Assessment of a new microsatellites panel for traceability in Italian inbreed pigs using parentage test

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Summary
The origin of meat and meat products can be traced by verifying the identity of an offspring from its parents' genotypes. Although there are many microsatellite panels applicable to swine population, efficiency of parental testing decreases when the population consists of consanguineous animals. The aims of the present study were to develop a new microsatellite panel for traceability using parentage test in inbreed pig population and to assess how hybridization can influence the efficiency of parental testing. A new genotyping assay, based on 20-microsatellite assay, was performed in 304 individuals consisting of related and unrelated animals. The results showed that the microsatellites used in this study display high level of polymorphism ensuring a parentage assignment of 100%. This genotyping panel can be a useful tool to test a 'parent-to-fork' traceability system based on 20 microsatellite loci and can overcome technical limitations in inbreed population.

Over the past decades, there has been a growing public interest in enhancing food traceability and transparency in food production. For these reasons, the EU's General Food Law was introduced in 2002 and made traceability mandatory for all food chain (Reg. EC 178/2002). DNA-based techniques, such as microsatellites, appear to be an ideal tracking tool to assess the origin of meat and meat products and allow the identification of the animal producer through a molecular fingerprint (individual tracking) (Scarano and Rao 2014, Orrù *et al.* 2006).

In contrast to the situation in cattle, where cows have on average three calves in their life, a sow produces about 40-60 piglets during her reproductive lifetime. Thus, it is not cost effective to record each pig in order to enable tracing of meat products by direct DNA fingerprints. In a real scenario, where the pig production is based on intensive breeding, it is therefore preferable to use the parentage test. Specifically, it is common to compare the maternal genotypes because very often the paternal lineage is unavailable, due to husbandry practices (Menéndez *et al.* 2015). A simple approach to parentage analysis relies on a process of exclusion. The genotype of candidate parent is compared to the offspring's genotype and is excluded as parent if a mismatch occurs at one or more loci (Jamieson and Taylor 1997).

During the last decades, the selection of high-performance pig breeds in combination with the displacement of extensive production systems has led to a dramatic decrease in the gene pool, resulting in a reduction of genetic variation among populations (Michailidou *et al.* 2014). Therefore, the effectiveness of a traceability system based on parentage test is linked to availability of an efficient panel of microsatellites, able to produce accurate results for both, pure breed pigs and commercial hybrids with high level of inbreeding. Although there are many microsatellites sets to be used in the swine population (Blasi *et al.* 2003, Costa *et al.* 2012, Guastella *et al.* 2010, Putnová *et al.* 2003, Lin *et al.* 2014, Oh *et al.* 2014, Nechtelberg *et al.* 2001), there is no data on the influence of inbreeding on the performance of microsatellite array.

The aims of this study were to test a traceability system 'from parents to fork', using new microsatellite markers and to verify how inbreeding may affect the efficiency of parentage test in this typology of breeding.

The study included 304 animals consisting of related animals (71 sows and 71 adult offspring) and 162 unrelated animals from different farms. These animals were commercial crossbreeds between Italian Duroc, Italian Large White and Italian Landrace. DNA was extracted from blood using DNeasy Mini spin column (Qiagen) according to the manufacturer's instructions.

