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Evaluation of 24 microsatellite markers for parentage exclusion in three indigenous pig types of India

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ABSTRACT

A set of 24 FAO recommended microsatellite markers was evaluated for parentage exclusion (PE) in three Indian pig types of Assamese, North-Indian and Ankamali. The genomic DNA from these three Indian pig types was amplified at these loci by polymerase chain reaction and resolved for alleles. The allelic frequency data was analysed to calculate the probability of paternity exclusion when one of the parents is to be excluded (PE_1), when both the parents are to be excluded (PE_2) and when only one parent is known and that is to be excluded (PE_3) in these three Indian pig types using a set of minimum 5 loci and then increasing the number of loci in increments of 5 upto maximum of 24 loci. The cumulative PE_1 values taking into consideration all the 24 loci varied from 1–2.07×10^{-10} in North Indian pigs to 1–3.95×10^{-11} in Ankamali pigs. The cumulative PE_2 values taking into consideration all the 24 loci varied from 1–4.57×10^{-16} in Assamese pigs to 1–3.17×10^{-18} in Ankamali pigs. Similarly, cumulative PE_3 values for all the 24 loci varied from 0.9999968 in Assamese pigs to 0.99999955 in Ankamali pigs. The cumulative PE values obtained, even with a set of 15 loci (CGA, IGF1, S0005, S0026, S0068, S0090, S0155, S0178, S0215, S0218, S0228, S0355, SW122, SW911, SW936), were clearly more than the required value of 0.9995 in all the three breeds with the minimum value of 0.99985 for PE_3 in Assamese pigs. Clearly, this set of 15 loci or the sets with 20 or 24 loci can be safely employed for parentage exclusion purposes in the Indian pigs.

Key words: Microsatellite markers, Parentage exclusion, Pigs

The term microsatellite, also short tandem repeats (STRs), refers to a class of highly polymorphic codominant markers. They are dispersed throughout the nuclear genomes and follow Mendelian inheritance (Litt and Luty 1989, Weber and May 1989). They can be amplified easily and efficiently by polymerase chain reaction (PCR) and scored on urea polyacrylamide gels or through automated resolution with fair degree of reliability. The microsatellites, depending upon annealing temperatures and allele sizes, can also be multiplexed into single reaction. Thus microsatellites are markers of choice for evaluation of genetic diversity and differentiation of closely related breeds (Takezaki and Nei 1996). Their usefulness for pedigree or parentage verification had been proven in almost all the livestock species (Glowatzki-Mullis *et al.* 2007, Monies *et al.* 2011, Kathiravan *et al.* 2012, Souza *et al.* 2012, Rahimi-Mianzi *et al.* 2015, Yu *et al.* 2015, Brenig and Schulz 2016). Correct parentage resolution is necessary for any breed improvement programme especially in the multiple mating species like pigs (Aguilera-Reyes *et al.* 2006). The utility of microsatellites for parentage analysis had been evaluated in Chinese, European, Czech and Taiwanese pig breeds (Putnova *et al.* 2003, Fan *et al.* 2005, Lin *et al.* 2014, Yu *et al.* 2015). If such a method could be standardized for parentage and pedigree verification for indigenous breeds of pigs, it would be of immense help to Indian pig breeders. Keeping this in mind, this study was undertaken to evaluate the utility of microsatellite markers for parentage exclusion in three Indian pig types of Assamese, North Indian and Ankamali. The microsatellite set evaluated also included 13 microsatellites recommended by International Society for Animal Genetics (ISAG) for pigs for parentage exclusion (www.isag.org.uk/comparisons.hlm).

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MATERIALS AND METHODS

The blood samples were collected from 25 pigs of Assamese type from Asom, 25 pigs of North Indian type from Haryana and 26 samples of Ankamali type from Kerala representing 3 pig types (Bhat *et al.* 1981). The DNA was isolated by standard procedure of digestion with proteinase K, extraction with phenol/chloroform and precipitation with ethanol. The stock DNA was stored at –20°C and the working dilutions were stored at 4°C.

The genomic DNA was amplified by PCR using 24 microsatellite primers. The primers were synthesized by Gemini Biotech (The Woodlands, TX, USA). Each 25 µl

reaction consisted of DNA (approximately 100 ng), primers (60 ng), dNTPs (40 mM each), 10× buffer (10 mM tris, 50 mM KCL, 0.1% gelatin, pH 8.4) (2.5 µl), $MgCl_2$ (1.5 mM or as specified in FAO 1998) and Taq DNA polymerase (0.75 units). The thermo-cyclic conditions were initial denaturation at 92°C for 2 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at the temperature given in FAO (1998) for 45 sec and extension at 72°C for 45 sec, with a final extension at 72°C for 10 min. The amplified fragments were analysed on 7% denaturing urea polyacrylamide gel and detected by silver staining (Bassam *et al.* 1991). The fragment sizes were calculated according to Sealey and Southern (1990).

