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Research Article

Investigation of Blood Protein Polymorphism and Estimation of Genetic Distances in Some Dog Breeds in Turkey*

Metin ERDOĞAN
Department of Medical Biology and Genetics, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyon - TURKEY
Ceyhan ÖZBEYAZ
Department of Animal Science, Faculty of Veterinary Medicine, Ankara University, Ankara - TURKEY

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Abstract: Phylogenetic relationships in some Turkish dog breeds were investigated to determine protein polymorphisms by electrophoretic analysis. Blood samples were collected from 276 dogs including the Kangal, Akbash and German Shepherd as well as Doberman Pinscher, Setter, Pointer and Labrador breeds. Enzymes and proteins were separated electrophoretically using isoelectrofocusing gel, starch gel and polyacrylamide gel. Polymorphism on albumin (Alb), postalbumin-1 (Poa-1), postalbumin-3 (Poa-3), transferrin (Tf), and esterase (ArE) loci was detected, while no polymorphism was observed on the hemoglobin (Hb) locus. These polymorphisms were used to estimate the average heterozygosity value ($\bar{h}_s$), *F*-statistics and the number of gene flow in each generation (N_m). The genetic distances (d_{ij}) were also compared among these dog breeds. Average heterozygosity values were in the range 0.32 (Doberman Pinscher) to 0.41 (Kangal Shepherd), and significant differences in heterozygosity were found among the breeds ($P < 0.05$). $\tilde{F}_{IS_w}$, $\tilde{F}_{IT_w}$ and $\tilde{F}_{ST_w}$ values were estimated as 0.085, 0.083 and 0.160, respectively, for whole loci in the breeds and these values were significant at $P < 0.001$. The estimated values of genetic distance in populations other than the Setter breed were between 0.013 and 0.242. Cluster analysis (UPGMA) results showed that the Pointer and Akbash breeds formed a cluster and then the German Shepherd joined this cluster. Finally, the Labrador breed also joined this cluster. However, the Doberman and Kangal Shepherd breeds formed a different cluster. The Setter breed did not join either and formed its own cluster. The formation of 2 distinct clusters in Kangal and Akbash Shepherd dogs reveals that these breeds have different genetic structures in terms of the investigated loci and they were not closely related to each other.

Key Words: Average heterozygosity, biochemical polymorphism, cluster analysis, dog, *F*-statistics, genetic distance

Türkiye'deki Bazı Köpek Irklarında Kan Protein Polimorfizmi ve Irklararası Genetik Mesafelerin Tahmin Edilmesi

Özet: Türkiye' de yetiştirilen bazı köpek ırkları arasındaki genetik ilişkiyi belirlemek amacıyla protein polimorfizminden yararlanılmıştır. Akbaş, Kangal, Alman Çoban Köpeği, Doberman, Labrador, Setter ve Pointer köpek ırlarına ait toplam 276 köpekten kan alınmıştır. Enzim ve proteinlerin belirlenmesinde izoelektrofokusing, nişasta ve poliakrilamid jel elektroforezi kullanılmıştır. Alb, Tf, Poa-1, Poa-3, ArE lokuslarında polimorfizm tespit edilirken Hb lokuslarında ise polimorfizm tespit edilmemiştir. Lokuslardaki bu polimorfizmden yararlanarak ortalama heterozigotluk ($\bar{h}_s$), *F*-istatistikleri ve her jenerasyon göç eden birey sayısı (N_m) hesaplanmıştır. Bu köpek ırklarını karşılaştırmada ise genetik uzaklık (d_{ij}) değerleri kullanılmıştır. Ortalama heterozigotluk değeri 0,32 (Doberman) ile 0,41 (Kangal Çoban köpeği) arasında tahmin edilmiş ve köpek ırkları arasında heterozigotluk bakımından herhangi bir farklılık bulunamamıştır ($P < 0,05$). Köpek ırklarında tüm lokuslar üzerinden sırasıyla 0,085, 0,083 ve 0,160 ($P < 0,01$) olarak hesaplanan $\tilde{F}_{IS_w}$, $\tilde{F}_{IT_w}$ ve $\tilde{F}_{ST_w}$ değerleri istatistiki olarak önemli bulunmuştur. Setter ırkı hariç diğer ırklar arasında genetik uzaklık değerleri bakımından büyük farklar bulunmamakla birlikte ırklararası genetik uzaklık değeri 0,013 ile 0,242 arasında tespit edilmiştir Yapılan kümeleme analizi sonucu çizilen dendogramda, Pointer ve Akbaş ırkları bir küme oluşturmakta, daha sonra bu kümeye Alman Kurt Köpeği ve Labrador ırkının da dahil olmasıyla başka bir küme oluşmaktadır. Doberman ve Kangal ırkları ise başka bir küme oluşturmaktadır. Setter ırkı ise bu iki kümeye de girmemiştir. Kangal ve Akbaş Çoban köpeklerinin farklı kümelerde yer almaları, her iki ırkın incelenen lokuslar bakımından farklı genetik yapıda olduklarını göstermekte, bu da bu iki ırkın yakın akraba oldukları düşüncesini zayıflatmaktadır.

