the average number of effective alleles is the same (3.532 – ZW, 3.431 – ZS). Similar estimates in each of the populations were found for the other variables. Both populations are heterozygous, H_e and H_o are above 0.5 for the whole panel of 11 STRs.

The specific to Zlotnicka breed alleles 22 and 23 at the *387A12F* locus were found at frequencies of 0.061 vs. 0.826 and 0.105 vs. 0.016, respectively. These alleles have not been found in commercial breeds that we have analysed with the panel, nor in the breeds included in Biotype Diagnostic GmbH worked out population study. The 22 allele was detected in the genetic resource in CZ (PC pig) at the frequency of 0.005 [Vrtková *et al.* 2016]. The 19.1 allele of the *387A12F* locus was found only in ZS at frequency of 0.024. The rate difference of alleles was recorded between ZW and ZS

Table 3. Alleles detected at each locus in Zlotnicka pigs

Locus	Alleles	
	ZW	ZS
387A12F	9, 12.1, 13, 14.1, 15, 16, 18, 20, 21, **22, 23**	13, 14.1, 15, 19.1, 21, **22, 23**
S0655	5, 11, 13, 22	5, 9, 10,11,12,13,14, 23, 24, 25
SBH1	10, 13, 14, 15, 16	10, 11, 12, 13, 14, 15, 16, 17
SBH2	6, 22, 23, 26, 29, 30	6, 9, 23, 26, 27, 28, 29, 30, 31, 33
SBH4	56, 57, 58, 59, 60, 62, 65.1, 66.1,	49, 56, 58, 60, 61, 62
SBH10	34, 35, 41, 42, 43, 45, 48, 49	40, 41, 42, 45, 46, 47, 48, 49, 50
SBH13	9, 12, 13, 15, 16	9,11, 13, 14, 15, 16, 17
SBH18	11, 12, 13, 14, 16, 17, 18	9, 11, 12, 13, 14, 15, 16, 17, 19
SBH19	11,14, 15, 16	11, 13, 14, 15, 16
SBH20	20, 22, 23, 31, 32, 36, 38, 39, 41, 42	20, 22, 23, 31, 35, 37, 39, 40
SBH22	18, 20, 23, 23.3	18, 20, 21, 22, 23, 23.3, 24.3

ZW– Zlotnicka White, ZS– Zlotnicka Spotted, bold – alleles specific for the Polish Zlotnicka breed.

(Tab. 3). Big rate difference between the populations might be affected by the number of individuals sampled in each of the breeds (ZW – 91, ZS – 250).

Comparison of the sexes

The genetic structure of the ZW and ZS boars and sows is illustrated in Table 4.

Table 4. Genetic variability among the sexes

Breed sex	n	TNA	MNA	MN_e	H_o	H_e	F
ZW all	91	71	6.455	3.532	0.659	0.684	0.023
ZW boars	13	53	4.818	3.192	0.688	0.699	-0.033
ZW sows	78	64	5.818	3.454	0.657	0.674	0.010
ZS all	250	85	7.727	3.431	0.637	0.659	0.040
ZS boars	20	61	5.545	3.319	0.673	0.670	-0.032
ZS sows	230	81	7.364	3.420	0.634	0.658	0.044

n – number of analysed samples, TNA – total number of alleles observed, MNA – mean number of alleles per locus, MN_e – mean number of effective alleles per locus, H_O – observed heterozygosity, H_e – expected heterozygosity, F – fixation index.

Between sexes comparison of variability in ZW

In the ZW population, the number alleles per locus in boars ranged from 3 (*SBH22, S0655*) to 8 (*387A12F*), in sows from 3 (*SBH22*) to 9 (*387A12F*). The smallest number of effective alleles (1.763) was detected in sows at the SBH1 locus. The number of effective alleles in boars was higher than 2.15 at all loci. The lowest observed heterozygosity in boars was find for the S*0655* locus (0.462) while in sows 0.417 (*SBH18*) and 0.462 (*SBH1*).

Between sexes comparison of variability in ZS

In the ZS population, the number alleles per locus in boars ranged from 4 (*SBH22, SBH18*) to 8 (*SBH1*) and in sows from 5 (*SBH19*) to 10 (*SBH2*). At the *387A12F* locus, low number of effective alleles was determined in both sexes (1.818, 1.422). Low N_e was detected in sows at locus *SBH4* (1.786). The lowest observed heterozygosity in boars – 0.450 – was found for the *SHB4* and *SBH22* loci. Very low heterozygosity was observed in sows at loci *387A12F* (0.283) and *SBH22* (0.373).

