

# Genetic diversity testing for the Shikoku

## Overview

The Veterinary Genetics Laboratory (VGL), in collaboration with Dr. Niels C. Pedersen and staff, has developed a panel of short tandem repeat (STR) markers that will measure genetic heterogeneity and diversity across the genome and in the Dog Leukocyte Antigen (DLA) class I and II regions for specified dog populations. This test panel will be useful to dog breeders who wish to use DNA-based testing as a supplement to in-depth pedigrees. Using in-depth pedigrees and DNA based diversity data, along with DNA testing results for desired phenotypes and health traits can aid in informing breeding decisions.

DNA-based testing of the Shikoku breed is now in the preliminary results phase with the objective of building a snapshot of individual- and breed-wide genetic heterogeneity and diversity. The first 27 dogs were from the USA (n=25) and The Netherlands (n=2). This data base will be progressively expanded as more dogs are added with the goal of characterizing all the known alleles for the breed at 33 STR loci across the genome as well as all existing DLA class I and II haplotypes identified by seven STRs. We are accepting Shikoku from all parts of the world with a goal of 100 individuals tested to complete the initial phase.

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### **Results reported as:**

Short tandem repeat (STR) loci: A total of 33 STR loci from across the genome were used to gauge genetic heterogeneity and diversity within an individual and across the breed. The alleles inherited from each parent are displayed graphically to highlight heterozygosity, and breed-wide allele frequency is provided.

DLA haplotypes: Seven STR loci linked to the DLA class I and II genes were used to identify genetic differences in regions regulating immune responses and self/non-self-recognition. Problems with self/non-self-recognition, along with non-genetic factors in the environment, are responsible for autoimmune disease.

Internal Relatedness: The IR value is a measure of the genetic relatedness of an individual's parents. The value takes into consideration both heterozygosity of alleles at each STR loci and their relative frequency in the population. Therefore, IR values heterozygosity over homozygosity and uncommon alleles over common alleles. IR values are unique to each dog and two individuals from different sources may have identical IR values but a very different genetic makeup.

## **I. Introduction**

### **A. Breed history [1-3]**

The **Shikoku**, or Shikoku Ken, is a native Japanese breed from Shikoku island and the mountainous districts of Kochi Prefecture, hence an alternate name Kochi-ken (Kochi dog). The Shikoku was bred over centuries mainly for hunting deer and boar, which along with the Kishu dog has led them to be called Japanese boar dogs. The Shikoku is one of the six ancient Japanese dog breeds that belong to the Spitz family. It is intermediate in size between the larger Akita Inu and the smaller Shiba Inu and similar in size to the Kai Ken, Ainu and Kishu Inu. These breeds are very similar to each other with overlapping colors and shared morphology.

There were three varieties of Shikoku- the Awa, Hongawa and Hata after their areas of origin. The Shikoku of Hongawa are said to maintain most of the original purity because the relative inaccessibility of their home area. The Shikoku is tough and agile, necessary qualities for the mountainous regions of Japan. The Japanese Crown recognized the Shikoku dog as a living natural monument" of Japan in 1937.

The Shikoku is recognized by the Japanese Kennel Club. The Japanese Kennel Club is recognized by the AKC, but only the Shiba Inu is cross-recognized by the AKC. However, the Shikoku has been recently recognized by the AKC standard Foundation Stock Service (FSS) [2]. The AKC provides this service to allow certain purebred breeds to continue to develop while providing them with the security of a reliable and reputable avenue to maintain their records. The Shikoku is also listed by the Canadian Kennel Club Hound group and the United Kennel Club, but also awaiting full recognition.

### **B. Breed characteristics [1-3]**

The Shikoku stands 17–21 inches (43–53 cm) high, with a square body, wedge-shaped head, pricked triangular ears and feathered curved (sickle) tail typical of the Spitz-type. The outward appearance, other than size and coat color, is similar to the Siberian husky. The outer coat is tending to be harsh and straight, and the undercoat soft and dense. The hair on his tail is long. The coat can be red, black and tan, or sesame, the latter being favored and a mixture of black, red, and white hairs. A rare cream color also exists. These base colors are broken by white around the underside of the body, near the eyes, snout, and legs.

### **C. Temperament of Shikoku [1-3]**

Shikoku are the ideal companion for active outdoor people, being very energetic and active outside and are calm and quiet indoors. The Shikoku is a very intelligent dog and a quick learner. They are purportedly not as stubborn and independent as the other native Japanese breeds. The Shikoku is cautious around strangers and sticks only to people that it trusts. This loyalty often translates to a protective nature. However, they are not prone to excessive barking. Shikoku owners, preferably adults, should remain the dominant figure. Shikoku's have a high prey drive and should not be left alone with small pets such as cats, ferrets or guinea pigs. Shikoku's are energetic and require a lot of exercise and interaction with family members to avoid negative

behaviors.

## II. Genetic studies of contemporary Shikoku

### A. Population genetics based on allele frequencies at 33 STR loci on 25 chromosomes

STR markers are highly polymorphic and have great power to determine genetic differences among individuals and breeds. The routine test panel contains 33 STRs consisting of those that are recommended for universal parentage determination for domestic dogs by the International Society of Animal Genetics (ISAG) and additional markers developed by the VGL for forensic Purposes [10, 11]. Each STR locus is made up of 7 to 27 different alleles (avg. 15.4 alleles/locus) when tested across many breeds of dogs and other canids. Each breed, having evolved from a small number of founders and having been exposed to artificial genetic bottlenecks will end up with only a portion of the total available diversity. Artificial genetic bottlenecks include such things as popular sire effects, geographic isolation, catastrophes, outbreaks of disease, and ups and downs in popularity and resulting increases and decreases in population size. The alleles identified at each of the 33 STR loci and their relative frequencies were determined for the 27 Shikoku are listed in Table 1. [Link to Table 1](#)