Anahtar Sözcükler: Ortalama heterozigotluk, biyokimyasal polimorfizm, kümeleme analizi, köpek, *F*-istatistik, genetik uzaklık,

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Introduction

Dogs (*Canis familiaris*) are thought to be derived only from wolves (*Canis lupus*), and are thought to be the oldest domesticated animal (1,2). The relationship between man and dogs should be called partnership or companionship, but not commensalism, which is usually observed between man and other domesticated animals (3). The evidence suggests that it is possible to trace the route of migration of man by tracing the route of dog populations in prehistoric times (4). Migrations, natural selection and conscious selection and mutations have been determined in the existence of different dog breeds from prehistoric times to the present.

It has been well understood from archeological discoveries that dogs lived in Mesopotamia domestically at 12,000–14,000 B.C. Although there is no definite information on the history of Turkish Shepherd dogs, archeological data indicated that some dogs, similar to Turkish Shepherd dogs, lived in Anatolia as early as about 7000 B.C. and in Tibet at about 4000 B.C. (5,6).

Some dog breeds (such as the Great Pyrenees, Kuvasz, Shar Planinetz, Kommondor and Maremma) seen from Central Asia to Europe resemble Turkish Shepherd dogs. (7,8). It is assumed that Turkish migration played a very important role in spreading Turkish dog breeds to Europe (5,6). Furthermore, Kırmızı (5) claimed that the origins of the German Shepherd dog and Turkish Shepherd dog were in Central Asia and these breeds spread to Europe and Anatolia by the migrations of Turks from Central Asia to Anatolia and Europe, in which they continued to spread.

Finding polymorphisms in blood proteins including enzymes in various domesticated animals has enabled us to elucidate the phylogenetic relationships of breeds or populations of animals. In contrast to the large number of investigations on enzymes and proteins in the blood of the common dog including European and Asian breeds (4,9-14), there is no corresponding comprehensive study concerning the biochemical genetic variability in Turkish dog breeds.

The aim of the present study was therefore to determine the biochemical polymorphism in Turkish Shepherd dogs and to estimate the genetic distance between this and other dog breeds.

Materials and Methods

The investigation was conducted on 276 blood samples collected from Turkish dogs including the Kangal, Akbash and German Shepherd as well as the Doberman Pinscher, Setter, Pointer and Labrador breeds. Their breeding distribution is shown in Table 1. Blood samples were collected into tubes containing sodium citrate from Vena cephalica antebrachii. The samples were stored at 4 ºC and utilized the same day. Plasma and erythrocytes were separated by centrifugation (3500 g x 10 min). Subsequently, the erythrocytes were hemolyzed by adding an equal volume of distilled water. The samples were then used either immediately or stored at –20 ºC until analysis. The starch gel electrophoresis system was employed to analyze albumin and hemoglobin (Sigma, S-4501) as described by Gelderman (15). Other plasma proteins were analyzed by polyacrylamide gel electrophoresis as described by Özbeyaz (16). Esterase was separated by isoelectrofocusing gel with a Multiphor II chamber. Investigated proteins and the original electrophoretical methods are shown in Table 2.

Table 1. Animal material.

Breeds	Number (n)
Kangal Shepherd	88
Akbash Shepherd	29
German Shepherd	63
Doberman Pinscher	26
Setter	18
Pointer	22
Labrador	30
Total	276

Table 2. List of 6 blood protein loci examined.

