Paternity Diagnosis using The Multiplex PCR with Microsatellite Markers in Dogs

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ABSTRACT

The number of abandoned dogs is increasing with the worsening of the economy and the rising of feed value. It was becoming a serious social problem because of the disease transmission and destruction of natural ecosystems by abandoned dogs been wild animal. In order to solve these problems, companion dogs necessary to secure its own genetic information and to establish the systematic tracking system. Using multiplex-PCR method with 27 microsatellite marker (MS marker) divided 3 set, various alleles occurring to 6 dog breed (Labrador Retriever, German Shepherd, English Springer Spaniel, Belgian Malinois, Jindo Dog, PoongSan Dog) make use of markers to determine allele frequency and heterozygosity. MS marker FH2834 and FH2790 have only two allele and most were found in 13 alleles at FH3381 and FH3399. Average heterozygosity of MS marker is 0.534 and especially, heterozygosity represented the highest value of 0.765 at FH3381. So, it was recognized appropriate allele frequency for individual identification and paternity diagnosis in companion dogs. Using multiplex-PCR method with MS marker, various alleles occurring to dog breed make use of markers to deter mine individual identification and paternity diagnosis, traits associated biomarkers and breed-specific marker for faster, more accurate and ways to reduce the analysis cost. Based on this result, a scientific basis was established to the existing pedigree data by applying genetics additionally. Animal registration system is expected to be conducted nationwide in future. The method expects to very useful this system.

(Key words : Microsatellite marker, Canine, Multiplex PCR, Individual identification, Paternity diagnosis)

INTRODUCTION

The dog as man's best friendly companion animal have a history lived with humans for 12,000 years or more in the world. They were estimated more than 800 species worldwide and are being raised more than 150 kinds in Korea. Domestic pet dog (companion dog) industry was growing by rising incomes, lifestyle of personalization and expanding of digital culture continuously. The number of domestic dogs estimated at approximately 5.3 million, and about 3.5 million people of dog fancier are expected in 2006. Also, the economic value of dog-related industries is expecting a minimum of about 1 trillion won in 2007.

However, the number of abandoned dogs is increasing with the worsening of the economy and the rising of feed cost. It was beco ming a serious social problem because of the disease transmission and destruction of natural ecosystems by abandoned dogs been wild animal. In 2008, companion animals (dogs) registration using bio-injection microchip for the first time in the country carried out a pilot by some local government. But it exist the antipathy of owners because of insertion of foreign object and possibility of disease at inserted region (Vascellari *et al*., 2006) and might be lost of all information if malicious removing of microchip. Their own genetic information and the necessity of a national integrated management were required in order to settlement of methodically history system at 4 million households of the domestic companion animal in 2011.

With the growth of the industry's pet dog, an international movement, lost during the breeding, the preferences of genetic traits and an excellent breed descent, a dispute over the authenticity of pure-blooded and cloning for medical research for the disease and for companion dog due to the relatively short life is very increased the importance of paternity diagnosis and individual identification in companion dogs. Additionally, gene profiling studies utilizing of mitochondria DNA, blood protein, minisatellite, microsatellite, single nucleotide polymorphism(SNP) and various genetic markers

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by the recognition of the value of conservation and utilization in many traditional livestock as genetic resources in various worlds carried out in the traditional livestock (Chung *et al*., 2001; Girish *et al*., 2007; Ichikawa *et al*., 2001; Kim *et al*., 2010; Oh M. Y. *et al*., 1992; Signer and Jeffreys, 1997). In particular, the microsatellite has a regular repeating sequence of 2～6 base pairs of DNA, are distributed approximately 50,000 to 100,000 across the entire mammalian genome and is non-coding DNA sequence of the wide range of high polymorphism (Debrauwere *et al*., 1997). It is widely used to analysis of the genetic diversity of livestock populations because of convenience and polymorphism of the experiment among the technology using DNA (Barker *et al*., 1997; Bjornstad *et al*., 2003; Laval *et al*., 2000; Li *et al*., 2000). Currently, multiplex PCR as well as simple PCR technique using microsatellite marker (MS marker) are also being developed to save time and costs (Jamsari *et al*., 2011; Koskinen and Bredbacka, 1999; Weissenberger *et al*., 2010).

This study exa mined to deter mine gene identification such as paternity diagnosis and individual identification through traits associated biomarkers and breed-specific marker for faster, more accurate and ways to reduce the analysis cost using optimized multiplex-PCR method with known MS marker.