**Table 1.** Alleles and their frequencies for 33 STR markers in 27 Shikoku

AHT121	AHT137	AHTH130	AHTH171-A	AHTH260	AHTk211
96 (0.39)	131 (0.72)	115 (0.76)	219 (0.93)	236 (0.31)	89 (0.81)
98 (0.06)	133 (0.11)	117 (0.02)	221 (0.02)	246 (0.17)	91 (0.04)
100 (0.13)	147 (0.11)	121 (0.22)	237 (0.06)	248 (0.52)	95 (0.15)
102 (0.11)	151 (0.06)				
106 (0.26)					
108 (0.06)					
AHTk253	C22.279	FH2001	FH2054	FH2848	INRA21
282 (0.35)	110 (0.31)	132 (0.09)	148 (0.02)	234 (0.02)	97 (0.46)
286 (0.11)	116 (0.41)	140 (0.33)	152 (0.04)	238 (0.96)	99 (0.15)
288 (0.39)	118 (0.26)	144 (0.22)	156 (0.24)	240 (0.02)	101 (0.39)
290 (0.11)	122 (0.02)	148 (0.20)	160 (0.06)		
292 (0.04)		152 (0.02)	164 (0.09)		
		154 (0.13)	168 (0.56)		
INU005	INU030	INU055	LEI004	REN105L03	REN162C04
110 (0.04)	144 (0.31)	210 (0.04)	95 (0.35)	227 (0.28)	200 (0.19)
124 (0.74)	146 (0.28)	214 (0.04)	97 (0.59)	231 (0.19)	206 (0.80)
126 (0.22)	152 (0.41)	220 (0.30)	107 (0.04)	233 (0.30)	210 (0.02)
		222 (0.61)	109 (0.02)	239 (0.07)	
		224 (0.02)		241 (0.17)	

REN169D01	REN169O18	REN247M23	REN54P11	REN64E19	VGL0760
202 (0.06)	160 (0.26)	268 (0.33)	230 (0.09)	145 (0.07)	12 (0.63)
212 (0.69)	164 (0.59)	270 (0.22)	236 (0.26)	147 (0.04)	13 (0.07)
216 (0.26)	168 (0.04)	272 (0.44)	238 (0.65)	151 (0.85)	14 (0.07)
	172 (0.11)			153 (0.04)	21.2 (0.04)
					23.2 (0.19)

VGL0910	VGL1063	VGL1165	VGL1828	VGL2009	VGL2409
17.1 (0.15)	13 (0.04)	20 (0.72)	18 (0.11)	10 (0.11)	13.3 (0.28)
18.1 (0.31)	14 (0.83)	22 (0.07)	19 (0.61)	11 (0.24)	15 (0.33)
19.1 (0.54)	15 (0.11)	26 (0.04)	20 (0.11)	13 (0.56)	15.3 (0.02)
	20 (0.02)	28.2 (0.17)	21 (0.07)	14 (0.02)	16 (0.24)
			22 (0.09)	15 (0.07)	18 (0.13)

VGL2918	VGL3008	VGL3235
13 (0.20)	11 (0.02)	12 (0.06)
14 (0.48)	12 (0.41)	15 (0.09)
15 (0.11)	13 (0.02)	16 (0.09)
17.3 (0.15)	15 (0.41)	18 (0.76)
19.3 (0.06)	17 (0.09)	
	18 (0.06)	

The average number of alleles per locus that have been discovered for all canids tested at the VGL is 15.4. Therefore, the most striking findings were the comparatively low number (3-6) of alleles found at each locus, and the high incidence of one or two alleles (Table 1). A single allele at two loci (FH248 and AHTh171-A) occurred 96 and 93% of dogs tested, respectively (Table 1). A single allele at four other loci (AHTk211, REN162CO4, REN64E19, VGL1063) was identified in  $\geq 80\%$  of dogs. These particular loci and alleles are linked to regions of the genome that have been highly conserved across the breed and most likely involve phenotypes linked to critical features of the breed standard. Although it is likely allele and allele frequencies may change as more dogs are tested, any additional alleles will be at low number and frequency and unlikely to significantly change these observations.

## B. Assessment of population heterozygosity using standard genetic parameters

Allele and allele frequencies at each of the 33 STR loci are listed in Table 1 and used to do a standard genetic assessments of the population as a whole (Table 2). These assessments include the average number of alleles found at each STR locus ( $N_a$ ); the average number of effective alleles ( $N_e$ ) per locus (i.e., the number of alleles that contribute most to genetic differences/heterozygosity; the observed or actual heterozygosity ( $H_o$ ) for the entire population; and the heterozygosity that would be expected ( $H_e$ ) if the existing population was in Hardy-Weinberg equilibrium (i.e., a state equivalent to random breeding).

**Table 2.** Summary of Standard Genetic Assessment for Shikoku using 33 STR loci

	<b>N</b>	<b>Na</b>	<b>Ne</b>	<b>Ho</b>	<b>He</b>	<b>F</b>
<b>Mean</b>	27	4.09	2.39	0.52	0.52	0.016
<b>SE</b>		0.18	0.15	0.03	0.03	0.023

The alleles identified in this group of 27 dogs (Na) represented 4.09 /15.4=26.5% of alleles known to exist at each of these loci in all canids tested at the VGL. Although this is higher than the Berger Picard (15.4%), it is somewhat lower than other small and less popular breeds such as the Swedish Vallhund (31.9%), Irish Red and White Setter (34.8%) and Flat Coated Retriever (38.6%); and considerably lower than popular and genetically diverse breeds such as the Golden Retriever (54.5%), Toy Poodle (55.6%) and Standard Poodle (58%).

The 27 Shikoku had an average of 4.09 alleles/loci (Na), but only 2.39/4.09=58% of the alleles were responsible for existing heterogeneity (heterogeneity=genotypic variation=phenotypic variation). This is typical for most pure breeds of dogs.

The observed (actual) heterozygosity of this group of 27 dogs was 0.52, while the expected heterozygosity (He) for a population in Hardy-Weinberg equilibrium (HWE) was nearly identical, yielding an average coefficient of inbreeding (F) that was near zero. If the 27 Shikoku selected for preliminary testing were representative of the breed, it can be concluded that Shikoku breeders have done a good job in selecting the least related parents from within population.