Table I. *List of microsatellite primers sequence.*

Locus	Primer sequence (5'-3')	Chr.	Reference
	Multiplex N. 1		
S0005	F: TCCTTCCCTCCTGGTAACTA-FAM R:GCACTTCCTGATTCTGGGTA	5	[13]
S0090	F:CCAAGACTGCCTTGTAGGTGAAA-VIC R:GCTATCAAGTATTGTACCATTAG	12	[13]
S0101	F:GAATGCAAAGAGTTCAGTGTAGG-PET R:GTCTCCCTCACACTTACCGCAG	7	[13]
S0155	F: TGTTCTCTGTTTCTCCTCTGTTTG-FAM R:GTTAAAGTGGAAAGAGTCAATGAT	1	[13]
S0355	F:TCTGGCTCCTACACTCCTTCTTGG -NED R:GTTTGGGTGGGTGCTGAAAAATAGGA	15	[13]
S0386	F: GAACTCCTGGGTCTTATTTTCTA-NED R:GTCAAAAATCTTTTTATCTCCAACAGTAT	/	[13]
SW24	F: CTTTGGGTGGAGTGTGTGC -FAM R:ATCCAAATGCTGCAAGCG	17	[13]
SW240	F: AGAAATTAGTGCCTCAAATTGG-VIC R:AAACCATTAAGTCCCTAGCAAA	2	[13]
SW857	F:TGAGAGGTCAGTTACAGAAGACC-PET R:GATCCTCCTCCAAATCCCAT	14	[13]
SW951	F: TTTCACAACTCTGGCACCAG-NED R:GATCGTGCCCAAATGGAC	10	[13]
	Multiplex N. 2		
SW72	F: ATCAGAACAGTGCGCCGT -PET R:GTTTGAAAATGGGGTGTTTCC	3	[13]
SW936	F: TCTGGAGCTAGCATAAGTGCC- FAM R:GTGCAAGTACACATGCAGGG	15	[13]
SW911	F: CTCAGTTCTTTGGGACTGAACC-HEX R:CATCTGTGGAAAAAAAAAGCC	9	[13]
S0228	F: GGCATAGGCTGGCAGCAACA -HEX R:GTTCCGCCCTCACAGACCCAAAT	6	[13]
	Multiplex N. 3		
SW1370	F: AGAGCAGTGGTCTGCTAAGATG-NED R:GAATTGCCTAAATTTACTTGTCC	2	[15]
SW1035	F: TATGGGGGCCCTAAAAAGAC-PET R:AACGGCCTTAACCTCCTCAG	16	[15]
SWR153	F: CCACGTTCTCCTTTTTGAGG- VIC R: ATGAGTTGTGGTGTAGGTCGC	4	[15]
SW2038	F: GCCGAGAAACCCTTCACC -VIC R:TAGCCTGTTCAGTGCCACC	14	[15]
S0017	F: CTAGGAGAAAATCTGAGGTT- FAM R: GTTTGAATGGAGGTGCTGTA	8	[15]
SW1823	F: CAGGTCATTGCTGTAGTGAAGG-NED R:GAGCCTTGGGCTACGTAGTG	6	[15]

Individual genotyping was performed using twenty microsatellite markers fluorescently labelled divided in 3 multiplex PCR (Table I), using Type-it Microsatellite PCR Kit (Qiagen). The first 14 microsatellite markers are suggested by Nechtelberg and colleagues (Nechtelberg *et al.* 2001) and reported in FAO Guidelines (FAO 2011); while the last six markers are present in the USDA MARC database[1]. The PCR conditions were as follow: 1) denaturation at 95 ˚C for 15 minutes; 2) 35 cycles of 95 ˚C for 30 seconds, 60 ˚C for 90 seconds and 72 ˚C for 30 seconds; 3) last step on 72 ˚C for 10 minutes. Fragment analysis was carried out with capillary electrophoresis on the ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) using GeneScan™ 600 LIZ® Size Standard. The allele size was assigned using the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA) and the allele nomenclature was standardized using reference samples according to International Society for Animal Genetics (ISAG).

Allele frequencies, number of alleles (na) and Polymorphism Information Content (PIC) were assessed by Excel Microsatellite Toolkit (Park 2001). GENEPOP package (Raymond and Rousset 1995) was utilized to calculate observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg Equilibrium (HWE) and F-statistics for each locus (Weir and Cockerham 1984).