MATERIALS AND METHODS

Table 1. List of microsatellite markers and primer

Set No.	Chr.	Marker	Primer		Annealing temp.	Product size	Dye
			Forward (5'→3')	Reverse (3'→5')			
Set 01	2	FH3005	ACTCATTTCCAAGGTGATTTG	GTACTCACCGCAAGTGCAAG	61℃	200~236	Fam
	10	FH2537	AAAAAGTGTAGAGCTTTCTTCAAA	ATTGAGACCCAAGACTGTTAGTG		146~176	Fam
	10	FH3921	CCTTCTTCTTAACACCTCTTCC	CTCTGTTTGCCAGATGATAACC		364~394	NED
	10	FH3381	CCCAGAAACTCAACTGATGC	AGCTCTTACACGCATTGAGG		276~312	Fam
	12	FH3116	GAGAAATCCTGTCATGTGCTG	CCTTTTCCCTTCTTTCCTTG		186~200	VIC
	19	FH3372	AGTGCCTTTGAATGTTAATGC	ACATCAAAATGGTTACACTTGG		142~162	VIC
	26	REN62M06	AAGTGGAATGGAGTCTGC	CATGAACCTGTCGTAAGC		243~255	NED
	27	REN277O05	CCTCCTCTCACTTGTCCTGC	AAATGGTGTCTTCAGCTCCG		331~338	VIC
	30	REN51C16	CAGTTCATCCTTCCCCCTCTC	GTGCTAGTCTGGCTGTGCTCA		246~264	VIC
	38	FH3399	TCTCTATGCCTGCAGTTTCC	TTCTGATGCCCTCATAAAGC		234~282	PET
	X	FH3027	GTTTCCTCACATGCAAAAGC	GCTGGAGGTCAAGGATAAGG		196~234	PET
Set 02	4	FH2097	CAATGTCGAATTCCATGGTG	ATGGAGCAAGATGTGTTTGTG	61℃	268~300	NED
	8	REN204K13	TCGGGATGTTTCTCTTCCAC	CTGCTTAAATTCTCCCAGCG		246~254	VIC
	9	FH1014	AGGCTATTAACCCCTGATCG	CGATGCCTTACTTAAACAAACC		242~250	Fam
	16	FH3058	GCCTTCCATAGATGAATGAGG	CCATACATGGTTTTGAGAACG		218~234	Fam
	18	FH2834	GCAAGCTTTAAAATACCTTTCC	GCCTGAACTGATTGATGACC		263~265	Fam
	31	FH2712	AAGGTAGTCCCACGATCCTC	GAGCCCTGTTCTCAGGTTG		170~186	PET
	35	REN112C08	ATGGCCCACCGATACACA	TCGGGGACATACTTGAACC		218~236	NED
	36	FH2998	GATTTTGATACCCTGAGAATGC	CTCACTGGCTCTCACATGC		196~228	PET
	X	FH2584	GTTAGGTTCACAGTGGGCGT	ACTCAAAGACCTGGAGGGGT		299~317	VIC
	Y	REN197E16	TGGGTGTGAGTCATCCAAGA	CGTTACTGTATGCTTAAGCTTTTGA		140~160	Fam
Set 03	12	FH2054	GCCTTATTCATTGCAGTTAGGG	ATGCTGAGTTTTGAACTTTCCC	61℃	148~180	Fam
	23	REN181K04	ACAAGCCGACTCTAGCGAAA	AGATGGGGCCTAACCAAAGT		214~228	Fam
	24	FH2079	CAGCCGAGCACATGGTTT	ATTGATTCTGATATGCCCAGC		269~293	NED
	26	REN01O23	TTCCCTGCAGCCCTTCCTCA	TGTGCCTCATTCCTTTTTAT		185~203	VIC
	31	FH2582	TGGAGTGTGTTCCAAGGTCA	GTTGTTCCCACAAAAGGCAG		342~386	NED
	33	FH2790	CCAATATTGTTAAGAAGTTCAAGC	AGGCCTTCTCTGTCCTCTTG		204~208	Fam

Animals and Extraction of DNA from Blood

DNA was extracted from blood of total 48 by 8 dogs at each breed (Labrador Retriever, German Shepherd, English Springer Spaniel, Belgian Malinois, Jindo-dog and PoongSan-dog) using Wizard genomic DNA purification kit (Promega, USA) and analyzed concentration and purity at absorbance of 260 nm and 280 nm using ND-1000 spectrophotometer (Nanodrop, USA).