For all other loci, in both subpopulations, the observed heterozygosity was higher than 0.5. Higher numbers of alleles per locus detected in sows was apparently caused by higher number of sows included in the data sets, which increased the probability of sampling more alleles from the pool of the alleles present in the population. Negative fixation index means surplus of heterozygotes, therefore, boars are heterozygous (even though there are just few of them) which is beneficial for sustainability of the breed. A lesser number of effective alleles in sows, is a prove of higher heterozygosity of boars, especially with regard to the numbers of animals of both sex (Tab. 4).

Assignment to breed of origin by the clustering method

Clustering method showed indeed two different clusters when the populations were clustered. With K=2, ZW and ZS were identified as independent collections (Fig. 1).

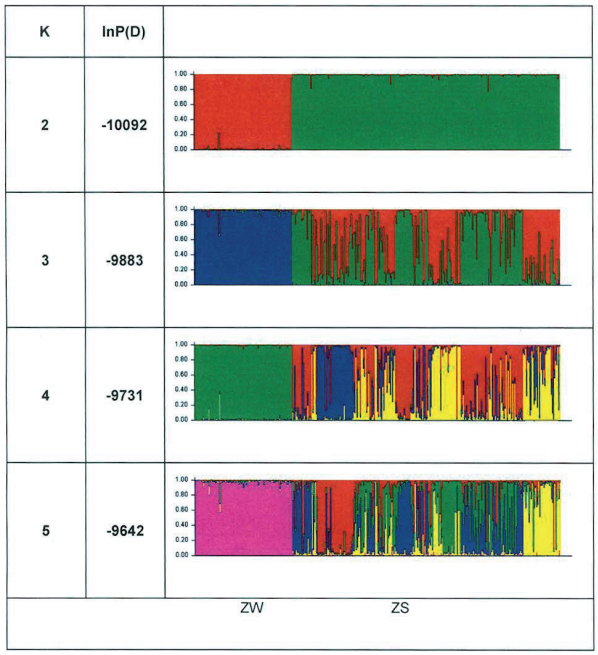

Fig. 1. Graphical representation of the estimated membership fractions of individuals of the breed analysed in each of the K inferred clusters, for K=2 to K=5.

For K=2, ZW (red) and ZS (green) were identified as separate populations. While the ZS population was further splitting up with the presumed K higher number, ZW remained unchanged up until K=5. Population ZW seems to be highly closed, separate population unless the smaller number of sampled animals had not limited the number of sampled alleles.

Table 5. Probability of identity by locus

Probability	Microsatellite / Loci											
ZW	*SBH1*	*S0655*	*SBH13*	*SBH2*	*SBH22*	*SBH10*	*SBH19*	*SBH18*	*SBH4*	*387A12F*	*SBH20*	
PI	0.316	0.233	0.225	0.199	0.192	0.162	0.142	0.110	0.078	0.061	0.040	
PISibs	0.592	0.509	0.499	0.488	0.470	0.473	0.434	0.409	0.376	0.366	0.334	
ZS	*387A12F*	*SBH4*	*SBH20*	*SBH22*	*SBH18*	*SBH13*	*SBH10*	*SBH19*	*S0655*	*SBH2*	*SBH1*	
PI	0.485	0.233	0.232	0.232	0.212	0.120	0.117	0.107	0.096	0.063	0.050	
PISibs	0.716	0.528	0.521	0.528	0.490	0.418	0.418	0.402	0.392	0.361	0.346	

PI – probability of identity of independent samples, PISibs – probability of identity among siblings.

Table 6. Probability of exclusion by locus

Probability	Microsatellite / Loci											
ZW	*SBH20*	*387A12F*	*SBH4*	*SBH18*	*SBH19*	*SBH10*	*SBH2*	*SBH22*	*SBH13*	*S0655*	*SBH1*	
P1	0.699	0.628	0.582	0.510	0.446	0.427	0.367	0.362	0.336	0.322	0.262	
P2	0.533	0.445	0.404	0.333	0.277	0.244	0.211	0.216	0.199	0.182	0.116	
P3	0.867	0.820	0.764	0.696	0.619	0.631	0.540	0.511	0.493	0.474	0.418	
ZS	*SBH1*	*SBH2*	*S0655*	*SBH19*	*SBH13*	*SBH10*	*SBH18*	*SBH20*	*SBH22*	*SBH4*	*387A12F*	
P1	0.663	0.623	0.538	0.512	0.495	0.492	0.355	0.339	0.337	0.336	0.179	
P2	0.491	0.446	0.361	0.335	0.318	0.316	0.211	0.181	0.172	0.172	0.052	
P3	0.836	0.802	0.719	0.690	0.678	0.682	0.518	0.510	0.519	0.516	0.316	

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.

