ANALYSIS OF GENETIC DIVERSITY AND DISTANCES OF SOME DOG (*Canis lupus familiaris*) BREEDS BASED ON DNA BARCODES

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ABSTRACT

DNA barcodes (*i.e.* cytochrome c oxidase subunit I or COI in the mitochondrial genome) obtained from 7 dog breeds sampled in the Philippines and 8 dog breeds in the United States retrieved from GenBank were analyzed using Neighbour-Joining method based on Kimura 2-parameter model in MEGA5. Based on 671 COI positions, overall genetic diversity of dog breeds was 2.9%. Average genetic distance was higher among dog breeds sampled in the Philippines (d=0.033) than among GenBank-derived samples (d=0.001). Average pair-wise distances between dog breeds sampled locally and those accessed from GenBank was 0.043 unit. Our results indicate that DNA barcodes can be effective in differentiating between breeds sampled in the Philippines, but not among dog breeds whose COI sequences where derived from GenBank. No genetic basis for grouping breeds based on their functional type is suggested. Dogs of the same breed from the two countries do not have the same COI sequences. Genetic distances between dog breeds from the United States were too small to identify introgressions and conclusively determine their origins and diversification. More COI sequences should thus be determined from distinct breeds and mixed-breed populations to improve reliability of using DNA barcodes to confirm breed origin of a dog.

Key words: DNA barcodes, dog breeds, evolutionary analysis, genetic diversity

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INTRODUCTION

About 400 breeds and varieties of dogs worldwide have been described and classified as traditional breeds with long histories as registered breeds, rare breeds with their own registries, or new breeds that may still be under development. Breeds are also categorized by the functional type from which the breed was developed. Examples are companion dogs, guard dogs, hunting dogs, herding dogs, and working dogs although there are many other types and subtypes (*e.g.*, hound, terrier, toy, and non-sporting).

Genetic, morphological and behavioural data indicate that the domestic dog originated from the wolf, although there is yet no consensus concerning in which geographical region the domestication of wolves occurred (Wayne, 1993; Clutton-Brock, 1995). Based on mitochondrial DNA studies, Vilà *et al*. (1997) suggested that dogs may have been domesticated from wolves on different occasions and at different places while Savolainen *et al*. (2002) specified a common origin from a single East Asian gene pool for all dog populations. Based on autosomal single nucleotide polymorphisms (SNPs), vonHoldt *et al*. (2010) pointed to the Middle East as the source of most genetic diversity in the domestic dog and a more likely origin of domestication events. On the other hand, Ding *et al*. (2011) using Y-chromosome data alluded to a single domestication region in the southern part of East Asia specifically south of the Yangtze River. More recently, Wayne and vonHoldt (2012) showed that the nuclear genome of dogs derives primarily from Middle Eastern or European wolves, a result more consistent with the archaeological record.

DNA typing methodologies have also been recommended to analyze forensic biological evidence for including or excluding an individual animal as a possible source of evidence in a criminal investigation (Himmelberger *et al*., 2008). Information from the HV1 region in the mitochondrial DNA for example, may now replace forensic examinations of animal hairs that have been limited to morphological studies. The microscopic analyses of hair rarely tell more than species, as hair can vary both between individuals of the same species as well as within an individual. In the United States, Webb and Allard (2009) showed that dog breeds were found to have similar sequences of the mitochondrial DNA control region, although not identical. While a single database comprised of purebred and mixed breed dogs is deemed sufficient for the continental United States, no genetic basis was found for grouping dogs by either purebred or mixed or geographic location.

DNA barcodes (*i.e.* cytochrome c oxidase subunit I or COI gene of the mitochondrial genome) were used to analyze the evolutionary relationships, genetic diversity and distances among dog breeds sampled in the Philippines and dog breeds from the United States whose COI sequences were retrieved from GenBank. Initially proposed as a standard

for rapid and inexpensive species identification method that is accessible to non-specialists (Hebert *et al*., 2003), this mitochondrial gene is short enough to be readily amplified and sequenced with broad-range primers, providing a reliable sequence read through a single pass in conventional cycle-sequencing platforms. Potential applications of the unique identifications based on DNA barcodes to forensic examinations and canine registry programs are also given.

MATERIALS AND METHODS

The taxonomic classification of domesticated dog is as follows: Kingdom- Animalia, Phylum- Chordata, Class- Mammalia, Order- Carnivora, Family- Canidae, Genus- *Canis*, Species/subspecies- *C. lupus familiaris*.

