Journal of Animal and Veterinary Advances 11 (22): 4236-4245, 2012
ISSN: 1680-5593
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An Identification Method for the Tokyo X Strain; The Pig Brand of the Tokyo Metropolitan Agriculture and Forestry Research Center

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Abstract: In this study, researchers developed a DNA-based identification method for the Tokyo X strain which is a pig brand developed by the Tokyo Metropolitan Livestock Experiment Station. To formulate the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Method, Single Nucleotide Polymorphisms (SNPs) were screened and a total of 104 SNP loci were fixed in the original Tokyo X strain of these, researchers selected 8 SNPs which are recognisable by restriction endonucleases and have high polymorphism within other major commercial breeds and market pork. The combined exclusion probability of general commercial pork and Berkshire pork from Tokyo X was 98.1 and 97.4%, respectively. The SNP marker set developed in this study will be useful in preventing fake Tokyo X brand pork from appearing on the market.

Key words: Tokyo X strain, identification method, SNPs, exclusion probability, PCR-RFLP

INTRODUCTION

Tokyo X is a synthetic pig strain developed by the Tokyo Metropolitan Livestock Experiment Station (now known as the Tokyo Metropolitan Agriculture and Forestry Research Centre) in 1995. This strain's origins, Beijing Black (male 2, female 5), Berkshire (male 5, female 16) and Duroc (male 4, female 15) were selectively bred for 5 generations to achieve uniform traits and genetic kinship in a herd of this strain. In 1997, Tokyo X was registered as a distinct strain by the Pig Breeders' Association of Japan.

Tokyo X is a medium-framed and early-maturing type. Coat colour varies; black, uniform red, red with black spots, black piebald and occasionally white occur in the herd. The meat quality is superior in particular, the high intramuscular fat content satisfies the requirements of the Japanese consumer. In a sensory evaluation by the university students in Tokyo, the Tokyo X pork was considered to have a better taste than that of commercial 3-way cross pigs of Landrace, Large White and Duroc (LWD) or Berkshire origin. As regards reproduction traits, the number of litters per sow per year is 1.7 and piglets per litter is 9.5 these values are inferior to those of the Large White/Landrace cross which is the most popular way to produce the sow for 3 ways crossing. Furthermore, the age at slaughter of the Tokyo X strain is 210 days which is almost 30 days more than that of the LWD 3 ways cross. Because of the superiority of meat quality and the inferiority of production and reproduction traits of Tokyo X, the meat of this strain is higher priced than that of general commercial pork.

At present, the original strain, represented by 23 boars and 62 sows is maintained at the livestock centre in Ome city (http://www.tokyo-aff.or.jp/syutiku/oume.html). A co-operative farming group has been established for the production of Tokyo X pork and the mating within purebred pigs which are supplied by the Ome livestock centre is obligatory. Approximately 9,000 pigs are shipped from this group annually. The higher price of Tokyo X pork will result in the appearance of general commercial pork, falsely labelled as Tokyo X pork in the market. Parentage testing by using DNA microsatellite markers is of practical use in the prevention of false labelling in this

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strain. However, microsatellite marker analysis requires expensive capillary sequencers and highly skilled assessment of the number of repeat units of the marker, so it is not practicable in conventional laboratories.

In this study, researchers present an identification system for the Tokyo X strain by using the PCR-RFLP Method for use in laboratories with basic equipment. First, researchers screened the SNP loci for those that were single allele in Tokyo X but showed polymorphism in the other commercial breeds. Second, researchers genotyped the selected SNPs from DNA extracted from >1000 commercial pigs. Third, researchers selected 8 SNP loci which showed higher minor allele frequencies in the commercial pig samples and were recognisable by restriction endonucleases. Last, researchers developed the PCR-RFLP System for distinguishing Tokyo X pork from other commercial pork.

MATERIALS AND METHODS

Sample collection and DNA extraction for genotyping by MALDI-TOF MS: Researchers collected blood samples from the 85 Tokyo X pigs (23 boars and 62 sows) maintained at the Ome centre to establish whether the selected SNPs were single allele in all of the conserved Tokyo X pigs. In addition, researchers collected blood samples from 784 general market pigs (mainly white LWDs) from 3 abattoirs and 464 Berkshire pigs from 1 abattoir. The Berkshire breed is used for Kurobuta (black hog) pork production and this pork has >5% share in the Japanese market. Researchers also purchased 96 fresh meat samples of general market pork from the stores. DNA was extracted by conventional phenol/chloroform Method after treatment with proteinase K (Sambrook and Russell, 2001). All animal experiments were performed in accordance with the institutional and Japanese government guidelines for the care and use of laboratory animals.

Screening of SNP loci for use in identifying the Tokyo X strain and genotyping by MALDI-TOF MS: Earlier, genotyping was performed on >2,000 SNPs using Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) on reference DNA from 384 individuals. This DNA panel was the same as that used in the earlier studies (Okumura *et al*., 2008, 2010) and included 26 Tokyo X pigs. A total of 1,760 SNPs was successfully genotyped and registered at the SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/) of the National Centre for Biotechnology Information (NCBI) with ss numbers of ss196000741 to ss1960002500 (Matsumoto *et al*., 2012). Of the 1,760 SNPs, 606 loci were single allele in the 26 Tokyo X individuals in the reference DNA panel. Then, the SNPs for genotyping the market pork samples and maintained Tokyo X samples were selected on either of the following criteria: The Minor Allele Frequency (MAF) was >20% in both Landrace and Large White samples of the reference DNA or the MAF was >10% in Duroc or Berkshire samples of the reference DNA.