Protein	Locus	Reference
Plasma albumin	Alb	Day et al. (9)
Hemoglobin	Hb	Tanabe et al. (10)
Plasma postalbumin-1	Poa-1	Reetz (17)
Plasma postalbumin-3	Poa-3	Reetz (17)
Plasma transferrin	Tf	Reetz et al. (18)
Plasma arylesterase	ArE	Braend (19)
		Braend and Andersen (20)

Gene frequencies at the loci with codominant alleles were estimated by gene counting. The genetic variability of each dog breed or population was calculated by the proportion of polymorphic loci and the average heterozygosity per locus over all loci examined ($\bar{h}_s$) (21). When inbreeding or selection was done in a population, changes occurred in the Hardy-Weinberg proportions in favor of homozygotes, and this is called the *fixation index* (*F*) (22). The method described by Weir and Cockerham (23) was used to calculate *F*-statistic values on the basis of all over loci and in one locus. To determine the statistical significance, bootstrap confidence gap analysis (24) and χ^2 tests (25) were used. The effective number of individual exchanges between populations per generation (N_m) was computed with $N_m = (1-\tilde{F}_{ST}) / 4\ \tilde{F}_{ST}$ (21,26). Mean genetic distance among the dog breeds was calculated using the method developed by Nei (27), in which data of 5 polymorphic loci and 1 monomorphic locus were used. From the matrix of genetic distance values, dendrograms of the dog breeds were drawn by the unweighted pair-group method (UPGMA) of clustering in numerical taxonomy (28). All computations for the statistical analysis were performed using the TPFGA program (29).

Results

The biochemical polymorphism was detected on 5 of 6 proteins examined in this study. Taking 95% as the criterion for polymorphism, the Hb locus was monomorphic for all populations. All the examined proteins followed codominant heredity. Many researchers have used different nomenclatures in biochemical polymorphism studies related to dogs. A diagram of the electropherograms or chromatograms of the 6 variable proteins used in this study is given in Figure 1.

The gene frequencies (X_i) for each polymorphic locus, the index of heterozygosity (h_o, h_{sj}) and the average heterozygosity with the standard error ($\bar{h}_s \pm S_{\bar{h}}$) of the dog breeds studied are given in Table 3. It can be seen from the examined dog breeds that the frequency of Alb^S is higher rationally compared to Alb^F. Tf^O and Tf^S were not found in this study. The Tf^F was recognized in only 1 German Shepherds and in 2 Doberman Pinschers. The ArE^O frequency in the Labrador and the Setter and the ArE^K frequency in the other breeds were found to be higher. The highest ArE^K frequency values were detected in the Pointer breed. ArE^D could not be detected in all examined breeds. The Poa–1^A frequency was high in all breeds. In the Labrador breed, the monomorphic structure was determined.

The polymorphism was 66.7% in the Labrador breed and 83.3% in the other breeds on the basis of all loci in all examined dog breeds. The rate of heterozygosity was 0.32 ± 0.15 (mean ± S.E.) and 0.41 ± 0.09 in the Doberman Pinscher and Kangal breeds, respectively. The

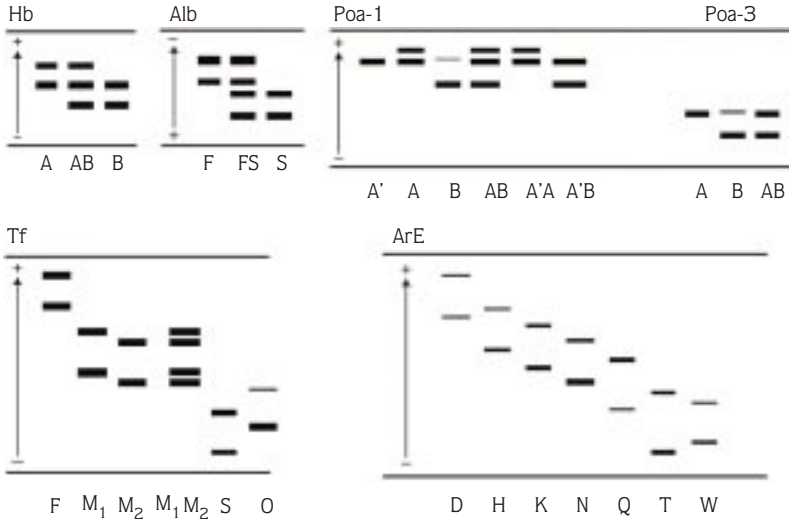

Figure 1. Diagram of the electropherograms or chromatograms of the 6 variable proteins.