Field sampling

Materials used for the present study were obtained from local private owners of registered purebred dogs who provided authoritative animal records and identifications. One specimen representing a registered dog breed was examined to ascertain COI sequence divergences within the domesticated dog species. Close relatives of the same breed were expected to have the same COI sequences. In addition, COI sequences of 8 other dog breeds from the United States were derived from the whole mitochondrial genome deposited by Webb and Allard (2009) in GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The classification of 7 dog breeds sampled in the Philippines and 8 dog breeds in the United States retrieved from GenBank based on their functional type, location of sampling and GenBank Accession Numbers are shown in Table 1.

Laboratory analysis

Most analytic methods followed those described by Hebert *et al*. (2004). DNA sources for this study included blood samples extracted from live specimens without harming them using gauge 20 or 22 hypodermic needle on the femoral vein, in accordance with institutional, local and national guidelines regarding animal care and use in experimentation. Fresh blood samples were placed in NucleoSave blood storage cards (Machery-Nagel, USA) and allowed to dry for three days under room temperature.

Laboratory protocols for DNA extraction, purification, elution, and amplification for mammalian specimens were developed at the Animal Biotechnology Laboratory, Animal and Dairy Sciences Cluster, College of Agriculture, University of the Philippines Los Baños.

Table 1. Classification of domestic dog breeds used in the phylogenetic analysis.

Name of breed	Functional type	Place of sampling (Philippines)	GenBank accession number	
			From the Philippines	From the USA
Chihuahua*	Toy	Bay, Laguna	JX280484	EU408262
Dachshund*	Hound	Lipa City, Batangas	JX280487	EU408272
Rough Collie	Working	Lipa City, Batangas	JX280485	-
Dalmatian	Non-sporting	Bay, Laguna	JX280486	-
Toy Poodle	Non-sporting	Quezon City, MM	JX280489	-
French Bulldog*	Non-sporting	Quezon City, MM	JX280488	EU408275
Shih Tzu*	Non-sporting	Quezon City, MM	JX280490	EU408302
Great Dane *	Non-sporting	-	-	EU408276
German Shepherd*	Working	-	-	EU408277
Pit Bull Terrier*	Terrier	-	-	EU408293
Pug*	Toy	-	-	EU408294

* Dog breeds from the United States whose COI sequences were derived from the whole mitochondrial genome deposited in GenBank by Webb and Allard (2009).

DNA extraction. Using a Harris 1.2 mm micropunch, at least 30 discs from each dried NucleoSave card or sample were collected and placed in labelled microcentrifuge tubes.

DNA purification. Sample discs were washed with 200 µl of FTA Purification Reagent (Whatman Inc., USA) for four to five times and rinsed with 200 µl sterile molecular biology grade water. Sample discs were then dried in a laminar hood overnight.

DNA elution. Six dried sample discs were transferred in a sterile PCR tube and added with 55 µl sterile nanopure water. DNA was eluted by incubation at high temperature specifically at 90^{o}C for 10 min using Veriti 96 Well Thermal Cycler (Applied Biosystems). Eluted DNA was stored at -20^{o}C for further use.

DNA amplification. The COI gene was amplified using primers LCO1490 (5' GGTCAACAAATCATAAAGATATTGG 3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA 3') from Hebert *et al*. (2004). The 20-µl PCR reaction mix included 13.44 µl sterile ultrapure water, 2.0 µl of 10X buffer, 1.0 µl of MgCl2, 0.8 units of Taq DNA polymerase, 0.4 µl (0.2 mM) of each forward and reverse primers and 2.0 µl of DNA template. The optimized PCR amplification program was composed of three min at 94°C followed by five cycles of 40 sec at 94°C, 30 sec at 52°C and 45 sec at 72°C, followed by another 30 cycles of 40 sec at 94°C, 30 sec at 54°C, and 45 sec at 72°C, and finally seven min at 72°C.

PCR products were visualized in a 1.0% agarose gel with ethidium bromide. Post stained gels are viewed using Molecular Imager® Gel DocTM XR System (Bio-Rad, USA). PCR products were purified using

GF-1 PCR Clean Up Kit (Vivantis, Malaysia). In cases where multiple bands occurred (*e.g.*, pseudogenes or short DNA sequences less than 200 bp), gels were excised and purified using GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia). The DNA amplification regime was repeated four times for each sample specimen. The final PCR product for each sample specimen (about 30 to 50 µl final volume) was obtained from pooled amplicons of all four PCR reactions (replicates).