The new assay was compared with the previous panel suggested by Nechtelberg and colleagues (Nechtelberg *et al.* 2001) to verify if the efficiency of the parentage test is directly linked to the number of microsatellites used. The probability of non-exclusion for one candidate mother was assessed in case the genotype of both parents was not known (1EX) and if only the parent's genotype of the opposite sex was known (2EX) according to the formula of Jamieson and Taylor (Jamieson and Taylor 1997). Further, the non-exclusion probability for identity analysis of unrelated and related individuals was calculated as previously described (Waits *et al.* 2001). The mother assignments were performed using Cervus 3.0 software (Marshall *et al.* 1998, Kalinowski *et al.* 2007).

The summary statistics results calculated for the assay used in this study were reported in Table II.

The total number of alleles for the twenty markers panel were 164 with a mean number of 8.2 alleles per locus, ranging between 4 (SWR153) and 15 (S0005) alleles. The expected heterozygosity (He) and observed heterozygosity (Ho) mean

[1] http://www.genome.iastate.edu/ pigs/maps/marc.html.

Table II. *Allele frequencies and F-Statistics of 20 microsatellite loci used in this study.*

Locus	na	He	Ho	PIC	HW	F(null)	FIS	FST	FIT
S0005	15	0.8355	0.8905	0.8795	NS	0.0275	0.0164	0.0815	0.0966
S0090	7	0.7072	0.6851	0.6344	NS	- 0.0166	- 0.0867	0.088	0.009
S0101	5	0.4671	0.4764	0.4464	NS	0.0097	0.0011	0.0332	0.0343
S0155	6	0.6678	0.6517	0.5892	NS	- 0.0181	- 0.0987	0.117	0.0298
S0355	8	0.5362	0.5056	0.5056	NS	- 0.0476	- 0.1195	0.926	- 0.0159
S0386	7	0.4803	0.5556	0.4748	NS	0.0746	0.1144	0.0434	0.1529
SW24	12	0.6875	0.7748	0.7371	*	0.0578	0.0395	0.1317	0.1659
SW240	13	0.6842	0.7188	0.6903	NS	0.0247	0.0143	0.0613	0.0748
SW857	9	0.7961	0.8115	0.7865	***	0.0072	- 0.088	0.0494	0.0411
SW951	5	0.4934	0.5474	0.4875	NS	0.0495	0.0946	0.0086	0.1023
SW72	14	0.7138	0.6819	0.6456	**	- 0.0278	- 0.0957	0.0686	- 0.0205
SW936	9	0.7697	0.7283	0.678	NS	- 0.0287	- 0.1066	0.0794	- 0.0187
SW911	8	0.6513	0.6271	0.5684	NS	- 0.0211	- 0.0641	0.043	- 0.0183
S0227	7	0.773	0.7265	0.6864	NS	- 0.0346	- 0.1064	0.062	- 0.0341
SW1370	7	0.5592	0.6602	0.6251	**	0.0821	0.1265	0.0547	0.1742
SW1035	5	0.7928	0.726	0.6791	NS	- 0.0477	- 0.1484	0.0864	- 0.0492
SWR153	4	0.5066	0.5796	0.5354	NS	0.0574	0.101	0.0502	0.1462
SW2038	7	0.6842	0.7961	0.766	**	0.0746	0.1054	0.0701	0.1681
S0017	7	0.4737	0.735	0.691	***	0.2167	0.3431	0.0351	0.3662
SW1823	9	0.4803	0.8	0.7712	***	0.2469	0.3764	0.0674	0.4185
all	8.2	0.6379	0.6839	/	/	/	0.0298	0.0681	0.0959

na = Number of alleles per locus; Ho = Observed heterozygosity; He = Expected heterozygosity; PIC = Polymorphic information content; HW = Deviation from Hardy-Weinberg equilibrium; F(null) = Frequencies of null alleles; FIS = Inbreeding coefficient; FST = Fixation index; FIT = Overall inbreeding coefficient; NS = Not significant; *Significant at the 5% level; **Significant at the 1% level; ***Significant at the 0.1% level.

values were 0.6839 and 0.6379, respectively. Three loci (S0101, S0386 and SW951) showed a value of Polymorphism Information Content (PIC) lower than 0.5. The probability of finding null alleles is significant for three loci (SW24, S0101, SW857), these values were highly influenced by inbreeding of sub structured populations.