Table 3. The gene frequence (X_i), heterozygosity indexes (h_o, h_{sj}), the average heterozygosity and standard errors ($\bar{h}_s \pm S_{\bar{h}}$) of examined dog breeds and loci.

Locus	Allel	Akbash n = 29			German Shepherd n = 63			Doberman Pinscher n = 26			Kangal n = 88			Labrador n = 30			Pointer n = 22			Setter n = 18		
		X_i	h_o	h_{sj}	X_i	h_o	h_{sj}	X_i	h_o	h_{sj}	X_i	h_o	h_{sj}	X_i	h_o	h_{sj}	X_i	h_o	h_{sj}	X_i	h_o	h_{sj}
Tf	F	0.00	0.00	0.48	0.01	0.02	0.45	0.04	0.08	0.38	0.00	0.00	0.39	0.00	0.00	0.50	0.00	0.00	0.47	0.00	0.00	
	M_1	0.38	0.41		0.32	0.44		0.77	0.31		0.74	0.30		0.43	0.60		0.36	0.36		0.50	0.78	0.51
	M_2	0.62	0.41		0.68	0.46		0.19	0.23		0.26	0.30		0.57	0.60		0.64	0.36		0.50	0.78	
Poa-1	A	0.79	0.41	0.34	0.92	0.16	0.15	0.90	0.19	0.18	0.73	0.49	0.42	1.00	0.00	0.00	0.91	0.18	0.17	0.58	0.72	
	B	0.21	0.41		0.07	0.14		0.10	0.19		0.19	0.38		0.00	0.00		0.09	0.18		0.39	0.78	0.52
	A'	0.00	0.00		0.01	0.02		0.00	0.00		0.08	0.16		0.00	0.00		0.00	0.00		0.03	0.06	
Poa-3	A	0.22	0.24	0.35	0.48	0.44	0.50	0.14	0.12	0.24	0.53	0.34	0.50	0.40	0.67	0.49	0.27	0.55	0.41	0.72	0.56	0.41
	B	0.78	0.24		0.52	0.44		0.87	0.12		0.47	0.34		0.60	0.67		0.73	0.55		0.28	0.56	
Alb	F	0.27	0.46	0.39	0.24	0.35	0.37	0.35	0.31	0.46	0.36	0.58	0.45	0.41	0.62	0.49	0.39	0.32	0.49	0.44	0.56	0.51
	S	0.73	0.46		0.76	0.35		0.65	0.31		0.65	0.58		0.59	0.62		0.61	0.32		0.56	0.56	
ArE	H	0.04	0.07	0.64	0.09	0.02	0.67	0.00	0.00	0.66	0.07	0.12	0.67	0.07	0.00	0.60	0.02	0.05	0.61	0.00	0.00	
	K	0.55	0.28		0.52	0.19		0.44	0.32		0.50	0.34		0.37	0.20		0.57	0.50		0.19	0.39	
	N	0.07	0.07		0.04	0.04		0.06	0.12		0.02	0.02		0.00	0.00		0.00	0.00		0.08	0.17	
	Q	0.22	0.38		0.20	0.10		0.38	0.44		0.24	0.31		0.52	0.23		0.23	0.46		0.72	0.22	0.45
	T	0.04	0.07		0.02	0.04		0.00	0.00		0.02	0.04		0.00	0.00		0.02	0.05		0.00	0.00	
	W	0.09	0.10		0.14	0.12		0.12	0.16		0.15	0.21		0.05	0.00		0.16	0.32		0.00	0.00	
$\bar{h}_s \pm S_{\bar{h}}$		0.37 ± 0.09			0.36 ± 0.10			0.32 ± 0.15			0.41 ± 0.09			0.35 ± 0.11			0.36 ± 0.09			0.40 ± 0.08		

differences in the mean heterozygosity values of each dog breed were statistically insignificant, based on the t–test ($P < 0.05$).