As for the screened SNPs, genotyping was performed by MALDI-TOF MS using the iPLEX reagent kit (http://www.sequenom.com) on the Sequenom MassARRAY platform according to the manufacturer's instructions as briefly described in the earlier study (Okumura *et al*., 2008).

Formulation of the PCR-RFLP identification method for the Tokyo X strain: Of the SNPs successfully genotyped on the conserved Tokyo X and market samples, the SNPs were selected for identification of the Tokyo X strain by PCR-RFLP. The criteria for SNP selection were SNP loci with single alleles in the conserved Tokyo X strain, a MAF of >10% in both general commercial and Berkshire pig samples and SNPs recognisable by restriction endonucleases. After applying the above-mentioned criteria, researchers attempted to select unlinked SNPs located on different chromosomes.

Researchers sequenced the flanking regions of the SNPs to eliminate polymorphisms such as SNPs, indels and repetitive sequences which disrupt annealing of primers and successful PCR. The DNA of 24 or 32 individuals was used for sequencing of about 800 bp that contained the relevant SNP markers in the centre of the sequences.

Multiple sequence alignment and identification of nucleotide polymorphism were done by ATGC Software Ver. 3.0 (Software Development Co., Ltd. Tokyo, Japan). After the sequences were obtained, screening for interspersed repeats and low complexity DNA sequences was performed using RepeatMasker (http://www.repeatmasker.org). Then, the primers for the PCR-RFLP assays were designed by Primer3 (http://primer3.sourceforge.net/) excluding SNPs, indels, interspersed repeats and low complexity DNA sequences on the primer-annealing sites.

PCR was performed in a 20 μL reaction volume with 0.5 μM final concentration of primers and 200 μM each of deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ and 1.0 U Taq DNA polymerase (AmpliTaq Gold, Biosystems, Foster City, CA, USA), together with 1×PCR buffer. The following PCR protocol was used for all primer sets: denaturation was performed at 94°C for 10 min followed by 40 cycles each at 94°C for 30 sec, 60°C for 30 sec and

72°C for 30 sec and finally, 1 cycle at 72°C for 5 min. PCR amplifications were verified by agarose gel electrophoresis (1%). Each amplified sample then underwent endonuclease digestion using 8 μL of PCR amplified product, 5 U of each corresponding endonuclease and 1×digestion buffer. For BsmFI digestion of SNP loci, 100 ng of bovine serum albumin was added as a reaction reagent. SNP loci, restriction endonucleases and reaction temperatures are listed in Table 1. Two restriction endonucleases, Hsp92II and BsrSI were purchased from Promega Corp. (Madison, WI, USA) and 4 restriction endonucleases, BbvI, BlpI, BsmFI and Tsp509I were from New England Biolabs, Inc. (Beverly, MA, USA). Restriction endonucleases and amplified product were mixed gently and incubated overnight for endonuclease digestion at the optimal temperature for each enzyme (Table 1).

Electrophoresis of 5 μL of the endonuclease-digested fragments was performed on a 3% agarose gel with 1×Tris-Borate-Ethylenediaminetetraacetate (TBE) buffer at a voltage of 135 V for 30 min by using a Mupid-exU electrophoresis system (Advance, Tokyo, Japan). After electrophoresis, the gels were stained with ethidium bromide and examined under ultraviolet light.

Researchers observed whether the consistent, identical fragment patterns appear in the 85 conserved Tokyo X pigs at the Ome centre using the established PCR-RFLP System.

Statistical analysis: The exclusion Probability (P_E) was calculated using the allele frequencies of the selected SNP loci in commercial pork samples. As the selected SNP loci were all on autosomes, the P_E of each locus was calculated as:

$$P_E = 1-p_i^2$$

where, pi is the allele frequency of the ith locus which is fixed single allele in the Tokyo X strain. Then, the combined P_E of the number of SNP loci (n) was calculated as:

$$P_E = 1-\prod_{i=1}^{n}(1-P_{Ei})$$

RESULTS AND DISCUSSION

Screening of SNP loci for use in identification of the Tokyo X strain: The results of the earlier study were used to select 156 out of 1,760 SNPs for use in genotyping the market samples and the Tokyo X pigs maintained at the Ome centre. Primer sets were designed and 149 were suitable for genotyping by MALDI-TOF MS. A total of 127 SNP loci were successfully genotyped and 104 of them were single allele in the Tokyo X strain. The allele frequencies of the SNP loci (having single alleles in the Tokyo X strain) in the 4 major breeds (Landrace, Large White, Duroc and Berkshire) in the reference DNA, general market pork samples and Berkshire pork samples are listed in Table 2.

Sequences of the flanking regions of selected SNP loci, primer positions and PCR-RFLP patterns: Of the 104 SNPs which were single allele in the Tokyo X strain, 14 SNPs were a MAF of >10% in both general commercial and Berkshire pig samples. Researchers selected 8 SNPs recognizable by restriction endonuclease (B00724, B01353, B01667, B01687, B01880, B02138, B02269 and B02344 corresponding to dbSNP ss nos. 196001149, 196001522, 196001697, 196001711, 196001824, 196001960, 196002054 and 196002096, respectively) to comprise the identification Method for Tokyo X. Researchers sequenced the flanking regions to eliminate polymorphisms such as SNPs, indels and repetitive sequences on primer annealing sites and then researchers designed the primers for PCR-RFLPs. The sequences, nucleotide polymorphisms and primer positions are shown in Fig. 1. PCR amplification and restriction endonuclease digestion were successfully completed on each of the SNP loci and the fragment patterns are shown in Fig. 2 and Table 1.