### **C. Standard genetic assessment values for individual STR loci**

The allele frequencies can be also used to do a standard genetic assessment of heterozygosity at each of the 33 autosomal STR loci (Table 3). This provides an estimate of genetic similarities in the specific regions of the genome that are associated with each STR marker. Phenotypic differences equate to genotypic differences. Therefore, alleles that are widely shared across the population are indicators that positive selection is occurring for certain desired traits. The Na values for an individual STR locus for this population of 27 ranged from a low of 3 to a high of 6 alleles per locus, while the Ne ranged from 1.08 to 4.39 alleles per locus. The observed heterozygosity (Ho) for an individual STR locus ranged from 0.15 to 0.89, while He ranged from 0.14 to 0.77 (Table 3). Loci with the lowest Ho and He values contributed the least to heterozygosity and are most likely involved with shared traits that are most important in maintaining standard breed characteristics. Loci with high Ho and He values are more genetically variable and associated with phenotypic variation within the breed

Twelve of the 33 loci had values of  $F > 0.00$  and 21 were negative with  $F < 0.00$  (Table 3). The loci with positive F values were under greater positive selection (i.e., more conserved) and therefore within regions of the genome that tend to be associated with desired breed-specific traits. However, the influences of these various inbred, neutral and outbred regions of the genome defined by these 33 STR loci have been kept in good balance by Shikoku breeders as evidenced by the nearly zero F value for the population as a whole (Table 2).

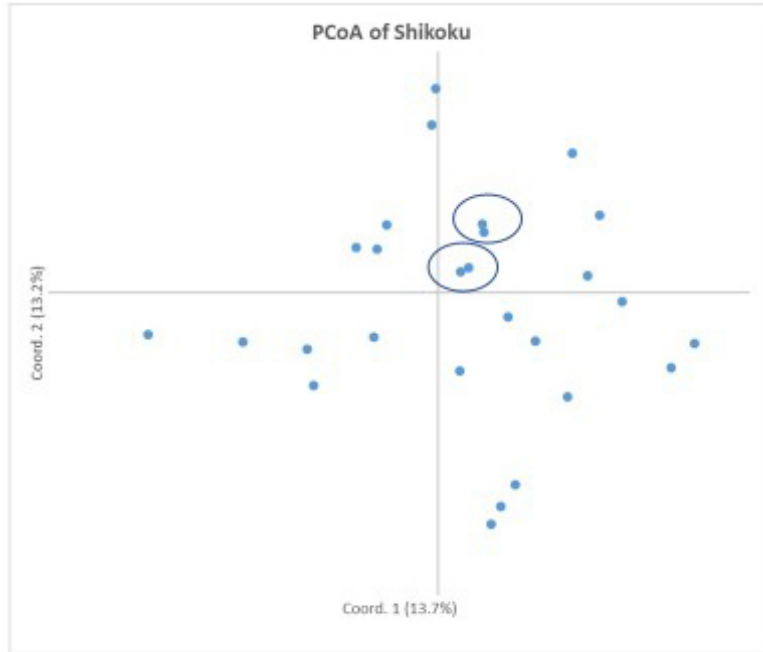
**Table 3.** Standard Genetic Assessment of each autosomal STR locus for 27 Shikoku

#	Locus	N	Na	Ne	Ho	He	F
1	AHT121	27	6	3.94	0.78	0.75	-0.042
2	AHT137	27	4	1.82	0.48	0.45	-0.068
3	AHTH130	27	3	1.60	0.41	0.37	-0.09
4	AHTh171-A	27	3	1.16	0.15	0.14	-0.064
5	AHTh260	27	3	2.53	0.63	0.60	-0.042
6	AHTk211	27	3	1.46	0.30	0.31	0.053
7	AHTk253	27	5	3.32	0.59	0.70	0.152
8	C22.279	27	4	3.01	0.70	0.67	-0.054
9	FH2001	27	6	4.39	0.78	0.77	-0.007
10	FH2054	27	6	2.63	0.48	0.62	0.223
11	FH2848	27	3	1.08	0.07	0.07	-0.029
12	INRA21	27	3	2.58	0.52	0.61	0.153
13	INU005	27	3	1.67	0.30	0.40	0.26
14	INU030	27	3	2.92	0.78	0.66	-0.182
15	INU055	27	5	2.15	0.48	0.54	0.101
16	LEI004	27	4	2.10	0.56	0.52	-0.062
17	REN105L03	27	5	4.30	0.89	0.77	-0.158
18	REN162C04	27	3	1.50	0.33	0.33	-0.006
19	REN169D01	27	3	1.85	0.33	0.46	0.276
20	REN169O18	27	4	2.31	0.59	0.57	-0.043
21	REN247M23	27	3	2.79	0.63	0.64	0.019
22	REN54P11	27	3	2.02	0.56	0.50	-0.102
23	REN64E19	27	4	1.36	0.30	0.27	-0.113
24	VGL0760	27	5	2.26	0.63	0.56	-0.131
25	VGL0910	27	3	2.44	0.70	0.59	-0.192
26	VGL1063	27	4	1.41	0.30	0.29	-0.016
27	VGL1165	27	4	1.80	0.37	0.44	0.165
28	VGL1828	27	5	2.43	0.52	0.59	0.118
29	VGL2009	27	5	2.60	0.63	0.62	-0.023
30	VGL2409	27	5	3.80	0.74	0.74	-0.006
31	VGL2918	27	5	3.22	0.63	0.69	0.087
32	VGL3008	27	6	2.90	0.67	0.66	-0.017
33	VGL3235	27	4	1.68	0.26	0.40	0.357

#### **D. Differences in population structure as determined by principal coordinate analysis (PCoA)**

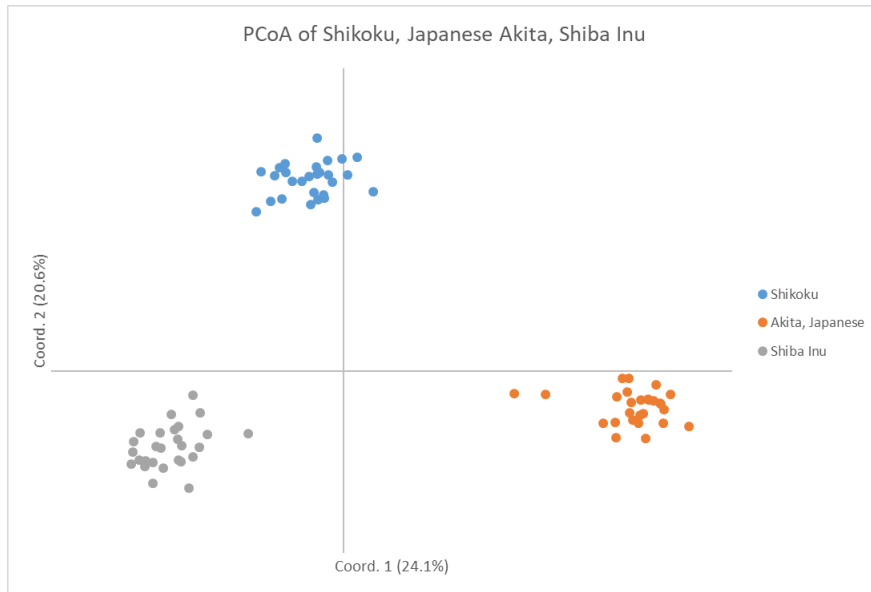
PCoA measures the genetic relatedness of individuals in a population. The data is computed in a spherical form but often presented in the two dimensions that most closely represent its multi-dimensional form (usually coordinates 1 and 2). The more closely individuals cluster together around the XY axis, the more related they are to each other.