MS Marker for Allele Analysis

MS markers utilized this study were firstly selected 228 MS markers based on microsatellite genetic loci of dog reported Mapviewer database of NCBI (National Center for Biotechnology Information) and were selected 45 MS markers considered annealing temperature of 61℃, product size and type of dye for Gradient PCR thereafter. The selected MS markers divided into 3 set of each 15 and were composed of the final set of 11, 10 and 6 that satisfied condition of multiplex PCR. Total 27 primers of 3 set are shown in Table 1.

Multiplex PCR and MS Analysis

Multiplex PCR was set up in 25 ul reaction volume consisting 6 ul (20 ng/ul) of genomic DNA, 0.4 ul (10 pmole) each of fluorescence dye primer of forward and reverse, 1 ul (Unit/ul) of Hot Start Taq DNA polymerase, 4 ul of 10× buffer and 3 ul of 2.5 mM dNTP. Conditions of Thermal Cycler PTC-0240 (MJ Research, Inc., MA, USA) were as follows: 15 min at 95℃ for initial denaturation, followed by 5 cycles with denaturation at

Table 2. Allele and heterozygosity at microsatellite marker

Set	MS marker	Allele(Total)	Allele(L)	Allele (G)	Allele (E)	Allele (B)	Allele (J)	Allele (P)	Heterozygosity
Set01	FH2537	8	3	1	2	2	6	5	0.499
	FH3005	8	4	2	4	3	5	5	0.588
	FH3027	11	3	2	4	4	6	5	0.664
	FH3116	4	2	1	1	2	2	2	0.183
	FH3372	6	4	1	3	3	4	5	0.521
	FH3381	13	7	4	5	3	7	8	0.765
	FH3399	13	8	3	4	3	8	6	0.725
	FH3921	11	5	2	1	3	5	8	0.578
	REN51C16	7	2	3	3	2	4	3	0.506
	REN62M06	5	1	1	3	2	2	4	0.267
	REN277O05	5	3	1	2	4	2	3	0.350
Set02	FH1014	5	1	2	2	4	4	3	0.465
	FH2097	9	5	4	3	5	8	4	0.658
	FH2584	8	3	1	2	3	8	3	0.560
	FH2712	8	3	3	3	4	7	5	0.686
	FH2834	2	1	2	2	2	2	2	0.335
	FH2998	9	6	5	5	6	6	6	0.803
	FH3058	7	3	2	2	3	5	5	0.610
	REN112C08	5	2	2	2	2	4	3	0.440
	REN197E16	6	3	4	2	4	3	4	0.597
	REN204K13	5	2	2	2	2	4	3	0.443
Set03	FH2054	9	4	5	4	6	6	6	0.743
	FH2079	6	4	1	2	3	2	3	0.491
	FH2582	12	5	4	5	3	8	5	0.784
	FH2790	2	2	2	2	2	2	1	0.301
	REN01O23	5	2	1	1	2	3	3	0.306
	REN181K04	5	3	3	2	4	3	3	0.567

L: Labrador Retriever, G: German Shepherd, E: English Spring Spaniel, B: Belgian Malinois, J: Jindo Dog, P: Poong San Dog

95℃ for 60 sec, annealing at 62℃ for 75 sec and elongation at 72℃ for 60 sec, 5 cycles with denaturation at 95℃ for 60 sec, annealing at 61℃ for 75 sec and elongation at 72℃ for 60 sec, 25 cycles with denaturation at 95℃ for 60 sec, annealing at 60℃ for 75 sec and elongation at 72℃ for 60 sec. The final had a extension temperature of 65℃ 30 min. PCR products were analyzed using the ABI-3730XL genetic analyzer (Applied Biosystems, USA) and GeneMapper version 4.0(Applied Biosystems, USA).

Statistic Analysis

Alleles of MS marker from Genotyper Software was organized individual and group by analyzing using Microsatellite Toolkit software (Park, 2000). The Heterozygosity of entire population, allele frequency and number of allele at each locus and at breed group were calculated. Also, it showed up variety of allele in marker about each breed through calculated the value of expected heterozygosity and observed heterozygosity about 6 dog breed.

