THE INHERITANCE OF SERUM ALKALINE PHOSPHATASE

IN THE PIGEON (*COLUMBA LIVIA*)

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THESIS

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CHAPTER I

INTRODUCTION AND PURPOSE

Since the development of the "one gene--one enzyme" theory by Horowitz (1951) and Beadle and Tatum (1941), research in enzyme inheritance, enzyme chemistry, and related disciplines has been greatly accelerated. The genetic basis for enzyme synthesis and biochemical action of enzymes have been and are now being studied in great detail. Since genes dictate enzyme specificity, which in turn regulates the speed and specificity of all metabolic reactions, special interest in enzyme research has been stimulated among geneticists.

In recent years many authors have reported finding multiple molecular forms of particular proteins during electrophoresis. These studies were first stimulated by the revolutionary results of the starch-gel technique of Smithes (1955), in which the separated and stained protein components of human serum were identified on the basis of electrophoretic mobility. When it was found that the lactate dehydrogenase of various human and animal tissues consisted of several fractions which could be separated by electrophoresis (Vesell and Bearn,

1957; Sayre and Hill, 1957; Wieland and Pfeiderer, 1957), interest in the study of the multiple molecular forms of enzymes was intensified. Hunter (1958), using a modification of Smithes' technique, produced zymograms of certain enzymes. Many cases are now known of a band or bands, termed isozymes (isoenzymes), which are under genetic control. Such control has been reported in many enzymes, including esterases, amylases, dehydrogenases, anhydratases, dehydratases, phosphatases, and several others.

Genetic variation in esterases has been studied in maize endosperm and seedlings (Schwartz, 1960), Tetrahymena cultures (Allen, 1960), the serum of mice (Popp and Popp, 1962), man (Harris, Hopkinson, and Robson, 1962), horses (Oki, Oliver, and Funnell, 1964), pigs (Augustinsson and Olsson, 1961), sheep (Lee, 1964); homogenates of fruit flies (Johnson, 1964; Wright, 1963) and house flies (Ogita and Tsutomu, 1965; Velthius and van Asperen, 1963); and in the red blood cells of the rabbit (Gruner, Sartore, and Stormont, 1965) and man (Tashian and Shaw, 1962). Several of these variations seem to be inherited as simple Mendelian traits functioning as codominant allelic systems, where each allele is expressed independently and no dominance is exhibited. Exceptions to this generalization have been found.

Popp and Popp (1962) found that in mouse serum the esterase patterns of the Es locus are each determined by a codominant allele at an autosomal locus. Sick and Nielsen (1964), also using the laboratory mouse, examined saliva and the aqueous extract of the pancreas and showed that each amylase variant is determined by an autosomal codominant allele. Ashton (1960, 1965), studying the same enzyme in cattle and pigs, found three genetic variants in the cow and two variants in the pig serum. Each appeared to be controlled by codominant alleles at one autosomal locus.

Henderson (1965) applied starch-gel electrophoresis and found that strains of mice can be classified into two groups on the electrophoretic mobility of isocitrate dehydrogenase. He suggested a codominant allele on an autosomal chromosome as the method of inheritance. By studying anemia in mice, Russell and Coleman (1963) discovered that several inbred strains vary in the hepatic activity of animolaevulinate dehydratase. Crosses between a strain with high activity and one with low activity produced heterozygotes with an enzyme activity midway between that of the two homozygotes, also suggesting codominance as the method of inheritance. Barnicot et al. (1964), working on the electrophoretic patterns of baboon erythrocyte haemolysates, identified

carbonic anhydrase as a slower or a faster moving fraction and suggested that each variant is determined by a codominant allele at an autosomal locus.