F-statistical analysis was performed without breed discrimination and the estimated *F*-values for each locus and from all over loci with the effective number of individual exchange between populations per generation are given in Table 4. $\tilde{F}_{IS_W}$, $\tilde{F}_{IT_W}$ and $\tilde{F}_{ST_W}$ values were estimated to be 0.085, 0.083 and 0.160, respectively, for whole loci in dog breeds and these values were significant ($P < 0.001$). Estimated exchange among populations per generation with the effective number of individuals was around 0.03.

From the values of the gene frequencies of the analyzed loci and by means of the application of several indices for genetic distance, dendograms of the dog breeds were obtained by cluster analysis. For the cluster analysis, the UPGMA algorithm was applied to the distance matrices obtained using Nei's index. The matrix of the genetic distances between every pair of breeds or populations computed from the gene frequencies is given in Table 5. The values of distance ranged from D = 0.013 for the Akbash-Pointer pair to D = 0.242 for the Setter-Doberman Pinscher pair. The average between-breed distance is D = 0.099. The Setter breed showed distance values with regard to the other breeds that are much higher than the average breed comparisons.

The dendrogram drawn from the matrix of the distances is shown in Figure 2. The formation of 2 large clusters was observed: The first group was Kangal and Doberman Pinscher, and the one formed by the rest of the breeds, except for the Setter, which separated from the hypothetical common trunk very early. The second group was Akbash, Pointer, German Shepherd and Labrador.

Discussion

In this study, polymorphism could not be found in the Hb locus. The reference sera for the Hb locus could not be found during this study. Therefore, these phenotypes were named HbB, since the Hb^A had been found in only Eskimo, Korean and Japanese dog breeds (30).

There are differences among the allele frequencies of the dog breeds in this study. In parallel to these results, previously reported allele frequencies in dogs were also different (10,11,14,20,31). Furthermore, the differences in allele frequencies were also different among different researchers (4). These differences in allele frequencies may arise from different breeding areas or mating in a non-randomized fashion or from gene flow from the other breeds.

Some breeds in the population showed disagreement with expected Hardy-Weinberg proportions for some loci. These changes in the Hardy-Weinberg proportions indicated that the mating was not random; therefore, the relationship or the similarities were increased in the population.

Table 4. *F*-statistic values and the effective number of individuals exchanged between populations per generation (N_m).

Locus	The measure of gene diversity ($G_{ST} = \tilde{F}_{ST}$)					N_m
	$\tilde{F}_{IS}$	$\tilde{F}_{IT}$	$\tilde{F}_{ST}$	χ^2	df	
Tf	0.094	0.062	0.150*	165.82	12	0.03
Poa-1	0.127	0.082	0.198*	218.59	12	0.04
Poa-3	0.079	0.076	0.149*	82.47	6	0.03
Alb	0.120	0.098	0.206*	113.19	6	0.04
ArE	-0.008	0.096	0.089*	230.99	30	0.02
Over all loci	$\tilde{F}_{IS_W} = 0.085$*	$\tilde{F}_{IT_W} = 0.083$*	$\tilde{F}_{ST_W} = 0.160$*	811.06	66	0.03

* $P < 0.001$; df: Degree of freedom.

Table 5. Genetic distances between every pair of the 7 dog breeds or population.

Breed/Population	Akbash	German Shepherd	Doberman Pinscher	Kangal	Labrador	Pointer	Setter
Akbash	*****						
German Shepherd	0.031	*****					
Doberman Pinscher	0.074	0.131	*****				
Kangal	0.093	0.081	0.069	*****			
Labrador	0.061	0.045	0.079	0.085	*****		
Pointer	0.013	0.025	0.077	0.089	0.035	*****	
Setter	0.213	0.174	0.242	0.129	0.118	0.211	*****

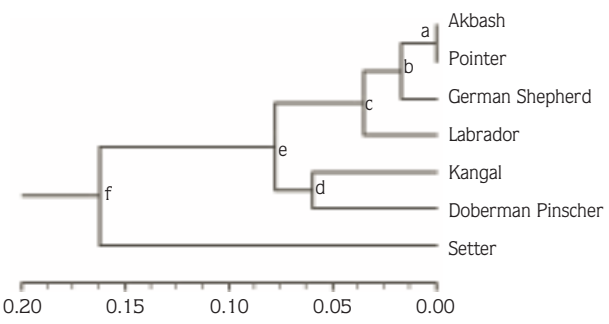

Figure 2. Dendrogram of the genetic distance matrix computed by the UPGMA method from Nei's distance (27).

