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DNA Polymorphism within *LDH-A* Gene in Pigeon (*Columba livia*)

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Pigeons (*Columba livia*) have long history of selective breeding for many purposes; one of them is pigeon racing using their homing ability. A total of 221 pigeon samples were sequenced for lactate dehydrogenase-A gene (*LDH-A*) including part of exon 5 and part of exon 6 and intervening intron 5. Six polymorphic sites were identified in intron 5; one indel and five SNPs. Statistical significant differences in allele frequencies were observed for 595bp and 600bp alleles between homing and non-homing groups in both Japanese and Egyptian pigeons. The frequency of 600bp allele was higher in both Japanese and Egyptian homing than in non-homing pigeons ($P<0.0001$). In Japanese pigeons; significant difference in allele frequency of three SNPs was observed between homing and non-homing groups, while in Egyptian pigeons, although similar tendency was observed, the difference in allele frequency was not significant. The DNA polymorphisms of pigeon *LDH-A* gene can be a potential genetic marker for homing ability in racing pigeon breeding.

Key words: DNA polymorphism, *LDH-A* gene, pigeon

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Introduction

Since the domestication of the wild rock pigeon (*Columba livia*) several hundred different domestic pigeon breeds have been established, most breeds are kept exclusively because of their appearance, flying capabilities or for the sport of pigeon racing (Traxler *et al*., 2000). The homing pigeon is a variety of domestic pigeon derived from the wild Rock Pigeon (*Columba livia domestica*) selectively bred to find its way home over extremely long distance (Levi, 1977). The wild rock pigeon has an innate homing ability meaning that it will generally return to its own nest and its own mate. This made it relatively easy to breed from the birds that repeatedly found their way home over long distances (Blechman, 2007). During pigeon racing, the competing birds are taken from their lofts to the racing start gate and then must race home. The time taken and distance are recorded and the fastest bird is declared the winner. Races are generally between 100 and 1800 km in distance (Walcott, 1996).

The L-lactate dehydrogenase (LDH, EC1.1.1.27) isozymes are encoded by three different genes: *LDH-A* (muscle), *LDH-B* (heart) and *LDH-C* (testis). The expression of these genes is developmentally regulated and tissue specific (Mannen *et al*., 1997). The *LDH* gene family is involved in aerobic and anaerobic metabolism; therefore it determines muscle endurance, recovery and aerobic capacity (Li, 1998). Skeletal muscle not only plays an important role in lactate production, but also in lactate removal from the circulation. Lactate is not an end product of skeletal muscle carbohydrate metabolism but instead seems to be an important fuel source for mitochondrial respiration in skeletal muscle. *LDH- A* catalyses the interconversion of pyruvate and lactate with nicotinamide adenine dinucleotide as a coenzyme (Van Hall *et al*., 1999; Van Hall, 2000). In human, mutation of *LDH-A* exon 6 greatly affects muscle causing painful muscle stiffness, cramps and easy fatigability after strenuous exercise (Kanno *et al*., 1988). In pigeon, two SNPs of *LDH-A* gene have been identified in intron 6 and showed significant difference for allele's frequencies between homing and non-homing groups. (Dybus *et al*., 2006).

Introns are non-coding sequences in a gene that are transcribed but spliced out of the precursor mRNA (Berget *et al*., 1977). Generally speaking, introns have little functional significance, although in some cases, introns polymorphism may influence the level of gene expression and consequently affect the phenotype (Wang *et al*., 2002; Kersting *et al*., 2008; Hejjas *et al*., 2009). Moreover, variations in introns have potential usefulness as genetic markers; it is thus possible to identify genotypes of certain interest to breeders prior

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to obtaining information on the performance (Dybus and Kmiec, 2002). Lessa (1992) introduced intron-targeted PCR, in which a non-coding intron was amplified using primers designed from highly conserved exon sequences; this approach is called Exon-Primed Intron-Crossing (EPIC)-PCR. This technique has been shown to yield substantial variability, mainly from intron length polymorphism, and was successfully used in several population genetic surveys (Hassan *et al*., 2003). In this study we reported the detection of DNA polymorphisms in pigeon's *LDH-A* gene intron 5 and analyzed the genotypes/alleles frequency in homing pigeons and a collection of other breeds summarized as non-homing pigeons.