Exclusion probabilities of SNPs for general commercial pork and Berkshire pork: The exclusion probability of

Table 1: Primers, restriction endonucleases and fragment sizes in PCR-RFLP. The RFLPs relevant to identification of Tokyo X were listed

SNP_ID	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product size (bp)[1]	Restriction Endonuclease (RE)	RE reaction temp. (°C)	Tokyo X RFLP (bp)	Non-TokyoX RFLP (bp)
B00724	gaaagagacaagctgttgtttgg	aaaggttttgtattcagctcatca	248	Hsp92II	37	52.80	60.000
B01353	catcagaagctggagattgc	cccagattactcggattgga	413	BsmFI	65	210.15	360.000
B01667	gggtgttttgaccagctcat	tcaggagaattgtccaaagtca	425	BsmFI	65	235.19	425.000
B01687	tgtgcgaggaaagtctaggg	agcctggagtcattttcttttg	411	Hsp92II	37	310.00	247.630
B01880	gccaccttgagggtgttatt	gtctgctgccggtacttctt	377	BbvI	37	229.50	279.000
B02138	tgagcttgttttcctgttttaca	cgagtgatgtgatgagtaaaattca	365	Tsp509I	65	156.79	235.000
B02269	tcaaggtgaggccatctttc	ctcccctgaggttctgttca	524	BsrSI	65	524.00	402.122
B02344	tatgaagcctgggaatttgc	tgtcctcctcaatggctttc	570	BlpI	37	570.00	356.214

[1]The annealing temperature of each PCR is 60°C

Table 2: SNP ID, dbSNP ss numbers, mapped chromosome number and allele frequencies in each pig breed and commercial market samples. Allele frequencies were those of the designated bases on the single allele in the Tokyo X strain