Both the proportion of polymorphic loci and the average heterozygosity per locus over all loci values in the dog populations studied were much higher than those reported from most wild species. They were also higher than those values from other domesticated species (4). As far as domesticated animals are concerned, extensive mating between breeds founded separately is the most likely cause of high genetic variability. The high proportion of polymorphic loci and $\bar{h}_s$ values in domesticated dog populations seem not to be confined to this case, although other mechanism(s) may also work to raise the genetic variability in the populations. The calculated average heterozygosity values for the Akbash, Kangal and German Shepherd breeds were higher than those of dog breeds raised in Spain (13), Bangladesh and Japan (4,32), while they were similar to those given by Lachmann (14).

The significant differences among estimated average heterozygosity values could indicate that the breeds had genetic variations at the same level on the basis of the loci examined and the possibility of determining breeds on the basis of average heterozygosity values is very low.

The $\tilde{F}_{IS}$ values are positive in the Tf, Poa-1, Poa-3 and Alb loci, and negative in the ArE locus. The positive $\tilde{F}_{IS}$ value indicated that the homozygote genotype frequency was high in the Tf, Poa-1, Poa-3 and Alb loci, whereas the negative findings indicated that the frequency of the heterozygote genotype was high in the ArE locus.

The $\tilde{F}_{IT}$ values were positive in all loci and were significant ($P < 0.001$). This is advantageous as increases in the frequency of homozygote genotypes in a population at breed level allow possible selection factors on these loci to be detected.

The estimated $\tilde{F}_{ST}$ value in the Tf, Poa-1, Poa-3, Alb and ArE loci were 0.150, 0.198, 0.149, 0.206 and 0.089, respectively, and these values were significant ($P < 0.001$), which indicates differences in genetic structure according to their loci. The estimated inbreeding coefficients ($\tilde{F}_{IT_W} = 0.083$ and $\tilde{F}_{IS_W} = 0.085$) were higher in our dog breeds than that of the Gos d'Atura dog population ($F = 0.044$) reported by Jordana et al. (13). The values of $\tilde{F}_{IT_W}$ and $\tilde{F}_{IS_W}$ were significant; the frequency of homozygotes increases genotypically and this increase could be higher than that of expected values. This unexpected increase could be due to non-random mating in dogs. Thus, the results of this study showed that the diversity of the estimated gene ($G_{ST} = \tilde{F}_{IS_W} = 0.160$) was significant in Turkish dogs ($P < 0.001$). This suggested that all populations have different genetic structures. The effective number of individuals exchanged between populations per generation ($N_m = 0.03$) was very low over all loci.

The values of distance ranged from D = 0.013 for the Akbash-Pointer pair to D = 0.242 for the Setter-Doberman Pinscher pair. The average between-breed distance was D = 0.099. The Setter breed showed distance values much higher than those of the average breed. The formation of 2 large clusters was observed. The first group comprised Kangal and Doberman Pinscher, and the one formed by the rest of the breeds, except for the Setter, which separated from the hypothetical common trunk very early. The second group comprised Akbash, Pointer, German Shepherd and Labrador. These values were similar to those of Gos d'Atura dog breeds (33), while they were higher than those of Spanish dog breeds (13).

Dendrograms on the basis of the genetic distances from gene frequencies have been widely used to illustrate the genetic relationships among animal populations. In fact, this procedure was also effective in dogs for classifying most breeds and populations into groups. They were convincing from the viewpoint of the geographical relation among the areas of their distribution or origin. However, some of the results contradicted this general rule and seem to be somewhat enigmatic.

F-statistics seem to be a very useful tool for the study of the phylogeny of animals, especially for domesticated animals in which mating between separated populations occurs frequently.

In conclusion, the formation of 2 distinct clusters in Kangal and Akbash Shepherd dogs reveals that these breeds have different genetic structures in terms of the investigated loci and they are not closely related to each other. Therefore, in order to determine the genetic structure and the degree of relativity, further research should be conducted at the molecular level.

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