Materials and Methods

Sample Collection and DNA Extraction

A total of 221 (123+36 for Japanese and 31+31 for Egyptian) feather and blood samples of two different groups of pigeons; 123 samples of Japanese racing pigeons representing Japanese homing group and 36 samples of free living wild rock dove, for simplicity termed non-homing group were genotyped. To avoid individuals with consanguinity and over-representation of popular sires and/or dams within the pedigree, a set (n=40) of Japanese racing pigeons (where the selected birds are expected independent each other for five generations) was selected from the genotyped racing pigeons (n=123). Japanese racing pigeon samples were collected from five different breeders, Chiba, Japan, whereas, Japanese wild Rock dove was collected from Rescue Center of Kyoto Municipal Zoo, Kyoto, Japan

Replication Samples: To validate the finding, we genotyped a total of 62 pigeon feather samples from two groups. Thirty-one samples were collected from nine Egyptian local breeds bred mainly for flying game (Ramadan *et al*., 2011) and racing purposes: Zagel (n=7), Safi (n=4), Rehani (n=3), Ablaq (n=3), Otatti (n=4), Morasla (n=4), Keshk (n=4), Messawed (n=1), and Karakandy (n=1) representing Egyptian homing group. Another thirty-one samples were collected from three Egyptian local breeds for ornamental and fancy purposes as Nemthawy (n=24) and Egyptian exhibition Tumbler (n=5) and for meat production in form of squabs as Romani (n=2), where these breeds termed for simplicity non-homing group. Egyptian samples were collected from seven breeders in four provinces (Cairo, Giza, Kaliobia and Zagazig) located in the Nile river delta in the northern part of Egypt. DNA was extracted from feather and blood samples using the QIAGEN DNeasy Tissue Kit (QIAGEN, Valencia, CA, USA).

Genotyping and Data Analysis

The direct sequence method was used for detecting polymorphism in the *LDH-A* gene. The cDNA sequence of the *LDH-A* gene of *Columba livia* was already known (Mannen *et al*., 1997; GenBank L76362). From this sequence, PCR primers were designed in exons by using Primer3 software to amplify part of exon 5 and 6 with an intervening intron. These primers were: LDH-A56F 5′-CCTGAAGGCTCTTC-ATCCAG-3′ and LDH-A56R 5′-TTGGGTGCACTCTTCT-CAAA-3′. The PCR was performed on a 15 μl reaction mixes including 20 ng of genomic DNA, 2x PCR buffer, each dNTP at 400 μM, each primer at 0.3 μM and 0.5 U of *LA-Taq* DNA polymerase (TaKaRa, Shiga, Japan). After an initial incubation at 95℃ for 2 min, PCR amplification was performed for 35 cycles consisting of 95℃ for 30 sec, 55℃ for 45 sec, 74℃ for 30 sec, followed by a final extension of 74℃ for 10 min. The amplified products were purified using PCR purification kit (Roche, Mannheim, Germany) and the resultant products were sequenced by using the same primers and the Big Dye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the standard protocol, and electrophoresed on an ABI PRISM 3130xl sequencer (Applied Biosystems). BLAST software (http://www.ncbi.nlm.nih.gov/) was used for sequence identification and confirmation. The MEGA 5 (Kumar *et al*., 2008), Finch TV 1.4.0 (http://www.geospiza.com/finchtv/) and Bioedit 7.0.5.3 (Hall, 1999) Softwares were used for sequences alignment and polymorphism detection. Genotypes and alleles frequencies in homing and non-homing pigeons were statistically tested by Fisher's Exact Test.

Results

Six polymorphic sites have been identified in *LDH-A* gene intron 5: one indel and five SNPs (Tables 1 and 2). The indel polymorphism was either 5 bp or 10 bp insertion/deletion resulting in three different allele's length; 605 bp, 600 bp and 595 bp (GenBank: AB744076, AB744077 and AB744078). The 605 bp allele mutation appeared with low allele and genotype frequencies in the all studied pigeon groups and was excluded from statistical analysis. High statistical significant differences in allele and genotype frequencies were observed in four (Indel, T182C, G249A, T297G) out of the six loci between Japanese homing and non-homing pigeons, whereas only one locus (Indel) showed significant differences in allele and genotype frequencies between Egyptian homing and non-homing pigeons (Tables 1 and 2). The frequency of 600 bp allele was higher in homing (nearly two-fold in Japanese and more than three-fold in Egyptian) than in non-homing pigeons (P<0.0001). On contrast, the *T* allele of T182C, *G* allele of G249A and *T* allele of T297G loci showed significant higher frequencies in Japanese non-homing than in homing pigeons, while it recorded an insignificant trend towards a higher frequency in Egyptian non-homing pigeons (Table 1).

The frequency of 600/600 bp genotype was higher in homing (both Japanese and Egyptian) than in non-homing pigeons (P<0.0001). The *T/T* of T182C, *G/G* of G249A and *T/T* of T297G loci showed significant higher frequency in Japanese non-homing than in homing pigeons, while it recorded a non-significant trend towards a higher frequency in Egyptian non-homing pigeons (Table 2). When all the genotyped samples of Japanese racing pigeons (n=123) were considered, the four loci (Indel, T182C, G249A, T297G) showed the same previous trend, (data not shown). The two loci (C442T and A443G) showed no significant differences in allele or genotype frequencies in both Japanese

Table 1. **Allele frequencies of Japanese and Egyptian homing and non-homing pigeons**