Multiple molecular forms of phosphatases have been described in a large variety of organisms. Menzel et al. (1963) found that the phosphatase activities on disodium beta-naphthyl phosphate in the tissue extract of house flies, after starch-gel electrophoresis, are more numerous in the insecticide-resistant than in the susceptible strains. Beckman and Johnson (1964) discovered an electrophoretic variation in the larval alkaline phosphatase of Drosophila melanogaster, the inheritance of which was controlled by a pair of codominant alleles on an autosomal chromosome.

Levinthal et al. (1962) showed that the alkaline phosphatase of Escherichia coli exhibited four and possibly five distinct bands after electrophoresis, and some of the mutants showed mobilities of all the bands different from those of the wild type. The authors suggested that the multiple bands were all products of the same gene. Differences in electrophoretic mobility have been found in human serum alkaline phosphatase (Boyer, 1961), and in human red cell phosphatase differences in the relative amounts of the components present have been discovered as well. Five distinct

phenotypes have been recognized (Hopkinson, Spencer, and Harris, 1963), and it has been suggested that these differences may be explained by postulating the occurrence of three allelic genes. Electrophoretic variations of phosphatases have also been reported in sheep and cattle sera (Rendel and Stormont, 1964).

Wilcox (1963, 1966), studying alkaline phosphatase in chicken serum, observed a recessively inherited electrophoretic variant. Similar findings have been reported by Law and Munro (1965).

The purpose of this work was to determine the manner of inheritance of serum alkaline phosphatase in the racing pigeon, (Columba livia).

CHAPTER II

MATERIALS AND METHODS

The serum samples used in this study were taken from domesticated homing pigeons (Columba livia). The birds varied in age from eight weeks to twelve years, and sexes were approximately of equal number. The birds along with their pedigrees were obtained from Russell V. Brown. Samples were taken by brachial venipuncture. This was facilitated by removal of several feathers from the axillary region. Three milliliter samples of whole blood from each bird were allowed to drain into dry glass tubes; these were permitted to stand at room temperature for one hour, then refrigerated at four degrees centigrade overnight to induce clotting. The samples were subsequently centrifuged at 3000 revolutions per minute. The supernatant serum was then decanted into four milliliter glass tubes and stored at minus twelve degrees centigrade.

The samples were subjected to acrylamide gel Disc Electrophoresis and stained specifically for alkaline phosphatase. Disc Electrophoresis, first elucidated by Smithes (1955), is based on the principle of two

discontinuous buffer systems, carrying different electrical charges, and separated by a gel. Electrophoretic separation of the serum components was accomplished in a medium consisting of three separately polymerized layers of polyacrylamide gel. From the origin (sample end) down, these are called (1) the sample gel, (2) the stacking gel, and (3) the separating gel. These solutions were prepared by mixing the stock solutions purchased from Canal Industrial Corporation in Unit Chemical Pack #300P (Table I).

The sample gel is composed of the serum sample mixed with a special sample solution (Table I). Its primary function is that of an anti-convection medium. The stacking gel has a larger pore size and a lower pH than the separating gel. In this layer the sample is concentrated into tightly packed fractions according to their electrophoretic mobility. As the ionized sample molecules cross the boundary between the stacking and the separating gels, a change in both physical and chemical conditions is encountered. In the separating gel these packed bands are separated and arranged in a new sequence based on both electrophoretic mobility and molecular weight. The latter occurs because of the physical sieving effect of the gel matrix.

Experimental Procedure

The stock solutions were refrigerated at four degrees centigrade, until needed to prepare working solutions. They were then warmed to approximately twenty-one degrees centigrade and prepared as indicated in Table I. The working solutions were used the same day as when prepared, to assure proper pH of the gel, and to prevent its partial polymerization. Since the stacking gel is photosensitive, it was prepared in a red Erlenmeyer flask to prevent photopolymerization.