The 27 Shikoku formed a single population (i.e., breed) in PCoA (Fig. 1). Individual dogs in the group were reasonably dispersed across all four quadrants of the graph, with the exception of two pairs of dogs that graphed together and were therefore more closely related to each other than to the others (Fig. 1). A number of other individuals appeared as outliers from the main population on the periphery of the graph. It can be assumed, therefore, that this group of 27 dogs, with the exception of these two pairs, were as unrelated to each other as possible given their relative lack of canid genetic diversity and this group of Shikoku is reasonably representative of the breed.



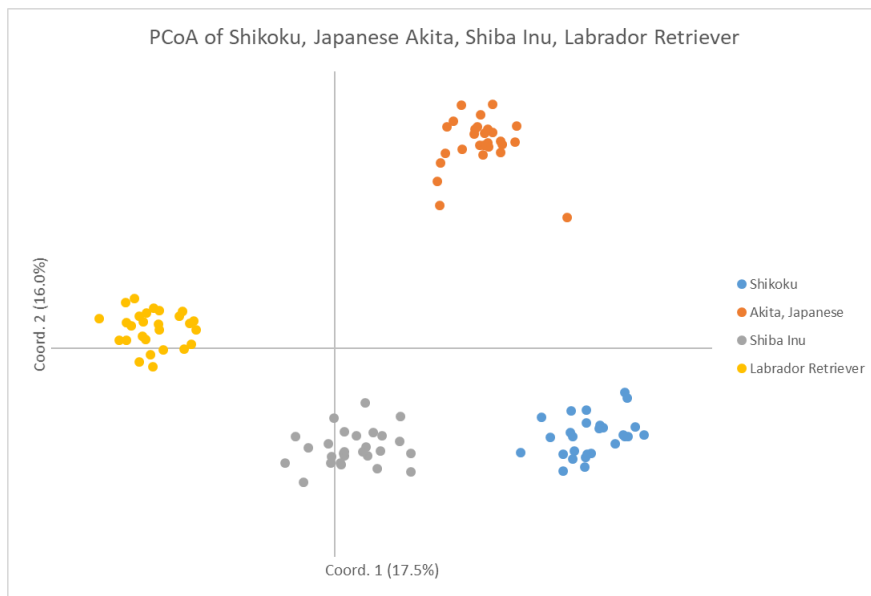
**Fig. 1.** PCoA of Shikoku (n=27) based on allele frequencies at 33 autosomal STRs. Two closely related pairs are circled.

The Shikoku are purportedly one of the heritage Japanese breeds and may therefore be closely related to breeds such as the Japanese Akita and Shiba Inu. This relationship was studied in PCoA (Fig. 2). PCoA comparing the three breeds show them to be genetically distinct. When comparing genetically distinct breeds in PCoA, the relationship of individuals within a breed is made closer (i.e., it deemphasizes genetic differences). Although this enhanced clustering is more evident for the Shikoku in a three-breed comparison, the population is still reasonably dispersed (Fig. 2), as demonstrated in the single breed PCoA (Fig. 1).



**Fig. 2.** PCoA showing relatedness of three Japanese breeds, Japanese Akita, Shiba Inu and Shikoku

In order to enhance the possible relatedness of these three Japanese breeds, a fourth unrelated Western breed, the Labrador Retriever, was added to the comparison (Fig. 3). In this comparison, the Shikoku and Shiba Inu breeds draw closer to each other in a single quadrant of the graph. This suggests that these two breeds are more closely related to each other than to Labrador retriever and Japanese Akita. This relationship is supported by significant sharing of a unique DLA class I/II haplotype (see section F). Interestingly, both Shikoku and Shiba Inu are more closely related to Labrador retrieves than to Japanese Akita.



**Fig. 3.** PCoA comparing three Japanese breeds (Akita, Shiba Inu, Shikoku) with an unrelated Western breed (Labrador retriever).



## E. Internal relatedness (IR) of individuals and the population as a whole

### 1. IR testing

Genetic assessments such as those presented in Tables 1-3 are indicators of population-wide (mean/average) heterozygosity and do not reflect the genetic diversity given to individual dogs by their parents. Internal Relatedness (IR) is a calculation that has been used to determine the degree to which the two parents of an individual dog were related. The IR calculation takes into consideration homozygosity at each locus and gives more importance to rare and uncommon alleles. Rare and uncommon alleles would presumably be present in less related individuals. IR scores of all individuals in a population can be graphed to form a curve ranging from -1.0 to +1.0. A dog with a value of -1.0 would have parents that were totally unrelated at all 33 STR loci, while a dog with an IR value of +1.0 has parents that were genetically identical at all loci. An IR value of +0.25 would be found among offspring of full sibling parents from a random breeding population. IR values  $>0.25$  occur when the parents of the full sibling parents were themselves highly inbred. The higher the IR value above 0.25 the more closely related were the parents and grandparents of the sibling parents.

Table 4 summarizes the IR values for the 27 Shikoku that were initially tested. The most inbred dogs had an IR score of 0.340, while the mean (average) IR score for the group was 0.015. One fourth of the population had IR scores between 0.105 and 0.340 and were significantly more inbred than the mean IR value (IR=0.015). In contrast, one fourth of the population had IR scores less than -0.075 to -0.194 and were significantly more outbred than the population mean (IR=0.015). Therefore, the population appeared to contain equal proportions of dogs that had parents that were as unrelated (most outbred) or related as possible (most inbred) given the genetic makeup of the population. The existence of both highly inbred and outbred individuals is a typical finding for almost all pure breeds of dogs tested at VGL.