Polymorphism	Allele	Japanese			Egyptian		
		homing (n=40)	non-homing (n=36)	*P value*	homing (n=31)	non-homing (n=31)	*P value*
Indel	*595*	0.213	0.583	1.30E-06[a]	0.435	0.823	2.58E-05
	600	0.712	0.334		0.533	0.177	
	605	0.075	0.083		0.032	0.000	
T182C	*T*	0.575	0.847	0.0003	0.855	0.887	N.S.[b]
	C	0.425	0.153		0.145	0.113	
G249A	*G*	0.775	0.972	0.0002	0.919	0.984	N.S.
	A	0.225	0.028		0.081	0.016	
T297G	*T*	0.775	0.944	0.005	0.903	0.952	N.S.
	G	0.225	0.056		0.097	0.048	
C442T	*C*	0.888	0.875	N.S.	0.952	0.984	N.S.
	T	0.112	0.125		0.048	0.016	
A443G	*A*	0.888	0.819	N.S.	0.952	0.984	N.S.
	G	0.112	0.181		0.048	0.016	

Positions of the SNPs were estimated according to 605 bp allele (Gen Bank: AB744076).
[a] 605 bp allele was excluded from statistical analysis.
[b] N.S. means not significant.

Table 2. **Genotype frequencies of Japanese and Egyptian homing and non-homing pigeons**

Polymorphism	Genotype	Japanese			Egyptian		
		homing (n=40)	non-homing (n=36)	*P value*	homing (n=31)	non-homing (n=31)	*P value*
Indel	*595/595*	0.050	0.417	2.99E-05[a]	0.194	0.710	0.0001
	595/600	0.325	0.333		0.484	0.226	
	600/600	0.475	0.111		0.290	0.064	
	600/605	0.150	0.111		0.000	0.000	
	605/605	0.000	0.028		0.032	0.000	
T182C	*T/T*	0.325	0.750	0.001	0.742	0.806	N.S.
	C/T	0.500	0.194		0.226	0.162	
	C/C	0.175	0.056		0.032	0.032	
G249A	*G/G*	0.550	0.944	7.68E-05	0.839	0.968	N.S.
	G/A	0.450	0.056		0.161	0.032	
	A/A	0.000	0.000		0.000	0.000	
T297G[b]	*T/T*	0.550	0.917	0.0001	0.839	0.936	N.S.
	G/T	0.450	0.056		0.129	0.032	
	G/G	0.000	0.027		0.032	0.032	
C442T[c]	*C/C*	0.775	0.778	N.S.	0.934	0.968	N.S.
	C/T	0.225	0.194		0.033	0.032	
	T/T	0.000	0.028		0.033	0.000	
A443G[d]	*A/A*	0.775	0.750	N.S.	0.934	0.968	N.S.
	A/G	0.225	0.139		0.033	0.032	
	G/G	0.000	0.111		0.033	0.000	

[a] 600/605 and 605/605genotypes were excluded from statistical analysis.
All the loci follow HWE except the following:
[b] T297G locus deviated from HWE in Japanese (P=0.022) and Egyptian (P=0.001) non-homing groups.
[c] C442T locus deviated from HWE in Egyptian homing group (P=0.002).
[d] A443G locus deviated from HWE in Japanese non-homing (P=0.006) and Egyptian homing (P=0.002) groups.

and Egyptian pigeons.

Discussion

SNP and EPIC have gained widespread use as effective markers in studying the genetic polymorphism of various animal populations (Vignal *et al*., 2002; Hassan *et al*., 2003). In this study, we investigated the DNA polymorphism within *LDH-A* gene intron 5 between homing and non-homing pigeons. During strong exercises, when oxygen is absent or in short supply and the rate of demand for energy is high; *LDH-A* enzyme converts pyruvate, the final product of glycolysis, to lactate with nicotinamide adenine dinucleotide as a co-enzyme (Van Hall *et al*., 1999; Van Hall, 2000). Lactate is considered as an important fuel source of energy for muscular activity, and homing pigeons have been strongly selected for rapid return from distant released places in their breeding history.

In this study, the allele and genotype frequencies of six polymorphic loci in *LDH-A* gene were compared between homing and non-homing pigeons in both Japanese and Egyptian populations. The frequency of 600bp allele was higher in both Japanese and Egyptian homing than in non-homing pigeons ($P<0.0001$). For our interest, this result is also corresponding to the previous knowledge studied by Dybus *et al*. (2006) using Polish and Chinese homing pigeons. The allele frequency difference of the *LDH-A* locus may reflect the history of the selection for speed and endurance of homing pigeons. Further studies like functional genomics and linkage analysis are necessary for confirming the relationship between homing ability and these genetic variants.

These findings has a potential not only to empower racing pigeon breeders, owners and trainers to make decisions that will maximize a pigeon's genetic ability, but also to understand genetic background of stamina for flying ability for migrating bird species.

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