					Allele frequency in each pig breed in the reference DNA panel				Allele frequency in each market sample			
SNP ID	dbSNP ss No.	SSC	Polymorphisms	Single allele in Tokyo X	Landrace (51)	Large white (69)	Duroc (68)	Berkshire (68)	General pork (879)	Berkshire (462)	Alleles not observed in Tokyo X	Restriction endonucleases used for PCR-RFLP
B00047	196000754	5	A/G	A	0.980	0.935	0.632	0.993	0.866	0.995	G	-
B00077	196000770	15	G/A	G	1.000	1.000	1.000	0.897	0.994	0.990	A	-
B00091	196000779	X	A/G	A	0.961	1.000	0.750	1.000	0.928	0.998	G	-
B00135	196000796	13	C/A	C	0.710	0.681	1.000	0.934	0.812	0.907	A	-
B00139	196000797	12	G/T	G	0.637	0.732	0.971	1.000	0.818	0.999	T	-
B00188	196000825	6	G/A	G	1.000	1.000	0.801	1.000	0.903	1.000	A	-
B00190	196000827	6	A/G	A	1.000	1.000	0.801	1.000	0.903	1.000	G	-
B00218	196000845	1	A/G	A	1.000	1.000	1.000	0.882	0.989	0.846	G	-
B00273	196000878	14	T/C	T	1.000	1.000	0.809	1.000	0.968	1.000	C	-
B00274	196000879	14	C/T	C	1.000	1.000	0.809	1.000	0.968	1.000	T	-
B00319	196000909	14	C/T	C	0.980	1.000	1.000	0.897	0.993	0.950	T	-
B00321	196000911	14	T/C	T	0.941	1.000	1.000	0.897	0.986	0.971	C	-
B00322	196000912	14	G/C	G	0.941	1.000	1.000	0.897	0.986	0.971	C	-
B00424	196000976	13	T/A	T	0.878	1.000	1.000	0.801	0.936	0.820	A	-
B00482	196001013	7	T/C	T	0.951	0.920	0.963	0.846	0.937	0.796	C	-
B00485	196001015	7	G/A	G	0.990	1.000	1.000	0.875	0.989	0.803	A	-
B00498	196001023	1	C/G	C	1.000	0.652	0.897	0.985	0.870	0.998	G	-
B00499	196001024	1	T/C	T	1.000	0.645	0.897	0.985	0.869	1.000	C	-
B00503	196001027	5	T/C	T	0.961	0.797	0.632	0.978	0.830	0.994	C	-
B00505	196001029	5	A/C	A	0.961	0.806	0.642	0.985	0.824	0.995	C	-
B00561	196001061	5	G/A	G	0.843	0.819	0.257	1.000	0.665	1.000	A	-
B00566	196001064	17	T/C	T	0.706	1.000	1.000	0.721	0.955	0.793	C	-
B00581	196001073	6	G/A	G	0.941	0.964	0.779	1.000	0.902	1.000	A	-
B00594	196001083	Unknown	G/A	G	1.000	1.000	0.866	1.000	0.984	1.000	A	-
B00626	196001099	4	T/G	T	0.980	1.000	0.912	0.868	0.960	0.933	G	-
B00724	196001149	13	A/G	A	0.598	0.920	0.765	0.824	0.774	0.814	G	Hsp92II
B00725	196001150	13	A/C	A	1.000	1.000	0.765	0.971	0.917	0.984	C	-
B00729	196001153	13	A/C	A	1.000	1.000	0.846	0.971	0.998	1.000	C	-
B00793	196001187	14	T/G	T	0.950	0.942	0.934	0.838	0.910	0.861	G	-
B00803	196001194	1	T/G	T	1.000	0.855	0.838	1.000	0.861	0.998	G	-
B00806	196001196	1	C/T	C	1.000	0.855	0.838	1.000	0.859	0.998	T	-
B00836	196001215	8	C/T	C	1.000	1.000	0.912	0.485	0.987	0.568	T	-
B00838	196001216	2	C/T	C	0.590	0.587	1.000	0.978	0.763	0.995	T	-
B00846	196001223	1	C/T	C	0.980	0.913	1.000	0.890	0.953	0.972	T	-
B00856	196001233	12	C/T	C	0.627	0.783	1.000	0.978	0.896	0.951	T	-
B00890	196001257	1	A/C	A	1.000	1.000	1.000	0.721	0.988	0.778	C	-
B00908	196001264	14	C/T	C	0.922	0.565	1.000	0.813	0.856	0.793	T	-
B00964	196001299	3	T/A	T	1.000	1.000	0.779	0.993	0.946	1.000	A	-
B01074	196001354	5	T/C	T	0.745	0.862	0.860	0.963	0.885	0.964	C	-
B01075	196001355	5	T/A	T	1.000	1.000	0.868	1.000	0.981	1.000	A	-
B01115	196001377	1	C/A	C	1.000	1.000	1.000	0.831	0.987	0.777	A	-
B01122	196001382	17	T/C	T	0.833	0.485	0.838	1.000	0.807	1.000	C	-
B01141	196001392	3	T/C	T	0.696	0.464	0.801	1.000	0.680	1.000	C	-
B01176	196001414	18	C/T	C	0.971	0.920	0.801	1.000	0.906	0.999	T	-
B01187	196001421	2	G/A	G	0.559	0.703	0.949	0.794	0.804	0.789	A	-
B01353	196001522	15	T/C	T	0.775	0.565	0.949	0.640	0.734	0.828	C	BsmFI
B01409	196001545	Unknown	T/C	T	0.878	1.000	0.875	1.000	0.792	1.000	C	-
B01414	196001549	Unknown	A/G	A	0.853	0.891	0.838	0.993	0.789	0.999	G	-
B01453	196001575	9	G/A	G	1.000	0.862	0.860	1.000	0.903	1.000	A	-
B01515	196001608	2	G/A	G	0.882	0.862	0.993	0.647	0.946	0.779	A	-
B01636	196001677	6	C/T	C	0.716	0.775	0.978	0.932	0.936	0.986	T	-
B01645	196001683	6	C/T	C	0.765	0.457	1.000	0.882	0.832	0.881	T	-
B01667	196001697	15	G/A	G	0.922	0.457	1.000	0.824	0.880	0.879	A	BsmFI
B01687	196001711	8	C/T	C	0.598	0.413	0.618	0.794	0.585	0.785	T	Hsp92II
B01688	196001712	8	C/T	C	0.598	0.413	0.618	0.794	0.584	0.782	T	-
B01744	196001746	12	C/T	C	0.990	0.891	1.000	0.448	0.972	0.482	T	-
B01877	196001822	17	G/A	G	1.000	1.000	0.956	0.779	0.911	0.662	A	-
B01880	196001824	17	A/G	A	0.892	0.964	0.941	0.779	0.870	0.663	G	BbvI
B01934	196001849	5	C/G	C	0.990	0.964	0.897	0.963	0.948	0.998	G	-
B02061	196001915	7	G/A	G	0.892	0.783	0.574	0.993	0.859	1.000	A	-
B02062	196001916	7	C/T	C	0.961	1.000	1.000	0.559	0.954	0.538	T	-
B02086	196001927	9	G/A	G	0.647	0.674	0.669	0.985	0.695	0.998	A	-