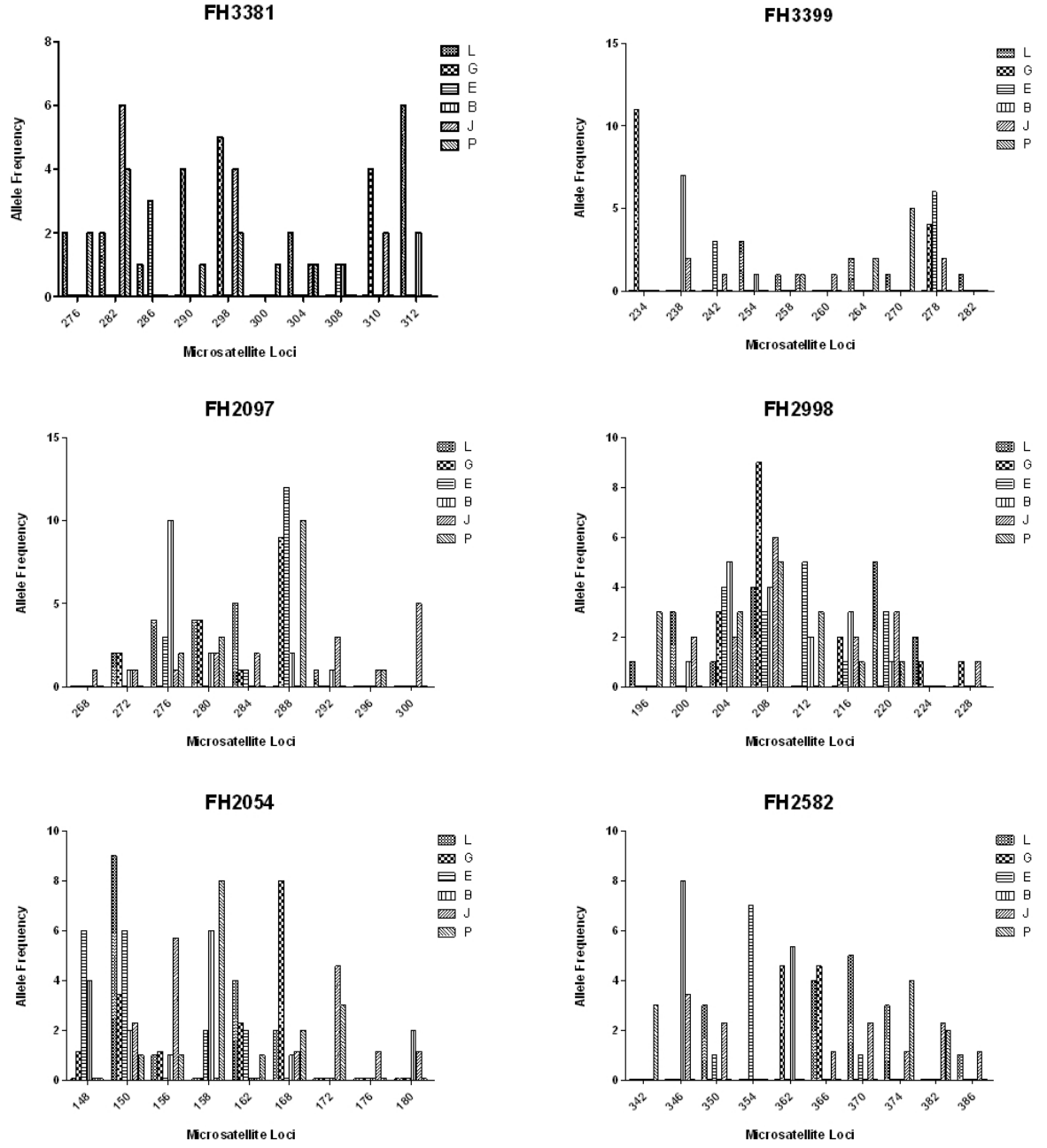

Fig. 1. Allele frequency distribution at microsatellite marker (MS marker) in 6 dog breed. L: Labrador Retriever, G: German Shepherd, E: English Spring Spaniel, B: Belgian Malinois, J: Jindo Dog, P: Poong San Dog.