The allele frequencies, observed (N_o) and effective (N_e) number of alleles, observed (H_o) and expected (H_e) heterozygosity were calculated using POPGENE computer program (Yeh *et al.* 1999). The polymorphic information content (PIC) was calculated as described by Botstein *et al.* (1980).

The parent exclusion probability for an individual microsatellite locus, when (i) given two parents and one offspring and one parent is to be excluded (PE_1), (ii) given two parents and one offspring and both parents are to be excluded (PE_2), and (iii) given one parent and one offspring and that parent is to be excluded (PE_3), was calculated as described by Jamieson and Taylor (1997). The three combined exclusion probabilities, to evaluate the number of loci required to achieve the required exclusion probabilities, were calculated as

$$P = 1 - (1–P_1)\,(1-P_2)\,(1-P_3)\ldots\ldots\ldots(1-P_n)$$

RESULTS AND DISCUSSION

The most important use of the molecular markers in pedigree verification is for parentage exclusion. Earlier, breeders relied on blood grouping and protein polymorphism as tools of genetic testing as part of their efforts to ensure pedigree integrity. The DNA genotyping especially with microsatellites is one of the most reliable and cost effective method of pedigree construction.

Although, in recent times, use of single nucleotide polymorphisms (SNP) have been suggested for parentage exclusion studies in livestock species including pigs (Fernandez *et al.* 2013, McClure *et al.* 2013, Yu *et al.* 2015), their absolute requirement for automated DNA sequencer reduces their cost effectiveness. Moreover, microsatellite loci can be easily chosen to be spread across the genome on different chromosomes to give a clearcut picture.

Due to their highly polymorphic nature, the use of microsatellite typing for parentage control and solving the problems of questionable paternity had been advocated in almost all livestock species (Behl *et al.* 2007, Souza *et al.* 2012, Monies *et al.* 2011, Jakhesara *et al.* 2012, Kathiravan *et al.* 2012, Brenig and Schulz 2016). Due to multiple mating nature of sows and so the probability of multiple paternity of the litter makes the use of such unambiguous paternity exclusion methodology absolutely necessary for pigs (Aguilera-Reyes 2006, Menéndez *et al.* 2015).

The diversity measures for all 24 loci evaluated in this study across the three Indian pig types is given in Table 1. The total number of alleles varied from 5 (S0178 and S0386) to 12 (S0005, S0355, SW936). The observed heterozygosity varied from 0.61 (S0227) to 0.89 (S0155) with an overall mean of 0.73±0.08. The overall mean effective number of alleles was 6.38±1.44 which was also selected in the overall mean expected heterozygosity of 0.84±0.04. The PIC values ranged from 0.69 (S0178) to 0.88 (CGA, S0005, S0355) with a mean of 0.83±0.05.

The total PE_1 values taking into consideration all the 24 loci varied from 1–2.07×10^{-10} in North Indian pigs to 1–3.95×10^{-11} in Ankamali pigs (Table 2). The total PE_2 values taking into consideration all the 24 loci varied from 1–4.57×10^{-16} in Assamese pigs to 1–3.17×10^{-18} in Ankamali pigs. Similarly, cumulative PE_3 values for all the 24 loci varied from 0.9999968 in Assamese pigs to 0.99999955 in Ankamali pigs. These values were clearly more than the generally accepted notion that such exclusion probabilities should be more than 0.9995 (Luikart *et al.* 1999, Cho and Cho 2004).

To calculate the minimum number of loci required for

Table 1. Annealing temperature (°C), PCR product size range, observed (N_o) and effective (N_e) number of alleles, observed (H_o) and expected (H_e) heterozygosity and polymorphism information content (PIC) at 24 microsatellite loci in three Indian pig types.

Micro-satellite locus	Annealing tempera-ture (°C)	PCR product size range (bp)	N_o	N_e	H_o	H_e	PIC
CGA	62	266-302	11	8.28	0.81	0.89	0.88
IGF1	58	278-296	8	5.77	0.72	0.83	0.82
S005	58	215-257	12	8.85	0.74	0.89	0.88
S0026	55	92-110	9	6.98	0.84	0.86	0.85
S0068	62	218-242	9	7.49	0.69	0.87	0.86
S0090	58	241-251	6	5.4	0.73	0.82	0.80
S0155	55	150-168	7	5.53	0.89	0.82	0.81
S0178	58	110-124	5	3.43	0.65	0.71	0.69
S0215	55	137-163	11	7.88	0.73	0.88	0.87
S0218	55	164-184	9	6.98	0.72	0.86	0.85
S0225	55	172-194	8	6.46	0.64	0.85	0.84
S0226	55	185-205	9	6.2	0.62	0.84	0.83
S0227	55	231-253	10	6.22	0.61	0.84	0.83
S0228	55	227-245	10	7.8	0.77	0.88	0.87
S0355	55	247-273	12	8.66	0.77	0.89	0.88
S0386	48	156-172	5	4.01	0.68	0.76	0.72
SW24	58	92-112	6	5.17	0.74	0.81	0.80
SW72	58	100-116	8	6.09	0.59	0.84	0.83
SW122	58	110-132	10	6.54	0.66	0.85	0.84
SW632	58	157-173	8	5.7	0.69	0.83	0.82
SW857	58	145-157	7	5.15	0.69	0.81	0.79
SW911	60	153-175	9	6.48	0.81	0.85	0.84
SW936	58	86-112	12	7.81	0.81	0.88	0.87
SW951	58	125-135	6	4.25	0.85	0.77	0.74
Mean			8.62± 2.14	6.38± 1.44	0.73± 0.08	0.84± 0.04	0.83± 0.05