Traceability

The estimates of probability of identity and probability of exclusions are important for the usability of microsatellites as identity and parentage testers. Probability of identity determination and power of exclusion varied across the loci. The estimates are shown in Tables 5 and 6.

Evaluation of effectiveness of the tetrameric STRs for traceability in ZW

Markers *SBH1*, *S0655* and *SBH13* (from the least efficient) were identified as markers of the smallest informative value and, therefore, the least suitable. *SBH4, 387A12F* a *SBH20* (from the least efficient) are the best markers in terms of suitability for identity and parentage testing. The probability of appearance of identical genotypes (PI) at locus *SBH20* in ZW is 4%. Compared to that, PI at locus *SBH1* is 31.7%.

In the case of recognition of siblings in the population, the probability of appearance of identical genotypes (PISibs) is 33.5% and 59.2% for the *SBH20* and *SBH1* loci, respectively.

In parentage testing for P1, when we have genotypes of both parents and one of them is given incorrectly, the effectiveness is higher than 50% for four loci (*SBH20* 69.9%, *387A12F* 62.8%, *SBH4* 58.2%, *SBH18* 51.1%). The effectiveness of these loci for P2 (when we have genotype of one parent only and this is wrongly identified) is from 53 to 33%. In the case that both presumed genotypes are known but parents are wrongly identified, the effectiveness is in range from 86.7 to 69.6%.

Evaluation of effectiveness of the tetrameric STRs for traceability in ZS

A smaller than 10 % probability of appearance of identical genotypes (PI) in ZS for loci *SBH1, SBH2* a *S0655* (5%, 6.3% and 9.6%) was recorded. The highest PI was found at locus *387A12F* – 48.5%. For all other loci, the probability of appearance of identical genotypes in population was below 24%. Higher than 50% probability of appearance of identical genotypes in siblings in the population was detected for four loci: *SBH4, SBH22, SBH10* (52%) and for the marker *387A12F*, in which it reached the level of 72%.

For P1, the effectiveness is higher than 50 % for four loci (*SBH1, SBH2, S0655, SBH19*; descending). The effectiveness for these loci and P2 is in range from 49.1 to 33.6 %. In the case that both presumed genotypes are given but parents are wrongly identified, the effectiveness is in range from 83.6 to 69.0%. The overall effectiveness of the panel of all 11 STRs loci is listed in table 7 for both breeds.

The probability of appearance of identical genotypes PI, PISibs using complete panel of 11 STR loci varies in similar way in both populations, however, more substantial assumption of appearance of identical genotypes exists for population ZS (Tab. 7). Vrtková *et al.* (2016) estimated in the PC breed PI and PISibs, using all 11 loci, at $4.037.10^{-11}$ and $8.315.\ 10^{-5}$, respectively.

The probability of exclusion of wrong parents under P1, P2, and P3, using the whole panel of 11 loci are above 97 % in both breeds. The estimates for ZW vs. ZS are: P1 – 0.999028 vs. 0.998867; P2 – 0.979980 vs. 0.976544; and P3 – 0.999991 vs. 0.999989.

Table 7. Probability of identity and probability of exclusions for combinations of 11 loci

Probability	ZW	ZS
PI	3.11777366480468 E-10	5.92143369493599 E-10
PISibs	1.33129593261900 E-4	1.74908996887315 E-4
P1	0.999028028006014	0.998866849228872
P2	0.979979763364943	0.976543972604964
P3	0.999991499126711	0.999989110205454

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.

In PC, Vrtková *et al.* (2016) report exclusion probabilities for loci combinations under P1, P2, and P3 as 0.999635, 0.989994, and 0.999998, respectively.

The 11 tetrameric Short Tandem Repeats panel proved to be useful for evaluation of diversity of the ZW and ZS breeds. The important practical finding is a detection of specific alleles at locus *387A12F* at high frequency in each population of Zlotnicka breed. The alleles 22 and 23 do not occur in commercial breeds, which were analysed with this panel, nor occurred in breeds included in the Biotype Diagnostic GmbH worked out population study. Allele 19.1 of the *387A12F* locus was found only in ZW. The studied subpopulations of the Zlotnicka pigs differ clearly as illustrated by clustering them based on the 11 tetrameric Short Tandem Repeats panel. ZW seems to be more unified within the subpopulation, compared to ZS, hence, it is possible to correctly assign individuals to adequate breed, with the use of tetrameric STRs.

On the basis of information obtained about Prestice Black Pied pigs and the results of the present paper we suggest using the panel of tetrameric STRs loci for parentage, traceability and differentiation of subpopulations studies in pigs of similar history (genetic resources closed in the second half of the 20th century).

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