**Table 4.** Internal relatedness (IR) values calculated using allele numbers and frequencies 27 Shikoku. The IR values can be adjusted to reflect how these same dogs would score if they were to exist in a large population of village dogs (IRVD).

	IR	IRVD
<b>Min</b>	-0.194	0.162
<b>1st Qu</b>	-0.075	0.306
<b>Mean</b>	0.015	0.382
<b>Median</b>	-0.011	0.370
<b>3rd Qu</b>	0.105	0.442
<b>Max</b>	0.340	0.631

### 2. Adjusted IR values based on village dogs (IRVD) as a measure of lost or retained genetic diversity

The IR values obtained from known alleles and their frequencies can be used to approximate the

amount of genetic diversity that has been lost as a breed evolves from its oldest common ancestors to the present day. Village dogs that exist throughout the SE Asia, the Middle East and the Island Pacific region are randomly breeding descendants of dogs from which most modern breeds evolved. The known alleles and their frequencies of a given breed can be compared with the same alleles and their frequency in modern village dogs to yield an adjusted IR score (IR-village dog or IRVD). Therefore, the IRVD score approximates how a Shikoku's IR score would compare to other village dogs if its parents were also village dogs.

The IR values listed in Table 4 are most easily studied in a graph form (Fig. 4). The graphic of IRVD scores for the 27 dogs (blue line) is shifted to the right of their IR scores. Almost all of the 27 Shikoku have IRVD values of 0.162 and greater (Table 4; Fig. 4) and if they were found today among village dogs, three-fourths of them would have IRVD scores  $>0.250$  and be judged offspring of at least full sibling parents. Most of them would be even more related, with parents that are even more related to each other than siblings from a large and genetically diverse random breeding dog population. It is noteworthy that the IR graph (red line) is biphasic with the major population peaking at around -0.1 and a secondary population that peaks at around +0.15. This second peak represents the most inbred portion of the population. The fact that there are two peaks suggests what is called "population stratification." Whether this stratification is an artifact resulting from how the test population was selected, characteristic of the breed in the USA, or typical of all contemporary Shikoku, will only be resolved with testing of more dogs from as wide a geographical area as possible.

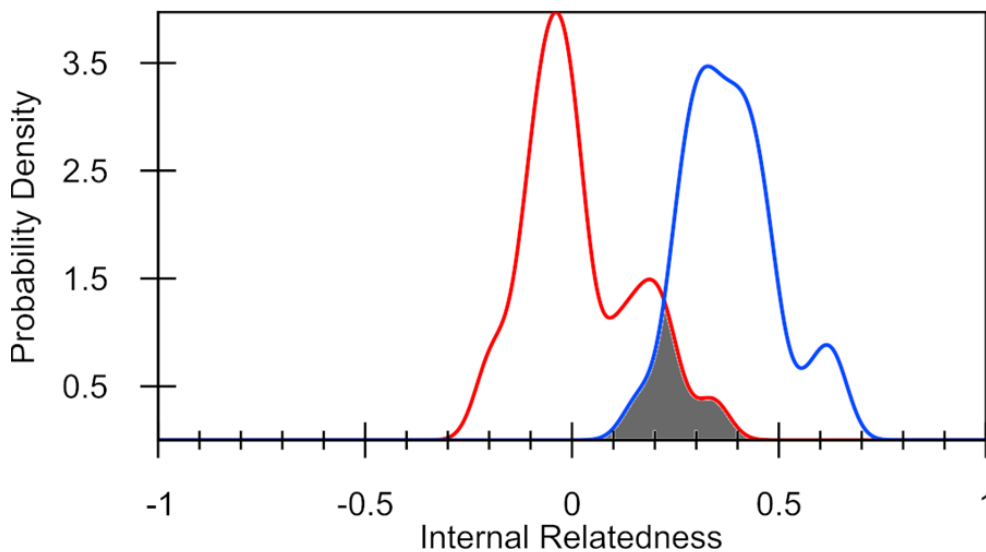


Fig. 4. Distribution of IR (red line) and IR-village dog (IRVD) (blue line) values for Shikoku (n=27). The area under the curve (black) represents the degree of allele sharing (14.9%) between Shikoku and village dogs.

The darkened area in Figure 4 representing the overlap of IR and IRVD curves is an estimate of the amount (14.9%) of genetic diversity in present-day randomly breeding village dogs that still exists in contemporary Shikoku. This figure is about one-half the 26.5% retained canid genetic diversity for the breed that was determined from allele and allele frequencies of the 33 autosomal

STRs (Tables 1, 2). Although both calculations are only estimates, and based on two different types of reference populations, it is reasonable to conclude that contemporary Shikoku have retained from 14.9-26.5% of the genetic diversity currently found in all canids. This loss of diversity has undoubtedly occurred over thousands of years of human selection (artificial genetic bottlenecks), which was slow at first but then rapidly accelerated over the last hundreds of years during which the breed rapidly attained its present appearance (phenotype). The greatest single genetic bottleneck for the breed was perhaps WWII.

## **F. DLA Class I and II haplotype frequencies and genetic diversity**

The DLA consists of four gene rich regions making up a small part of canine chromosome 12. Two of these regions contain genes that help regulate normal cell-mediated (Class I) and antibody-mediated (Class II) immunity. Polymorphisms in these regions have also been associated with abnormal immune responses responsible for autoimmune diseases, allergies, and resistance/susceptibility to infectious diseases.

The Class I region contains several genes, but only one, DLA-88, is highly polymorphic (i.e., with many allelic forms) and is the most important for immune regulation. Specific alleles at the four STR loci associated with the DLA-88 are linked together in various combinations, forming specific haplotypes (Table 5). Groups of genes and their alleles inherited as a block, rather than singly, are called haplotypes.