The sample gels were prepared in the sample column base caps by mixing the serum with the sample solution in a ratio of one to four. Because of the need to measure in microliters, capillary tubes, in which five millimeters represented three microliters, were fitted onto the needles of Tuberculin syringes. Through the use of this device, accurate quantities of serum and sample solutions were measured, then mixed in a ratio of 33 microliters to 132 microliters, respectively. Glass sample columns were then inserted into the base caps, and both were placed on a loading rack which was adjusted so that the columns were exactly vertical. A small amount of water, three or four millimeters deep, was added to the column to form a layer over the sample gel

mixture, eliminating the meniscus which would otherwise cause curved bands of the separated components. Then the sample gels were photopolymerized for approximately forty minutes by placing them as close as possible to a fluorescent light source. After polymerization the water layer was removed by inverting and blotting with an absorbent tissue. The sample columns were layered with a rinse solution and coated by tilting the loading rack. The rinse solution was removed by inverting and blotting with absorbent tissue, and the procedure repeated a second time.

Upon completion of the rinsing process, two-tenths of a milliliter of stacking gel solution was added. The columns were again layered with a three to four millimeter deep column of water, and the loading rack placed about thirty centimeters from a photopolymerizing light source for fifteen minutes. After polymerization began, the rack was moved close to the light source for an additional fifteen minutes to insure total polymerization. This was indicated by progressive cloudiness. If polymerization had been too rapid, a gel of uneven pore size with poor sample separation would have been the result.

When the polymerization of the stacking gel was completed and the water layer drained from the sample columns, equal parts of the separating gel and catalyst

were combined and mixed by gentle swirling. The inner surface of the glass columns was rinsed twice with this mixture, and each of the columns was filled to the top with enough excess solution added to form an inverted meniscus at the top. Each column was sealed by laying a ten by ten millimeter patch of cellophane paper on the meniscus, forming a flat surface without air bubbles. The gel columns were placed in the dark and allowed to stand undisturbed for thirty minutes while chemical polymerization occurred. To insure satisfactory results, electrophoresis was started within thirty minutes following chemical polymerization.

After the chemical polymerization was completed, the patch was removed from the top of each column, and the sample columns were carefully separated from the sample base caps to avoid deformation of the gels. The sample ends of the sample columns were inserted into the column adapters of the upper bath of the electrophoresis unit. Then the upper bath was placed in the lower bath, which contained the anode and approximately 250 milliliters of 10X buffer solution. Approximately 250 milliliters of 10X buffer solution, containing one milliliter of tracking dye, was slowly added to the upper bath. Finally, the lid, which held the cathode, was brought into position on the upper bath.

After allowing the power supply a one minute warm-up period, the two safety interlock jacks were connected and the current adjusted to the quantity desired--two and one-half milliamperes for each column. Within a few minutes a thin disc of tracking dye was observed. As this disc passed through the interface between the stacking and separating gels, it divided into two distinct discs. The leading disc was the free bromophenol blue dye; the trailing disc was a dye-albumin complex. Electrophoresis was continued until the leading disc reached the bottom of the sample columns. This usually required slightly more than ninety minutes.

At the conclusion of the process, the power was stopped, and the columns were removed from the bath. A needle and syringe filled with propylene glycol was inserted between the glass and the gel at both ends of the column. The needle was held flat against the glass surface, and as the propylene glycol was injected, it was rotated completely around the gel. After the gels were removed from the columns, they were placed in glass tubes, one millimeter by seven and one-half millimeters. The sample gels were stained for alkaline phosphatase overnight by adding stain to the tubes. The stained gels were stored in a seven per cent acetic acid

solution until visually compared. The stain was a modification of the Beckman and Johnson (1964) method for alkaline phosphatase and was made by adding 100 milligrams of sodium alpha-naphthyl phosphate and Blue RR salt, 10 drops of a 10 per cent magnesium chloride solution, 10 drops of a 10 per cent manganous chloride solution, 0.5 grams of polyvinylpyrrolidone, and 2 grams of sodium chloride to 100 milliliters of a 0.5 molar Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer at a pH of 8.65. The mixture was stirred for thirty minutes or until it became homogenous.