Table 2. Probability of paternity exclusion when one of the parents is to be excluded (PE_1), when both the parents are to be excluded (PE_2) and when only one parent is known and that is to be excluded (PE_3) in three Indian pig types using a set of minimum 5 loci and then increasing the number of loci in increments of 5 up to maximum of 24 loci and 13 loci that were common with parentage verification kit for pigs recommended by ISAG

Number of loci employed	Pig type		
	Assamese	Desi	Ankamali
		PE_1	
5	0.9944	0.9967	0.994
10	0.999966	0.999986	0.999965
13 (ISAG)	0.999982	0.9999934	0.9999966
15	0.99999944	0.9999998	0.99999971
20	0.99999999	$1\text{-}3.351\times10^{-9}$	0.999999998
All 24	$1\text{-}7.09\times10^{-10}$	$1\text{-}2.07\times10^{-10}$	$1\text{-}3.95\times10^{-11}$
		PE_2	
5	0.99985	0.999938	0.99983
10	0.999999972	0.99999999	0.999999972
13 (ISAG)	0.999999989	$1\text{-}1.8537\times10^{-9}$	0.999999999
15	$1\text{-}3.11\times10^{-11}$	$1\text{-}5.01\times10^{-12}$	$1\text{-}9.21\times10^{-12}$
20	$1\text{-}3.49\times10^{-14}$	$1\text{-}5.26\times10^{-15}$	$1\text{-}3.25\times10^{-15}$
All 24	$1\text{-}4.57\times10^{-16}$	$1\text{-}4.89\times10^{-17}$	$1\text{-}3.17\times10^{-18}$
		PE_3	
5	0.9597	0.9724	0.9571
10	0.9983	0.99908	0.9982
13 (ISAG)	0.9984	0.99926	0.99952
15	0.99985	0.999929	0.9999
20	0.999986	0.9999935	0.9999943
All 24	0.9999968	0.9999986	0.99999955

developing a set of loci to achieve the specified minimum cumulative exclusion probabilities, the PE values were calculated for a selected set of minimum of 5 loci, then increasing the number of loci in increments of 5. The minimum cumulative PE values obtained with selected set of 5 loci (CGA, S0026, S0228, S0355, SW936) with N_o>9, H_o>0.75 and PIC>0.85 were 0.994 for PE_1 (Ankamali), 0.999828 for PE_2 (Ankamali) and 0.95705 for PE_3 (Ankamali). Clearly, only the PE_2 values reached the required stringency. The cumulative PE values with selected set of 10 loci (CGA, S0005, S0026, S0215, S0218, S0228, S0355, SW122, SW911, SW936) with N_o>9, H_o>0.65 and PIC>0.8 were of the order 0.9999 for PE_1 and 0.9999999 for PE_2 thus achieving the required stringency. However, minimum PE_3 values with these 10 loci were of the order of only 0.998. However, with a set of 15 loci (CGA, IGF1, S0005, S0026, S0068, S0090, S0155, S0178, S0215, S0218, S0228, S0355, SW122, SW911, SW936), the PE values obtained were clearly more than the required value of 0.9995 in all the three breeds with the minimum value of 0.99985 for PE_3 in Assamese pigs. Similarly, set of 20 loci could clearly achieve the required cumulative PE values. However, 13 loci that were common with the set proposed by the ISAG, clearly achieved the required minimum values for PE_1 and PE_2 but showed a minimum PE_3 of 0.9984 in Assamese pigs. Moreover, in the recent times, the DNA genotyping has become most cost effective method for pedigree maintenance in large populations of animals because of the decrease in the price of reagents and instruments (Dimsoski 2003). The microsatellites have an added advantage of multiplexing over SNP based methods thus saving time and energy. In conclusion, the microsatellite marker set of 15 loci or the sets with more number of loci evaluated in this study can be safely employed for the parentage exclusion purposes in Indian pigs.

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