The class II region also contains several genes, three of which are highly polymorphic, DLA-DRB1, DLA-DQB1 and DLA-DQA1. Specific alleles at the three STR loci associated with the three class II genes are strongly linked and are often inherited as a single block or haplotype (Table 6). One haplotype comes from each of the parents. The STR-based haplotype nomenclature used in this breed diversity analysis is based on numerical ranking with the first haplotypes identified in Standard Poodles being named 1001, 1002, ... for class I haplotypes and 2001, 2002, ... for class II haplotypes. It is common for various dog breeds to share common and even rare haplotypes, depending on common ancestry

### ***1. DLA class I and II haplotypes existing in Shikoku***

The 27 Shikoku that were tested possessed 6 DLA class I and 7 DLA class II haplotypes. This is higher than the Berger Picard (2, 2), similar to other small and less popular breeds such as the Swedish Vallhund (6, 4) and Shiloh Shepherd (7, 6), but lower than small breeds such as the Giant Schnauzer (14, 15), Samoyed (13, 12) and Shiba Inu (16, 15). These latter breeds were, in turn, much lower than popular breeds such as the Golden Retriever (26, 23) and Miniature Poodle (33, 23).

The breed possessed four unique DLA class I haplotypes (1229-1232) and two unique class II haplotypes (2122, 2123). The presence of so many unique DLA class I and II haplotypes is atypical for among the breeds which have been studied by VGL. Interestingly, the Shiba Inu also had a greater proportion of unique haplotypes.

Two DLA class I (1133, 1191) and two class II haplotypes (2018, 2077) were found in 82% of the dogs tested. Two class I and II haplotypes were in linkage disequilibrium forming extended 1133/2077 and 1191/2018 haplotypes. All of the remaining haplotypes occurred at low and almost equal frequencies. It would appear that these two extended haplotypes have been inherited by descent from two important founders or closely related founder lines.

**Table 6:** DLA class I and Class II haplotypes and their frequencies, as identified in 27 Shikoku.

<b>DLA1 haplotype</b>	<b>STR alleles</b>	<b>Haplotype frequency</b>
1133	378 365 287 172	0.56
1191	388 373 260 186	0.26
1229	381 369 277 188	0.07
1230	386 369 289 184	0.04
1231	389 371 277 186	0.06
1232	380 365 260 186	0.02
<b>DLA2 haplotype</b>		
2012	345 322 280	0.02
2018	339 324 284	0.26
2056	339 323 286	0.06
2071	339 322 286	0.04
2077	347 325 286	0.56
2122	351 325 286	0.06
2123	339 323 292	0.02

## ***2. DLA class I and II haplotype sharing with other breeds***

DLA haplotypes are more conserved than other regions of the genome and inherited as blocks of linked genes, one from each parent, and passed from one generation to the next by descent. Recombination within and between these blocks of genes tends to be low, allowing them to remain much the same over the generations. Therefore, the DLA haplotypes found in a breed can be used to estimate the founder/founder lines that were used to create a breed and the importance of these various founders in subsequent breed evolution. The DLA class I and II regions are frequently shared between breeds, reflecting common distant ancestry (Table 6).

Because of the disproportionate number of unique DLA haplotypes, breed sharing in the DLA region was not as prominent as it is in other breeds. The strongest sharing is of the extended DLA 1191/2018 haplotype with the Shiba Inu. This supports the distant relationship found with PCoA (Fig. 3). Only one DLA class I haplotype was shared with Shiba Inu and Havanese, while class II sharing, in addition to 1191/2018, was also with the Flat Coated Retriever, Havanese,

Giant Schnauzer, Alaskan Klee Kai, Magyar Agar, Irish and Llewellyn Setters, American and Japanese Akita, Golden Retriever, and the Poodles (Table 6).

**Table 6.** Sharing of specific DLA class I and II haplotypes between Shikoku and other breeds tested at the VGL.

DLA Class I Haplotype Frequencies (Updated Oct 14, 2019)																	
DLA1 #	STRtypes	Irish Red and White Setter (n=60)	Flat Coated Retriever (n=546)	Havanese (n=443)	Shiba Inu (n=105)	Giant Schnauzer (n=214)	Shikoku (n=27)	Alaskan Klee Kai (n=541)	Magyar Agar (n=60)	Irish Setter (n=49)	Llewellyn Setter (n=91)	American Akita (n=99)	Japanese Akita (n=357)	Golden Retriever (n=712)	Miniature Poodle (n=287)	Poodle (n=2825)	Toy Poodle (n=142)
1133	378 365 287 172	--	--	0.021	0.01	--	0.56	--	--	--	--	--	--	--	--	--	--
1191	388 373 260 186	--	--	--	0.21	--	0.26	--	--	--	--	--	--	--	--	--	--
1229	381 369 277 188	--	--	--	--	--	0.07	--	--	--	--	--	--	--	--	--	--
1230	386 369 289 184	--	--	--	--	--	0.04	--	--	--	--	--	--	--	--	--	--
1231	389 371 277 186	--	--	--	--	--	0.06	--	--	--	--	--	--	--	--	--	--
1232	380 365 260 186	--	--	--	--	--	0.02	--	--	--	--	--	--	--	--	--	--
DLA Class II Haplotype Frequencies (Updated Oct 14, 2019)																	
DLA2 #	STRtypes	Irish Red and White Setter (n=60)	Flat Coated Retriever (n=546)	Havanese (n=443)	Shiba Inu (n=105)	Giant Schnauzer (n=214)	Shikoku (n=27)	Alaskan Klee Kai (n=541)	Magyar Agar (n=60)	Irish Setter (n=49)	Llewellyn Setter (n=91)	American Akita (n=99)	Japanese Akita (n=357)	Golden Retriever (n=712)	Miniature Poodle (n=287)	Poodle (n=2825)	Toy Poodle (n=142)
2012	345 322 280	0.192	--	0.006	--	0.012	0.02	0.0601	0.1	0.06	0.038	--	0.001	0.0007	0.063	0.005	0.053
2018	339 324 284	--	0.1401	0.018	0.2	--	0.26	--	--	--	0.027	--	--	--	--	--	--
2056	339 323 286	--	--	--	--	--	0.06	--	--	--	--	0.591	0.165	--	--	--	--
2071	339 322 286	--	--	0.006	--	--	0.04	--	--	--	--	0.005	--	--	--	--	--
2077	347 325 286	--	--	0.021	--	--	0.56	--	--	--	--	--	--	--	--	--	--
2122	351 325 286	--	--	--	--	--	0.06	--	--	--	--	--	--	--	--	--	--
2123	339 323 292	--	--	--	--	--	0.02	--	--	--	--	--	--	--	--	--	--

### 3. Heterogeneity in the DLA region

The 7 loci that define the DLA class I and II haplotypes are in stronger linkage disequilibrium than other parts of the genome that are measured by the 33 autosomal STR markers. However, the expectation is that these loci have achieved an equilibrium with other loci in the genome through random mating and over enough time. This can be tested by doing a standard genetic assessment of each locus (Table 7) and across all loci (Table 8).