DNA sequencing. PCR products were sent to Macrogen Inc., Seoul, Korea for unidirectional sequencing using appropriate forward primer and analyzed using 3730L DNA analyzer (AB, USA) and BigDye (AB, USA).

COI sequence analysis

Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The COI sequences were aligned using ClustalW (Thompson *et al*., 1994), (http://www.ebi.ac.uk/clustalw/). The evolutionary distance between a pair of sequences was measured by the number of nucleotide substitutions (*i.e.* transition and/or transversion) or differences occurring between them. The evolutionary divergence over COI sequence pairs were estimated between two domestic dog groups, *i.e.* dog breeds sampled in the Philippines and dog breeds from the United States whose COI sequences were derived from GenBank.

Diversity analysis. Diversity analysis involved the calculation of sequence divergence using the Kimura 2-parameter or K2P model (Kimura, 1980) which corrected for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the nucleotide frequencies were the same and that the rates of substitution do not vary among sites. Standard error estimates were obtained by a bootstrap procedure (1000 replicates) according to Nei and Kumar (2000).

Distance analysis. To estimate genetic distances among different dog breeds, the evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) with their variances estimated by a bootstrap approach. The average distance between sequence pairs were in the units of number of base substitutions per site (*i.e.* d units). All positions containing gaps and missing data were eliminated. Within (or between) group mean distance was estimated as the average evolutionary divergence over sequence pairs within (or between) groups.

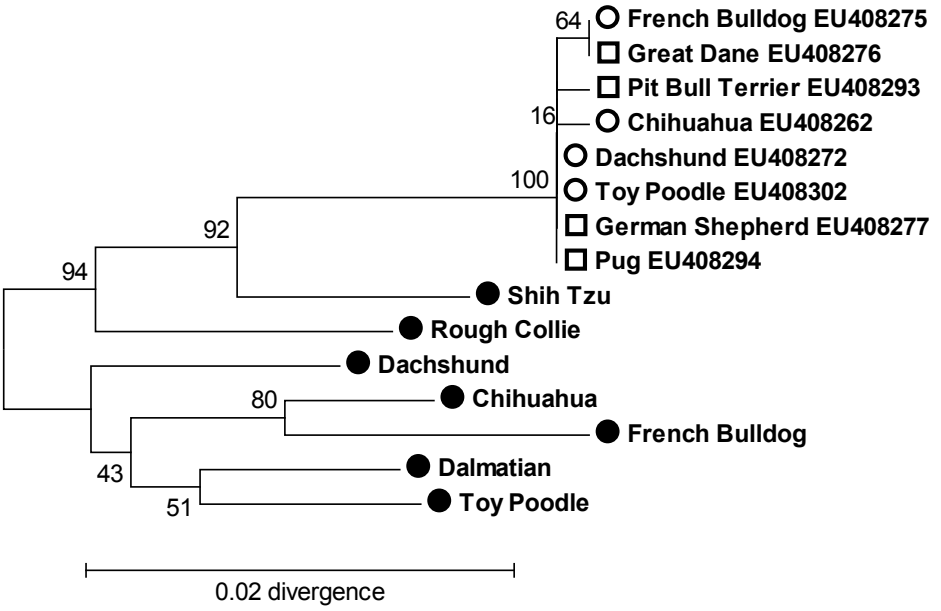
Phylogeny analysis. The Neighbour-Joining (NJ) method was used to infer the evolutionary history (Saitou and Nei, 1987). The nearest-neighbour distance, the minimum genetic distance between a dog breed and its closest relative were examined to test the discriminatory power of

COI barcodes. A bootstrap consensus NJ tree of K2P distances was inferred from 1000 replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

The rooted Neighbour-Joining tree representing DNA barcodes (Figure 1) showed that dog breeds diverged into two distinct evolutionary clades. One clade included local samples of Rough Collie and Shih Tzu, and all COI sequences retrieved from GenBank. Historically, the Rough Collie and Shih Tzu breeds originated from Scotland (early 19th century) and China (early 20th century), respectively. The second clade consisted of all other dog breeds sampled in the Philippines. The breeds in the latter group are widely known to be developed from other countries, i.e. Chihuahua (Mexico – 100 A.D.), Dachshund (Germany – early 18th century), Dalmatian (Croatia – late 19th century), French Bulldog (France – mid 19th century), and Toy Poodle (England – late 18th century). The

Figure 1. Neighbour-Joining tree with bootstrap support showing the evolutionary relationships of 7 dog breeds sampled in the Philippines (■) and 8 dog breeds from the United States whose COI sequences were derived from Genbank (○ or □ with GenBank accession numbers), (N=15 COI sequences; 671 positions)



divergence of COI sequences may be related to the findings of Savolainen *et al*. (2002) who reported three phylogenetic groups which suggested several maternal origins of the domestic dog from wolves, but a common origin from a single gene pool for all dog populations from East Asia about 15,000 years ago. Their work was based on the analysis of portions of mitochondrial DNA of 654 domestic dogs representing all major dog populations worldwide. In another analysis of mitochondrial DNA from 102 Swedish dogs of 52 different breeds, Savolainen *et al*. (1997) found no general correlation between dog breed and sequence variants.