Table 2: Continue

SNP ID	dbSNP ss No.	SSC	Polymorphisms	Single allele in Tokyo X	Allele frequency in each pig breed in the reference DNA panel				Allele frequency in each market sample		Alleles not observed in Tokyo X	Restriction endonucleases used for PCR-RFLP
					Landrace (51)	Large white (69)	Duroc (68)	Berkshire (68)	General pork (879)	Berkshire (462)		
B02089	196001930	9	A/G	A	0.627	0.558	0.647	0.993	0.648	0.998	G	-
B02138	196001960	11	A/G	A	0.980	1.000	0.821	0.757	0.854	0.795	G	Tsp509I
B02152	196001970	5	C/T	C	0.137	0.225	0.838	0.904	0.535	0.887	T	-
B02231	196002031	17	G/A	G	1.000	1.000	1.000	0.794	0.994	0.752	A	-
B02269	196002054	Unknown	T/C	T	0.716	0.486	1.000	0.904	0.752	0.848	C	*Bsr*SI
B02330	196002089	13	T/C	T	0.902	0.906	1.000	0.779	0.933	0.823	C	-
B02344	196002096	1	C/T	C	0.873	0.703	1.000	0.721	0.838	0.771	T	BlpI
B02356	196002103	6	A/G	A	1.000	1.000	0.816	1.000	0.939	1.000	G	-
B02357	196002104	6	C/T	C	0.265	0.565	1.000	0.971	0.769	0.968	T	-
B02403	196002133	14	C/T	C	0.941	0.739	0.662	0.890	0.744	0.916	T	-
B02508	196002188	6	G/A	G	0.922	1.000	0.838	1.000	0.870	1.000	A	-
B02509	196002189	6	T/G	T	0.922	1.000	0.836	1.000	0.870	1.000	G	-
B02606	196002217	7	C/T	C	0.990	0.743	0.873	0.956	0.919	0.966	T	-
B02720	196002248	17	C/G	C	1.000	1.000	1.000	0.794	0.994	0.745	G	-
B02753	196002257	5	A/T	A	0.588	0.536	1.000	0.978	0.828	0.997	T	-
B02764	196002263	9	A/T	A	1.000	1.000	1.000	0.735	0.994	0.631	T	-
B02781	196002274	1	T/C	T	0.265	0.884	0.985	0.701	0.802	0.761	C	-
B02801	196002289	3	C/G	C	1.000	1.000	0.888	0.993	0.949	0.987	G	-
B02818	196002296	14	G/A	G	0.588	0.699	0.985	0.933	0.790	0.945	A	-
B02828	196002305	Unknown	G/A	G	1.000	1.000	0.956	0.735	0.954	0.886	A	-
B02869	196002324	6	C/G	C	0.970	0.986	1.000	0.846	0.950	0.958	G	-
B02871	196002326	6	T/G	T	0.961	0.978	0.993	0.868	0.950	0.959	G	-
B02877	196002329	10	C/T	C	1.000	1.000	0.882	0.971	0.961	1.000	T	-
B02878	196002330	10	A/G	A	0.892	0.899	0.882	0.809	0.906	0.846	G	-
B02890	196002336	17	T/C	T	0.922	0.978	1.000	0.897	0.949	0.882	C	-
B02894	196002338	17	C/T	C	0.922	0.978	1.000	0.897	0.949	0.883	T	-
B02898	196002340	17	T/C	T	0.755	0.449	0.993	0.985	0.828	0.984	C	-
B02913	196002346	13	C/G	C	0.892	1.000	0.640	0.941	0.918	0.963	G	-
B02921	196002350	3	G/A	G	0.696	0.935	0.809	1.000	0.739	1.000	A	-
B02928	196002357	2	G/A	G	1.000	1.000	0.882	1.000	0.987	1.000	A	-
B02936	196002364	1	C/T	C	0.990	1.000	0.993	0.897	0.961	0.967	T	-
B02938	196002366	1	T/C	T	0.990	1.000	0.978	0.897	0.952	0.967	C	-
B02966	196002377	6	C/T	C	1.000	1.000	0.787	1.000	0.901	1.000	T	-
B03007	196002400	18	G/A	G	0.775	0.884	0.882	0.868	0.866	0.908	A	-
B03011	196002401	18	G/A	G	0.775	0.882	0.882	0.868	0.868	0.906	A	-
B03013	196002403	18	C/T	C	0.775	0.884	0.882	0.868	0.868	0.909	T	-
B03065	196002431	8	G/A	G	0.941	0.833	1.000	0.772	0.940	0.584	A	-
B03093	196002453	6	C/T	C	0.843	0.362	0.875	0.971	0.813	0.999	T	-
B03095	196002454	6	T/A	T	0.843	0.360	0.875	0.971	0.813	0.999	A	-
B03104	196002460	2	G/A	G	0.990	0.819	0.890	1.000	0.924	1.000	A	-
B03114	196002468	Unknown	G/A	G	1.000	1.000	0.801	1.000	0.908	1.000	A	-
B03171	196002498	3	G/A	G	0.941	1.000	0.735	0.941	0.809	0.949	A	-
Identification probability[1]		-	-	-	-	-	-	-	$6.59E^{-13}$	$3.37E^{-10}$	-	-

[1]The identification probability was that 1 animal of Tokyo X and 1 other pig will have identical genotypes for all SNPs and was calculated on the supposition that all SNPs were on the autosomes because 6 SNPs were not mapped on the porcine genome and the influence on the probability of 1 SNP on the X chromosome is negligible

each SNP for the 4 major pig breeds of Landrace, Large White, Duroc and Berkshire in the reference DNA and general commercial pork and Berkshire pork samples from the market were calculated and listed in Table 3. The combined exclusion probability of general commercial pork and Berkshire pork from Tokyo X was 98.1 and 97.4%, respectively.

Confirmation of single alleles in Tokyo X maintained at the Ome centre: The Tokyo X pigs maintained at the Ome centre were tested for a single allele at each locus by using the established PCR-RFLP System. Endonuclease-digested DNA fragments from corresponding loci of all the Ome centre pigs have identical electrophoresis patterns which confirms the presence of a fixed allele at each locus of the Tokyo X herd.