Standard genetic assessment of the 7 STR loci associated with the DLA class I and II regions was different than expected. The expected heterozygosity ( $H_e$ ) was always much higher at each locus than the observed heterozygosity ( $H_o$ ), yielding strongly positive  $F$  values of 0.19 to 0.41 (Table 7), which translates to 19-41% excess of homozygosity (or lack of heterozygosity) in the DLA region. The average  $F$  value for all 7 STR loci was 0.24 (Table 8). The only explanation for these findings was that the test population of 27 dogs contained an excess of dogs with certain DLA haplotypes. The most likely haplotypes contributing to this imbalance were the prevalent 1133/2077 and 1191/2018 haplotypes. Hopefully, this disparity will be resolved with careful selection and testing of more dogs and is not due to a more recent and widespread artificial bottleneck in the breed as a whole.

**Table 7.** Standard Genetic Assessment for Shikoku using 7 STRs in the DLA region

#	Locus	N	Na	Ne	Ho	He	F
1	DLA I-3CCA	27	6	2.59	0.48	0.61	0.22
2	DLA I-4ACA	27	4	2.43	0.44	0.59	0.24
3	DLA I-4BCT	27	4	2.48	0.48	0.6	0.19
4	DLA1131	27	4	2.34	0.44	0.57	0.23
5	5ACA	27	4	2.23	0.44	0.55	0.19
6	5ACT	27	4	2.23	0.44	0.55	0.19
7	5BCA	27	4	1.78	0.26	0.44	0.41

**Table 8.** Summary of Standard Genetic Assessment for Shikoku using 7 STRs in the DLA region

	N	Na	Ne	Ho	He	F
Mean	27	4.29	2.3	0.43	0.56	0.24
SE		0.27	0.09	0.03	0.02	0.03

### III. What does DNA-based genetic testing tell us about the Shikoku

The 27 Shikoku that were tested constituted a single breed based on allele and allele frequencies for the 33 autosomal STR loci and PCoA. The breed is clearly distinguishable from Japanese heritage breeds such as the Akita and Shiba Inu but shares at least one founder line with the Shiba Inu. The breed has retained 15-27% of the genetic diversity that still exists in all canid populations. This is similar to retained canid diversity in many smaller and less popular breeds but is only one half or less of the genetic diversity still present with large and popular breeds such as the Golden and Labrador Retrievers and various types of Poodles. More diversity is likely to be discovered as more dogs are tested, but this extra diversity will not greatly improve these figures.

Standard genetic assessment for heterozygosity and PCoA graphing indicates that the average Shikoku in the preliminary test population is a product of parents that are as unrelated as possible. However, internal relatedness (IR) scores also show that average values can be misleading, as some individuals were much more inbred or outbred than others and these two groups cancelled each other out when calculating breed-wide heterozygosity.

A lack of genetic diversity is not in itself bad, providing the founder population was relatively free of deleterious genetic traits and breeders have been judicious in avoiding a loss or imbalance in the original diversity. The Shikoku is surprisingly clear of simple breed-specific heritable disease traits and enjoys a reasonable lifespan compared to other dog breeds. The health problems that exist are of a complex genetic basis and are common to many dog breeds and even mongrels [7]. These traits common to modern dogs were most likely inherited from generation to generation as dogs underwent progressively more intense human-directed artificial selection.

The greatest problems with low genetic diversity often occur when a breed becomes popular, either for pets or shows, or both [9-11]. Popularity creates a rapid demand for new dogs, which in turn leads to less attention to proper breeding practices, and in particular overuse of certain sires. Likewise, showing often leads to subtle and not so subtle changes in interpretation of the breed standards and bouts of inbreeding to fix the desired trait [9]. Such changes often involve popular sires, but also popular dams and related bloodlines. If the gene pool is small, spontaneous mutations that often occur in areas of high selection pressures can be inadvertently swept up and amplified [9-11].

The lack of genetic diversity in the DLA class I and II region of these 27 Shikoku was noteworthy. It appears that two founders or founder lines have played an important role in the evolution of contemporary Shikoku. One of these founder lines is shared with the Shiba Inu, but not with Japanese or American Akita. It also appears that dogs that possess these haplotypes are possibly under some sort of positive selection, but whether that was due to non-random sampling or current inbreeding remains to be determined. A lack of genetic diversity in the DLA region is troublesome, but it is uncertain what it means, if anything, for the future of the breed. Certain DLA class I and II haplotypes have been associated with specific autoimmune diseases in certain breeds [8], but autoimmune disorders are not viewed as a serious problem for Shikoku. Therefore, the strongly shared DLA regions of the Shikoku do not appear to have a strong negative effect on self/non-self-recognition. Nevertheless, it is important that breeders maintain as much diversity and heterozygosity in the DLA region as possible.

The history of North American Shikoku lends support to the role of several key founders in breed genetics [14]. Contemporary Shikoku have descended mainly from the Hongawa and Hata lines, as the third Awa line was decimated as a result of World War II. One of the foundation dogs of the Hata line was "Gomago," who was born in 1934. In contrast, the Hongawa line is attributable to the foundation dog "Chousungo." Dogs belonging to each of these lines had contrasting and complimentary phenotypic traits. However, Hongawa Shikoku have had the most influence on the breed. Two other Shikoku from the same period, Kusugo and Kumago, were also significant show winners of the late 1930s. These four dogs are presumed to be at the foundation of contemporary Shikoku Ken.

Breeds that lack genetic diversity must be managed much more closely to avoid further loss of genetic diversity and have less leeway in dealing with simple recessive or complex polygenic disorders that might arise [10, 11]. Disease mutations are usually autosomal recessive in nature and frequently linkage with a region of the genome that is suddenly becomes subject to strong positive selection for a phenotype that is deemed desirable in the show ring [9-11]. Elimination of such deleterious mutations may result in loss of genetic diversity, especially when diversity is already limited [9-11].