RESULTS AND DISCUSSION

Because specific allele appearing to comparing different species-specific alleles can be used as a measure of genetic distinction within species and between species, therefore, we were calculated heterozygosity and the number of allele of locus and breed group about each MS marker (Table 2). The first set consists of 11 MS marker comes out 91 alleles. Especially, FH3381 and FH3399 have the highest of 13 alleles and FH3116 has the smallest of 4 alleles. The second set consists of 10 MS marker comes out 64 alleles. FH2097 and FH2998 have the highest of 9 alleles and lowest (FH2834) emerged as having 2 alleles. The final set consists of 6 MS marker shows 39 .alleles. FH2582 have the highest of 12 alleles and lowest (FH2790) emerged as having 2 alleles. As a result, total 194 alleles were genotyped to deter mine. The average heterozygosity of marker locus was 0.534 Especially, FH2998 represented the highest rate of heterozygosity of 0.803 has been recognized as suitable alleles for paternity diagnosis and individual identifica-

Table 3. Expected and observed heterozygosities and PIC value at 27 microsatellite in 6 dog breed

Locus	Population																	
	Labrador Retriever			German Shepherd			English Springer Spaniel			Belgian Malinois			Jindo Dog			PoongSan Dog		
	Ex H	Ob H	PIC	Ex H	Ob H	PIC	Ex H	Ob H	PIC	Ex H	Ob H	PIC	Ex H	Ob H	PIC	Ex H	Ob H	PIC
FH2537	0.508	0.375	0.427	0.000	0.000	0.000	0.400	0.500	0.305	0.458	0.625	0.337	0.783	0.625	0.702	0.842	0.875	0.755
FH3005	0.650	0.500	0.559	0.125	0.125	0.110	0.575	0.250	0.483	0.750	0.250	0.582	0.708	0.500	0.618	0.717	0.750	0.629
FH3027	0.492	0.375	0.398	0.525	0.125	0.371	0.742	0.500	0.645	0.592	0.375	0.510	0.833	0.625	0.748	0.800	0.250	0.708
FH3116	0.325	0.375	0.258	0.000	0.000	0.000	0.000	0.000	0.000	0.325	0.375	0.258	0.125	0.125	0.110	0.325	0.125	0.258
FH3372	0.517	0.500	0.443	0.000	0.000	0.000	0.592	1.000	0.456	0.567	0.750	0.468	0.675	0.625	0.570	0.775	0.750	0.682
FH3381	0.842	0.750	0.765	0.792	1.000	0.694	0.800	1.000	0.708	0.442	0.375	0.387	0.817	1.000	0.735	0.900	1.000	0.825
FH3399	0.842	1.000	0.766	0.492	0.625	0.398	0.758	0.875	0.658	0.592	0.750	0.456	0.850	0.750	0.776	0.817	0.750	0.730
FH3921	0.767	0.625	0.670	0.525	0.875	0.371	0.000	0.000	0.000	0.508	0.625	0.427	0.758	0.571	0.657	0.908	0.750	0.835
REN51C16	0.400	0.500	0.305	0.608	0.500	0.496	0.608	0.625	0.496	0.233	0.250	0.195	0.675	0.625	0.570	0.508	0.500	0.427
REN62M06	0.000	0.000	0.000	0.000	0.000	0.000	0.708	1.000	0.590	0.125	0.125	0.110	0.125	0.125	0.110	0.642	0.625	0.547
REN277O05	0.433	0.500	0.371	0.000	0.000	0.000	0.500	0.750	0.359	0.700	0.750	0.605	0.125	0.125	0.110	0.342	0.375	0.294
FH1014	0.000	0.000	0.000	0.458	0.625	0.337	0.233	0.250	0.195	0.742	0.875	0.636	0.725	0.625	0.622	0.633	0.500	0.511
FH2097	0.808	0.750	0.717	0.642	0.875	0.547	0.425	0.500	0.354	0.608	0.500	0.539	0.875	0.750	0.799	0.592	0.375	0.510
FH2584	0.575	0.375	0.447	0.000	0.000	0.000	0.525	0.375	0.371	0.675	0.500	0.556	0.925	0.500	0.852	0.658	0.250	0.544
FH2712	0.658	0.500	0.544	0.658	0.750	0.544	0.425	0.500	0.354	0.750	0.875	0.644	0.858	0.875	0.779	0.767	0.750	0.679
FH2834	0.000	0.000	0.000	0.400	0.500	0.305	0.500	0.500	0.359	0.525	0.875	0.371	0.125	0.125	0.110	0.458	0.625	0.337
FH2998	0.833	0.750	0.748	0.667	0.875	0.586	0.817	0.875	0.727	0.833	0.875	0.748	0.825	0.750	0.744	0.842	0.875	0.758
FH3058	0.575	0.375	0.447	0.500	0.750	0.359	0.500	0.750	0.359	0.575	0.750	0.482	0.742	1.000	0.642	0.767	0.625	0.679
REN112C08	0.400	0.500	0.305	0.400	0.250	0.305	0.525	0.375	0.371	0.458	0.625	0.337	0.517	0.625	0.443	0.342	0.375	0.294
REN197E16	0.508	0.375	0.427	0.525	0.250	0.458	0.458	0.625	0.337	0.650	0.875	0.530	0.658	1.000	0.544	0.783	1.000	0.685
REN204K13	0.325	0.375	0.258	0.533	0.750	0.375	0.400	0.500	0.305	0.325	0.375	0.258	0.650	0.750	0.559	0.425	0.500	0.354
FH2054	0.642	0.750	0.547	0.725	0.857	0.632	0.733	1.000	0.630	0.808	1.000	0.723	0.813	0.857	0.719	0.733	0.875	0.654
FH2079	0.780	0.286	0.674	0.000	0.000	0.000	0.458	0.625	0.337	0.575	0.875	0.447	0.440	0.286	0.325	0.692	0.375	0.575
FH2582	0.817	0.875	0.727	0.758	1.000	0.646	0.700	1.000	0.595	0.667	0.333	0.535	0.923	1.000	0.841	0.842	0.750	0.755
FH2790	0.233	0.000	0.195	0.527	0.286	0.370	0.458	0.625	0.337	0.325	0.125	0.258	0.264	0.000	0.215	0.000	0.000	0.000
REN01O23	0.233	0.250	0.195	0.000	0.000	0.000	0.000	0.000	0.000	0.525	0.625	0.371	0.538	0.571	0.427	0.542	0.250	0.428
REN181K04	0.508	0.125	0.427	0.714	0.000	0.555	0.233	0.000	0.195	0.742	0.625	0.645	0.538	0.143	0.427	0.667	0.000	0.555