The locus SW24 showed a significant deviation from Hardy-Weinberg Equilibrium at 5% significance level after Bonferroni correction; the loci SW72, SW1370 and SW2038 at 1% significance level and SW857, S0017 and SW1823 at 0,1%. Deviations from Hardy-Weinberg equilibrium at many loci might be caused by the inbreeding. Eleven out of 20 markers showed positive inbreeding coefficient (FIS) value indicating that exist of inbreeding in these loci. Furthermore, two loci S0017 and SW1823 showed very high FIS value of 0.3431 and 0.3764, respectively. The overall FIS coefficient for the loci was 0.0298, indicating a significant ($p < 0.001$) excess of homozygotes in the whole samples. Meanwhile, the overall inbreeding coefficient (FIT) value of an individual relative to the total population was 0.0959. The fixation index (FST) value for all samples was 0.0681, confirming the presence of inbreeding in our study group.

The results of the comparison between the new assay and the previous panel suggested by Nechtelberg and colleagues (Nechtelberg *et al.* 2001) are shown in Figure 1. The probability of non-exclusion in executing the parentage test if there are no data on parental genotypes (1EX) was 5.84E-04 for the 20 microsatellites assay showing a high performance; the other panel showed a lower performance (Figure 1a). We found similar results among the panels in the case of probability of assigning the putative parent incorrectly and knowing the genotype of the other parent (2EX) as shown by the trend of the graph (Figure 1b).

The results of simulation of parentage test confirmed the effectiveness of the microsatellites assay for establishment of parentage in inbreed pig population.

The identity test showed different results along the panels tested, whereas the related test with the 20-microsatellites assay performed well and showed improvement of the relationship assignment compared with the other panel (Figure 1c). The probability of non-exclusion for identity test using 14 microsatellite markers was 8.25E-13 and for 20 markers was 1.83E-18. The non-exclusion probability for full-sib test using