Based on 671 COI positions, the overall genetic diversity of dog breeds was about 2.9% (Table 2). Coefficient of differentiation which estimates the proportion of interpopulational diversity out of all dog samples was 42.03%. Average pair-wise genetic distance was higher among dog breeds sampled in the Philippines (d = 0.033 ± 0.005) than among GenBank-derived samples (d = 0.001 ± 0.001), implying greater diversity in COI sequences of dog breeds found in the Philippines (Table 3).

Table 2. Mean diversity between breeds and within domestic dog groups of the Canidae family

Diversity measures	No. of nucleotide sequences	N positions	Diversity (%)	
			Mean	Standard Error
Within population	15	671	1.73	0.27
Interpopulation			1.25	0.25
Entire population			2.98	0.47
Coefficient of differentiation			42.03	4.34

Following Hebert *et al*. (2003), a genetic diversity within the taxa of 2% may justify the effectiveness of COI barcodes as an identification tool to discriminate among members of the taxa. Similarly, a genetic distance value (d) less than 0.020 is considered low. DNA barcodes will therefore be effective in differentiating between dog breeds sampled in the Philippines, but not among dog breeds whose COI sequences where derived from GenBank. It is also noted that diversity of COI sequences from dog breeds reported in this study was higher than those reported by Bondoc (2012) where the analysis of DNA barcodes from different livestock species and breeds was based on 513 COI positions only.

Table 3. Pair-wise distances (d units) between dog breeds sampled in the Philippines and other dog breeds derived from GenBank

		Dog breeds sampled in the Philippines					
		1	2	3	4	5	6
Dog breeds sampled in the Philippines	2	0.034					
	3	0.026	0.035				
	4	0.023	0.037	0.023			
	5	0.021	0.041	0.026	0.035		
	6	0.045	0.034	0.035	0.035	0.049	
	7	0.034	0.037	0.020	0.035	0.038	0.035

		Dog breeds with COI retrieved from GenBank						
		8	9	10	11	12	13	14
Dog breeds with COI retrieved from GenBank	9	0.001						
	10	0.003	0.001					
	11	0.001	0.000	0.001				
	12	0.001	0.000	0.001	0.000			
	13	0.003	0.001	0.000	0.001	0.001		
	14	0.003	0.001	0.003	0.001	0.001	0.003	
	15	0.001	0.000	0.001	0.000	0.000	0.001	0.001

		Dog breeds sampled in the Philippines						
		1	2	3	4	5	6	7
Dog breeds with COI retrieved from GenBank	8	0.048	0.037	0.048	0.043	0.056	0.027	0.046
	9	0.046	0.035	0.046	0.041	0.054	0.026	0.045
	10	0.048	0.037	0.048	0.043	0.056	0.027	0.046
	11	0.046	0.035	0.046	0.041	0.054	0.026	0.045
	12	0.046	0.035	0.046	0.041	0.054	0.026	0.045
	13	0.048	0.037	0.048	0.043	0.056	0.027	0.046
	14	0.048	0.037	0.048	0.043	0.056	0.027	0.046
	15	0.046	0.035	0.046	0.041	0.054	0.026	0.045

Legend: 1= Chihuahua, 2= Rough Collie, 3= Dalmatian, 4= Dachshund, 5= French Bulldog, 6= Shih Tzu, 7= Toy Poodle, 8= Chihuahua EU408262, 9= Dachshund EU408272, 10= French Bulldog EU408275, 11= Toy Poodle EU408302, 12= German Shepherd EU408277, 13= Great Dane EU408276, 14= Pit Bull Terrier EU408293, 15= Pug EU408294.