Ex H: Expected Heterozygosity, Ob H: Objectived Heterozygosity, PIC: polymorphic information content

tion of companion dogs. Each of these breed, heterozygosity by various alleles also derived the value of a relatively wide range, but are distributed for each set.

Many studies conducted in cattle and pig, were known that discri mination of the breed over 96% showed by marker of a similar level (Fan *et al*., 2005; Oh J. D. *et al*., 2008). Allele number and heterozygosity will improve discri mination of each set because of various distribution of each set. Concretely, comparison using the frequency of allele expression by genetic marker of analysis target was able to detect specificity of breed group. When compared with graph of allele frequency of MS marker having relatively many alleles, it increased confidence of genetic discri mination because they represent significant differences (Fig. 1). At representative allele at each set, allele of 6 dog breed at each locus had many difference, therefore specificity of breeding was decided easily by combination of various alleles.

The observed and expected heterozygosity, and Polymorphic Informative Content (PIC) were calculated to deter mine the genetic diversity, the results are shown in Table 3. Observed and expected heterozygosity had various values from the minimum to the maximum, except only one allele and showed relatively high value in case of PIC. View of these results, the selected MS marker set can be understood as markers for analysis using usefulness of individual identification based on breed specificity within each group.

Based on these results, through a multiplex PCR technique using a combination of three types of MS marker, we can utilize breed-specific marker by faster, more accurate and cost-effective way by analyzing of multiple alleles showed in dog breed an can be used to parentage diagnosis and individual identification, in addition can be used as traits associated bio-marker. Therefore, MS marker having very high value in the field of molecule breeding used to prevent the extinction due to inbreeding, as well as, was a technology using improvement by selecting a specific trait. (Kaul *et al*., 2001). In addition, Multiplex PCR technique using various MS marker at a time than a single PCR by conventional methods to confirm only one MS marker can take advantage faster, more accurate and cost-effective way for analysis charge at the genetic paternity diagnosis and individual identification (Lim *et al*., 2009). To date, the demerits of studbook represent only documents were to break through providing a scientific evidence of companion dog registration system and supplementary the history tracking system, and thereby can build up to based on integrated management system at the national level of companion dog.

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