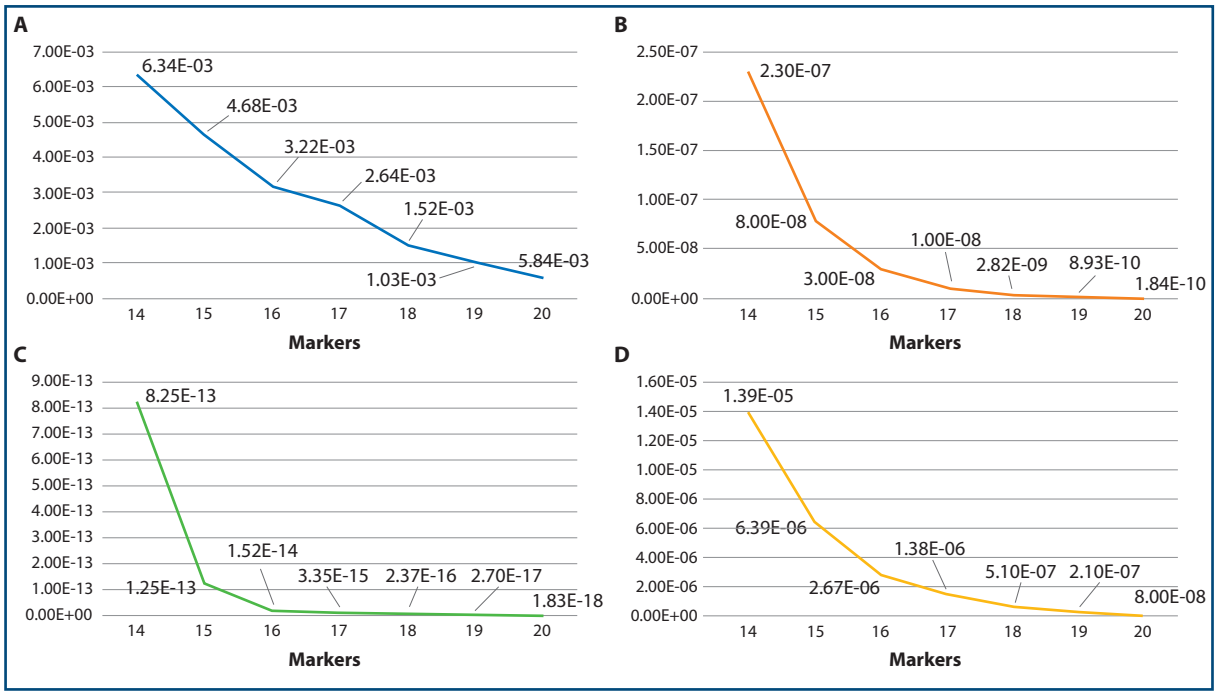

Figure 1. *Graphic representations of the comparison between the new assay and the previous panel suggest by Nechtelberg* et al. The following graphs report the non-exclusion probability if the parental genotype is unavailable (1EX).

Table III. *Results of the mother's assignments using the 14 and 20 microsatellite panels.*

	20 markers			14 markers		
	correct assignment	wrong assignment	no assignment	correct assignment	wrong assignment	no assignment
unrelated animals	/	/	162	/	13	149
piglets	71	/	/	68	2	1

14 and 20 microsatellite markers was 1.39E-05 and 8.00E-08, respectively (Figure 1d). The different results obtained from the probability values in our study confirmed that the new marker set should have good discriminatory power to resolve any situation, including parentage test with multi-putative mothers.

Based on our observational data, we tested 233 putative mother-offspring relationships (Table III). We have compared the results obtained in our study with those derived from only 14 microsatellites, as suggested by Nechtelberg and colleagues (Nechtelberg *et al.* 2001), and verified the accuracy of assigning the mother to each offspring in real conditions, because probabilities of parentage in animals with certain degree of inbreeding is lower than the probabilities calculated (Putnòva *et al.* 2003). In both of cases, a strict confidence (95%) level has been applied and the results of the assignments of parentage were reported in Table III. In addition to 162 unrelated animals, we tested 71 offspring and their respective mothers, assuming an unknown relationship.

Using the 20-microsatellite panel, it was possible to correctly assign each of the 71 offspring to their mother and none of the external individuals were attributed to the group of mothers (Table III). When we analysed the same group of animals using the 14 microsatellites, we found that two offspring were incorrectly assigned, and a son-mother combination remained unresolved, while 13 individuals, from the unrelated population, were wrongly assigned to putative mothers (Table III). These results confirmed that the offspring attribution to their mother using 14 markers only are insufficient and can result in incorrect traceability, as individuals outside are included in the related population.

In conclusion, in the populations where genetic variability is limited, an accurate traceability which would be based exclusively on parentage test, is possible only by using a very large number of markers. Our results support the efficacy of the described 20-microsatellites assay as a valuable tool for parentage testing in inbreed pigs. Although many microsatellites have been described for domestic pigs, in a population in which there is a high level of

consanguinity, a percentage of kinship testings may not be resolved by using a limited set of loci, and for this reason we recommend to amplify the panel with more microsatellites markers. Since the offspring in question shared common alleles across all loci, the comparison with maternal genotypes only might be challenging. The new set of microsatellite loci shown in this study overcomes these technical limitations and therefore has a potential to become a new more effective alternative for a reliable 'parent-to-fork' traceability system in inbreed populations.

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Statement of animal rights

The samples analyzed in this study were taken along the National eradication program for the swine vesicular disease. Therefore, the farm animal-welfare bodies didn't point out any animal welfare issue and considered unnecessary the authorization from the ethics committee.

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