The intent of this study was not to study the possible relationships between the main Japanese breeds. However, we did note inconsistencies between past DNA-based studies and our findings. Early work suggested that the six native Japanese breeds differ mainly by size - small, medium and large [1]. This assessment led to several more recent studies using polymorphisms within mitochondrial DNA and the cytochrome b gene to differentiate the six heritage Japanese breeds. These DNA-based studies indicated that "differences between breeds are small" and that

"individuals native to Japanese breeds cannot be unambiguously identified" [12, 13]. Therefore, it has been assumed that genetic variations observed among Japanese native dog breeds have resulted from extensive interbreeding and/or intrabreeding between indigenous Japanese breeds with limited introgressions from Western breeds. We did recognize the predominance of uniquely Asian DLA class I and II haplotypes in Japanese breeds, but only one of these Asian haplotypes was shared by the Shikoku and Shiba Inu, clearly not supporting extensive interbreeding. Moreover, using STRs instead of mitochondrial polymorphisms, we had no difficulty in genetically distinguishing American and Japanese Akita, Shiba Inu and Shikoku from each other and to identify DNA markers (STRs) that could potentially separate Japanese breeds and individuals within each breed.

## **IV. Health problems of heritable nature**

### **A. Life expectancy**

The average life expectancy of the Shikoku is assumed to be 11-13 years [6].

### **B. Health problems [4-6]**

#### ***1. Diseases of a complex heritable nature***

Shikoku are perceived as being comparatively healthy and those disease problems that exist appear to occur at a low frequency [4-6]. Most of the health conditions described in Shikoku are also of a complex heritable nature and occur in many other breeds as well as among random bred dogs [7]. Therefore, the heritable factors for these disorders have probably occurred as a result of human selection over a long period and passed down by descent as breeds were created.

Orthopedic problems described in the breed include panosteitis, hip dysplasia, elbow dysplasia, and luxating patella. Hip dysplasia was observed in 12.2% of Shikoku reported to the OFA, and luxating patella in 7.9% [5]. These problems manifest at a young age and predispose to mild to severe degenerative joint disease (osteoarthritis) over time.

Dental problems of undefined nature were described in 20% of Shikoku reported to the OFA [5].

Immunological problems include upper respiratory allergies. Some owners describe vague intestinal problems that are often ascribed to food allergies or intolerances.

Neurological problems include epilepsy and a rare fatal neurological disorder that has not been defined

Eye problems consist mainly of entropion and allergic conjunctivitis.

Heart problems appear to be uncommon and include dilated cardiomyopathy.

Reproductive problems include split or abnormal heat period, pyometra and male infertility. Therefore, it is often said that Shikoku are difficult to breed.



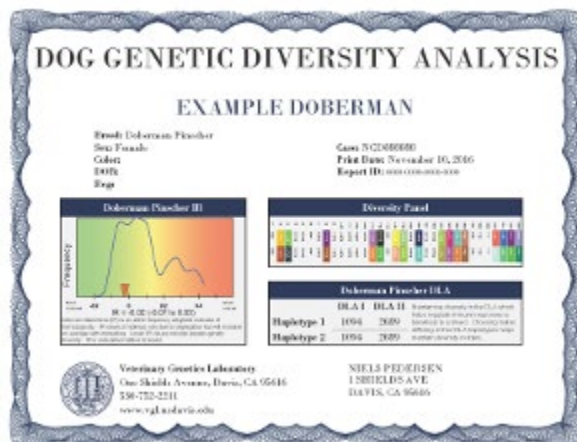
## 2. Diseases of Mendelian inheritance

There is no disease in Shikoku that is linked to a specific simple recessive or dominant mutation.

## V. Results of Diversity Testing

### A. How will you be given the results of DNA-based genetic diversity testing on your dog?

After a sample is submitted for genetic testing, the identity of the dog and owner will be replaced by a laboratory barcode identifier. This identifier will be used for all subsequent activities and each owner will be provided with a certificate that reports the internal relatedness, genomic STR genotypes and DLA class I and II haplotypes for the dog(s) tested. The internal relatedness value for the dog being tested is related to the entire population.



### B. What should you do with this information?

The goal for breeders should be to continue to produce puppies with IR scores less than 0. Although this initial population appeared to be outbred on average, there was a subpopulation of dogs that were much more inbred than the rest of the breed. Therefore, there is a possibility to better balance genetic diversity in the breed by DNA testing. Mates should be selected to avoid homozygosity at any genomic loci or DLA class I and II haplotype and encourage the use of dogs with less common genomic alleles or DLA haplotypes. Maintaining existing genomic diversity will require using IR values of potential mates based on the 33 STR loci to assure puppies of equal or greater overall diversity, like what is being done by many Standard Poodle breeders. However, IR values, because they reflect the unique genetics of each individual, cannot be used as the criteria for selecting ideal mates. Mates with identical IR values may produce puppies significantly more or less diverse than their parents. Conversely, a mating between dogs with high IR values, providing they are genetically different, may produce puppies having much lower IR scores than either parent. A mating between a dog with a high IR value and a low IR value, providing the latter has few alleles and DLA haplotypes in common, will produce puppies much more diverse than the highly inbred parent. Breeders should also realize that a litter of puppies may have a wide range of IR values, depending on the comparative contributions of each

of the parents. The more genetically diverse and different the parents, the greater the range of IR values in their offspring.

The next step is to compare the DLA class I and II haplotypes. You want to avoid breeding pairs that will produce puppies that will be homozygous for the same haplotypes. It is also prudent to use uncommon DLA haplotypes whenever possible. The objective should be to rebalance DLA diversity to minimize the contributions of a small number of common haplotypes. Also, less common haplotypes may offer more genome-wide genetic diversity than common ones.

Breeders who do not have access to computer programs to predict the outcome of matings based on IR values of sire and dam can also compare values by manual screening. Potential sires and dams should be first screened for genetic differences in alleles and allele frequencies for the 33 genomic STR loci. Some extra weight should be given to rare vs common alleles. This information is included on all certificates and on the breed-wide data on the VGL website.

Puppies, once born, should be tested for their actual IR values, which will reflect the actual genetic impact of each parent on internal diversity. Considerations of mate choices for genetic diversity should be balanced with other breeding goals but maintaining and/or improving genetic diversity in puppies should be paramount.

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