Current methods for individual identification and parentage testing were developed using DNA markers such as microsatellites and SNPs in many domesticated animals (Hirota *et al*., 2010; Luikart *et al*., 1999; Rohrer *et al*., 2007; Werner *et al*., 2004). All of these methods calculate the parentage exclusion or individual identification probabilities using high polymorphic DNA markers such as microsatellites and SNPs with high Minor

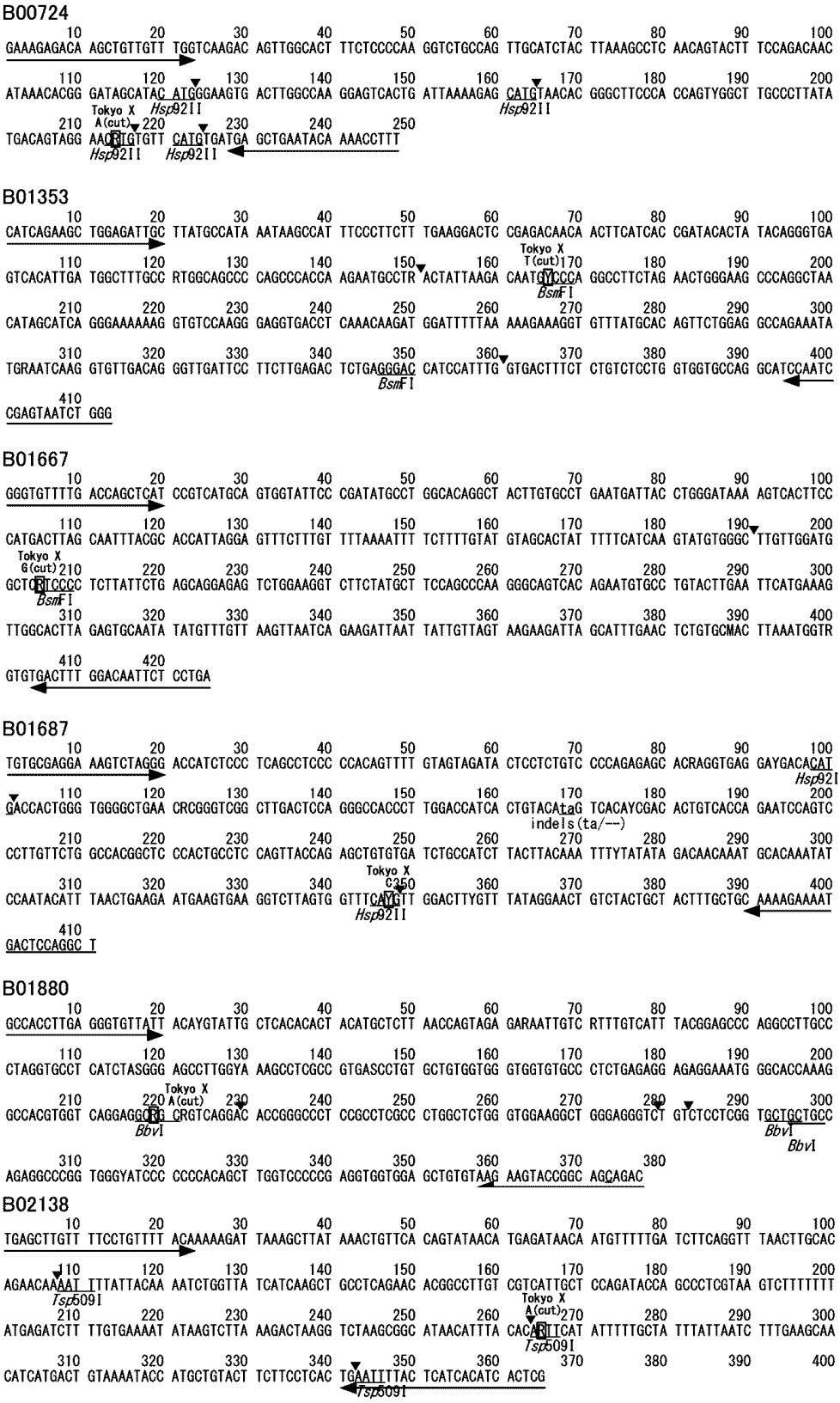

Fig. 1: Continue

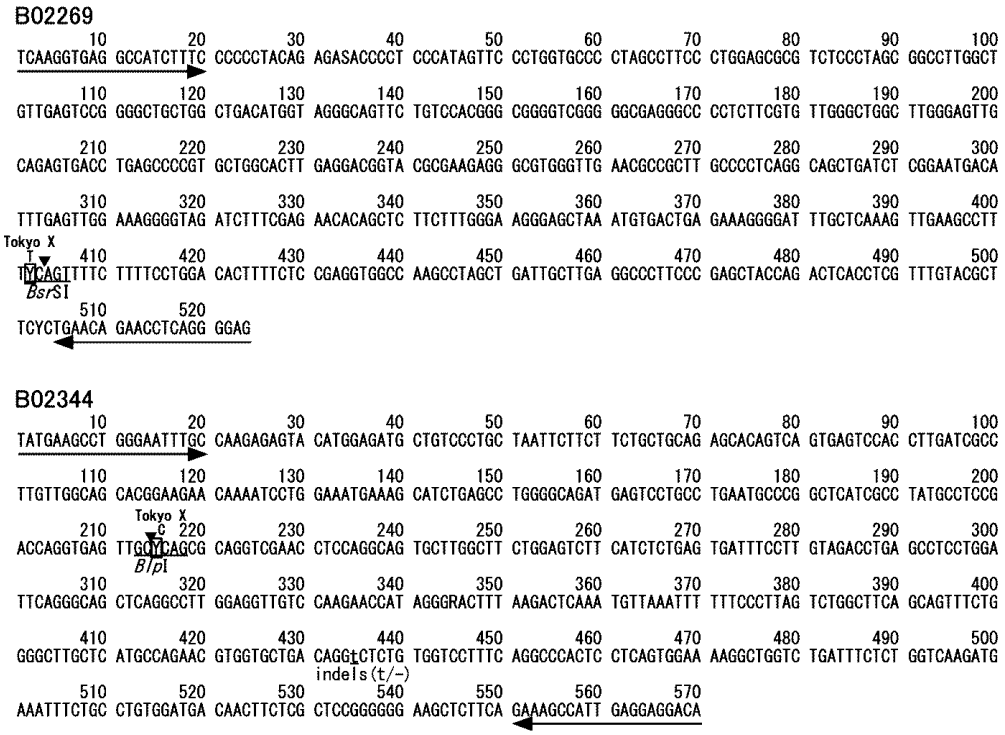

Fig. 1: Primer positions and cutting sites of restriction endonucleases. The arrows below the DNA sequences represent the primers used for PCR-RFLP. The recognition sites of each endonuclease are underlined and the cutting sites are indicated by the arrowheads. The SNP loci used for Tokyo X identification are enclosed by a box and the designation of cut in parentheses denotes that the Tokyo X samples are cut by the corresponding restriction endonucleases. Nucleotide polymorphisms such as SNPs and indels within each sequence are detected by sequencing of 24 samples for the flanking regions of 3 SNPs; B00724, B01880 and B02138 and 32 samples for 5 SNPs; B01353, B01667, B01687, B02269 and B02344, respectively

Allele Frequencies (MAFs). The advantage of using these polymorphic markers with high polymorphism is the general application of traceability to any animals and meat products.

Individual identification is done by genotyping the individual and the meat product produced by that individual and the resultant genotypes are compared for a perfect match. Thus, the implementation of DNA-based traceability requires the collection of DNA samples from the individual animal to the carcass or meat product of that individual in the meat supply chain. The DNA typing is expensive, estimated at about US $35.00 (Webb, 2004) and this cost does not include the costs of labour and the Physical Traceability System.