Comparisons of COI sequences between dog breeds (within dog groups)

Comparisons between dog breeds sampled in the Philippines

All new DNA barcodes from 7 dog breeds sampled in the Philippines were different. Table 3 shows that the genetic distances between dog breeds sampled in the Philippines ranged from d = 0.020 (*i.e.* between Dalmatian and Toy Poodle) to 0.049 (*i.e.* between French Bulldog and Shih Tzu). The apparently close genetic distances in general may be attributed to greater and repeated gene flow and extensive transportation routes among dog breeds as the "man's best friend" has often accompanied human emigrations. It may also imply recently diverged sister breeds where COI has not yet accumulated sequence differences.

Among the sampled dog breeds in the Philippines, Chihuahua had the closest genetic distance which separates it from French Bulldog (d = 0.021), Dachshund (d = 0.023), and Dalmatian (d = 0.026). Both French Bulldog and Dalmatian are considered as non-sporting dog breeds while Chihuahua is an example of a toy and hound breed, respectively. On the other hand, the non-sporting Shih Tzu was most distantly associated with the toy breed Chihuahua (d = 0.045) and French Bulldog (d = 0.049), also a non-sporting breed. Our results found no genetic basis for grouping dog breeds based on their functional type.

Comparisons between dog breeds whose COI sequences were derived from GenBank

Average genetic distances among purebred dog breeds from the United States whose COI sequences were derived from GenBank was small, ranging from d = 0.000 to 0.003. The genetic distances were too small to identify introgressions and conclusively determine their origins and diversification. The GenBank-derived DNA barcodes were taken from dog breeds sampled from the United States included the Chihuahua, Dachshund, French Bulldog, and Shih Tzu and other popular dog breeds historically known to be developed from different countries such as Great Dane (Denmark – mid 18th century), German Shepherd (Germany – end of 19th century), Pit Bull Terrier (England – end of 19th century), and Pug (China – mid 5th century).

Comparisons between dog breeds sampled locally and those retrieved from GenBank

The average pair-wise distances between dog breeds sampled in the Philippines and those accessed from GenBank was 0.043 ± 0.007 unit, ranging from d = 0.026 to 0.056. Slightly wider genetic distances were found for some dog breeds that were sampled in the Philippines and those found in the United States. Comparisons of COI sequences of

purebred dogs obtained from the two countries revealed genetic distances of 0.041, 0.045, 0.048, and 0.056 units for Dachshund, Toy Poodle, Chihuahua, and French Bulldog, respectively. This implies that (unrelated) dogs of the same breed do not have the same COI sequences and that DNA barcodes may be used to distinguish a purebred dog such as Dachshund, Toy Poodle, Chihuahua, and French Bulldog sampled in the Philippines from its counterpart breed found in the United States.

CONCLUSION

The COI sequences generated in this study provide further evidence to the effectiveness of DNA barcoding to analyze genetic diversity and relationships among dog breeds. Our results show that DNA barcodes may assign dog specimens to their correct breed or source provided that a reference data set has been defined for various breeds and sources. More COI sequences should therefore be determined from distinct breeds and mixed-breed populations to improve the reliability of using DNA barcodes to confirm the breed origin of a dog. It may be difficult to distinguish the DNA barcodes of crossbred (mixed) dogs or mongrels from their dam's breed because mitochondrial DNA is maternally inherited, but this could be clarified with an expanded DNA barcode reference collection. Other genes aside from COI may also be used to supplement the mitochondrial DNA COI sequences to clarify more conclusively the genetic diversity and relationships of various dog breeds.

Recommended applications of DNA barcodes in molecular traceability tests include: (1) to complement the inherent limitations of morphology-based systems and combine with phenotypic performance characteristics and history of dog populations to quickly assess local biodiversity of dogs, and (2) to protect dogs and promote and encourage the love for them. For example, DNA barcodes that are easily recovered from blood, tissue, hair and buccal swab samples from dogs can be used as forensic biological evidence in a criminal investigation to protect owners of these valuable pets from theft. DNA barcoding may also promote the integrity of a purebred dog registry and could provide insights into evolutionary processes that contributed to the development of the breed. DNA barcodes are thus suggested to be used as a major source of information, in addition to conventional identification and cataloguing methods, to verify, tag and certify their classification as distinct pure (or mixed) breed in local canine registries and stud books.

Moreover, based on the higher inter-species than intra-species variability of COI sequences found among livestock breeds sampled in the Philippines (Bondoc, 2012), DNA barcodes could be used to detect the presence of dog meat (and even identify its breed) in a livestock food specimen. This may be useful to prosecute violators of Republic Act 8485

or Animal Welfare Act of 1998, which among others aim to protect dogs and prohibit commercial trade of dog meat.

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