In cattle, the price of the meat product does not increase even if the cost of DNA typing is added to the carcass price because the carcass price is much higher than the cost of DNA typing. On the other hand, the price of the pig carcass is low, around US $5.0/kg in Japan (http://lin.alic.go.jp/alic/statis/dome/data2/i_pdf/3050a-3115a.pdf in Japanese). Therefore, the addition of the DNA genotyping cost to the carcass price of pigs is in fact, not practical because this will increase the price of the pork product by at least 10%. Although, several DNA-based traceability methods have been developed for pigs using high polymorphic markers (Ballester *et al*., 2007; Goffaux *et al*., 2005; Rohrer *et al*., 2007), these methods are not in general use probably because of the high cost of DNA genotyping and the complexity of DNA-based traceability. Furthermore, it is problematic to identify individual animals or to relay individual information reliably between the farm, abattoir, distribution outlet and especially, the consumer by using the physical traceability systems such as tags or barcode labels (Notermans, 2003).

Alternatively, the controlling genes of the porcine coat colour differences can be used for pig breed identification. Previously reported polymorphism in 2 genes, i.e., *MC1R* (Kijas *et al*., 1998, 2001) and *KIT* (Giuffra *et al*., 2002; Marklund *et al*., 1998; Pielberg *et al*., 2002) can explain a large proportion of coat colour diversity in domestic pigs, at least for Euro-American pig

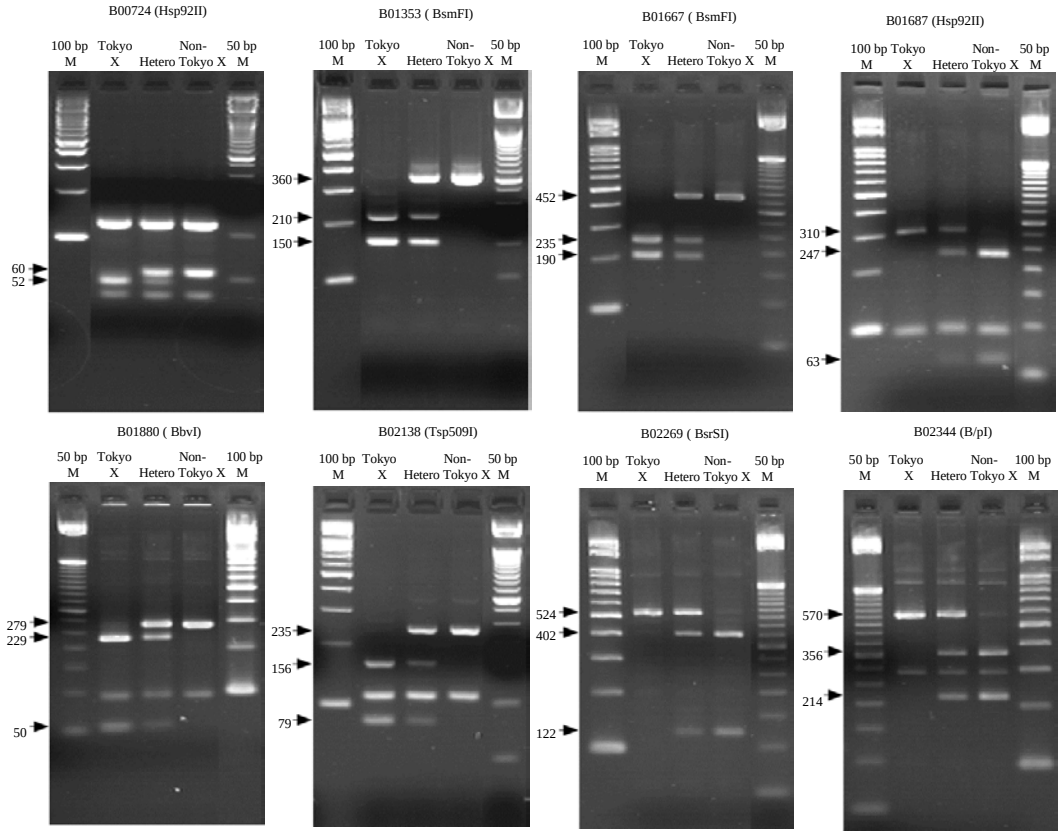

Fig. 2: Restriction Fragment Length Polymorphism (RFLP) digestion by each restriction endonuclease (designated in parentheses). The molecular marker lanes are designated as 100 bp M for 100 bp ladder marker and 50 bp M for 50 bp ladder marker, respectively. The designation Tokyo X represents the Tokyo X pork samples. The RFLPs relevant to the identification of Tokyo X are indicated by arrows. All of the conserved Tokyo X individuals have identical digestion patterns to those shown in this figure. The 2 digestion patterns designated as Hetero and Non-Tokyo X never appeared in the Tokyo X samples

Table 3: Allele frequencies and combined exclusion probabilities (P_E) in each pig group

				Allele frequency in each pig breed in the reference DNA panel[1]				Allele frequency in each market sample[1]	
SNP ID	Polymorphisms	Single allele in Tokyo X	Tokyo X (85)	Landrace (51)	Large white (69)	Duroc (68)	Berkshire (68)	General pork (879)	Berkshire (462)
B00724	A/G	A	1	0.598	0.920	0.765	0.824	0.774	0.814
B01353	T/C	T	1	0.775	0.565	0.949	0.640	0.734	0.828
B01667	G/A	G	1	0.922	0.457	1.000	0.824	0.880	0.879
B01687	C/T	C	1	0.598	0.413	0.618	0.794	0.585	0.785
B01880	A/G	A	1	0.892	0.964	0.941	0.779	0.870	0.663
B02138	A/G	A	1	0.980	1.000	0.821	0.757	0.854	0.795
B02269	T/C	T	1	0.716	0.486	1.000	0.904	0.752	0.848
B02344	C/T	C	1	0.873	0.703	1.000	0.721	0.838	0.771
P_E expected	-	-	0	0.981	0.999	0.880	0.982	0.981	0.974

[1]Allele frequencies were those of the designated bases on the single allele in the Tokyo X strain and the numbers in parentheses denotes the number of individuals used in this study

breeds. Using the polymorphism of the *MC1R* and *KIT* genes, the dominant white of the Landrace and Large White, red of the Duroc and black with 6 white points of the Berkshire are readily identifiable (Carrion *et al*., 2003). However, Tokyo X coat colour varies within the herd, so polymorphism of coat colour related genes cannot be utilised for strain identification.

The pure strain of Tokyo X is preserved by the livestock centre at Ome city. The piglets born at this centre are supplied to the co-operative farming group and

meat pig production by crossing purebred pigs is obligatory in this group. Therefore, the analysis of genetic polymorphism of the pure strain at the Ome breeding centre will provide a reference with which to establish a reliable DNA-Based Identification System for the Tokyo X strain.

In this study, researchers screened and collected more than a hundred SNP loci that were single allele in Tokyo X but show polymorphism in the other commercial breeds and market pork samples. When all selected 104 SNPs are used for Tokyo X identification, the identification probabilities of general commercial pork and Berkshire pork as Tokyo X pig are 6.59×10^{-13} and 3.37×10^{-10}, respectively (Table 2); therefore, the combined exclusion probabilities are almost 100%.

The genotyping of multiple SNP loci simultaneously needs optimised genotyping systems such as MALDI-TOF MS and SNP array detection and this equipment is generally expensive. Therefore, researchers selected 8 SNPs to construct a PCR-RFLP System which is practicable in conventional laboratories. Although, a high level of accuracy of genotyping is achieved by MALDI-TOF MS, the misclassification probability has earlier been estimated at 0.00-0.35% (Heid *et al*., 2008). So, the single allele properties of the conserved Tokyo X strain were reconfirmed by the constructed PCR-RFLP Method using 8 SNPs.

This method employs SNPs which are single allele in Tokyo X but show high polymorphism in the other breeds and commercial pork samples and the combined exclusion probabilities of general commercial pork and Berkshire pork were 98.1 and 97.4%, respectively. These values are comparable to those of the method developed to distinguish between Japanese domestic and imported beef where the combined exclusion probabilities of United States cattle and Australian cattle were 98.7 and 96.3%, respectively (Sasazaki *et al*., 2011).

CONCLUSION

In this study, researchers developed a DNA-based identification method for the Tokyo X strain and the ability to distinguish between Tokyo X pork and general market pork was evaluated. The selected 8 SNPs were recognizable by the restriction endonuclease and the developed PCR-RFLP System is suitable for conventional laboratories because there is no need of expensive equipment for SNP genotyping. The SNP marker set developed in this study will be useful in preventing fake Tokyo X brand pork from appearing on the market.

ACKNOWLEDGEMENTS

Researchers are greatly indebted to Ms. Ikuyo Nakamori (JATAFF Institute) for her technical assistance. Researchers are grateful to Dr. Taro Obata (JATAFF Institute) for their administration of the project. This research was supported by the Ministry of Agriculture, Fisheries and Forestry of Japan [grant number II-304] and the Japan Racing Association and Livestock Promotion Foundation.

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