TABLE I

COMPOSITION OF GELS AND SOLUTIONS

Working Solution	Parts	Canalco Stock Solution	pH
Sample Solution			8.3
	1	B	
	1	D	
	1	E	
	2	Conc. F*	
Stacking Gel			8.3-8.6
	1	B	
	1	D	
	1	E	
	4	F	
	1	Distilled Water	
Separating Gel			9.5
	1	A	
	1	C	
Rinse Solution			8.3-8.6
	1	B	
	1	E	
	6	Distilled Water	
Buffer			8.2-8.4
	250	Conc. Buffer	
	1000	Distilled Water	
Catalyst	.14 gms.	100 ml. Water	

*Add water to 40 gms. of Sucrose to make 50 ml. volume.

CHAPTER III

RESULTS

During the course of this investigation, serum samples were taken from twenty-one different parent birds (Columba livia) and fifty-one of their offspring. After electrophoresis of the serum, visual analysis revealed seven different banding patterns or phenotypes (Figure I). The phenotypes of the parents and offspring, including the mating types, are listed in Table II.

The phenotypes and genotypes were assigned to the different banding patterns according to the rules established by Ashton et al. (1966). In this paper it was recommended that the locus symbol for the phenotype and genotype be an abbreviation of the gene name and begin with a capital letter, the genotype being italicized. Also, it was suggested that the symbol for an allele should be a capital letter. Therefore, the locus symbol for alkaline phosphatase was abbreviated as Alp.

Phenotypic descriptions were arbitrarily assigned on the basis of migration distance or speed of the bands. The enzyme migrated in four different zones. Each zone was assigned a letter, with the shortest migration

distance being A and the longest being D. When only one zone was present, it was assumed to be a homozygous condition. The homozygous phenotypes were designated as Alp A, Alp B, and Alp D. No homozygous C phenotypes were observed. The heterozygous phenotypes were assigned letters on the basis of the migration pattern of the zones present; a band exhibiting both the A and B zones was designated as Alp A_B. This convention was followed to assign letters to the remainder of the phenotypes. A complete listing of birds and assigned phenotypes will be found in the Appendix. The genotype, corresponding to the phenotype Alp A, was designated as $\underline{Alp^A/Alp^A}$, and the one for the phenotype Alp A_B was listed as $\underline{Alp^A/Alp^B}$. This convention was followed in assigning the remainder of the genotypes (Figure 1).

Of the seven phenotypes exhibited by the adults, only five were found in the offspring (Table I). A mating between an Alp A_B and an Alp B_C produced one AB, one BB, and two offspring with the BC phenotype. No offspring with the AC phenotype were observed. Five matings between the AB and BB phenotypes produced thirteen Alp A_B and six Alp B offspring. Three offspring with a BB phenotype and two with the BC were observed from a cross of a Alp B_C with an Alp B. Two crosses between heterozygous AB parents resulted in thirteen

Alp A_B and three Alp B offspring. No phenotypic AA offspring were observed. When the serum from a cross between homozygous A and B parents was studied, only AB offspring were found. In a mating between AB and AD phenotypes, only Alp A_B and Alp A_D offspring were produced. Neither AA nor BD phenotypes were observed. In the final mating a phenotypic DD was crossed with an AB, and both AD and BD offspring were observed.

Since the ratios of the offspring in all of the matings suggested codominance as the mechanism of inheritance, a Chi-square analysis was performed on each family line (Table III). It was noted that none of the matings produced phenotypes or numbers of offspring which would deviate significantly from those expected for codominant inheritance. The numbers under the probability column (P) are an estimation of the per cent deviation that one could expect due to chance alone.

TABLE II

PHENOTYPES OF PARENTS AND OFFSPRING

Parent Phenotypes	Number of Matings	Offspring Phenotypes				
		Alp A_B	Alp B	Alp B_C	Alp A_D	Alp B_D
Alp A_B X Alp B_C	1	1	1	2	..	..
Alp A_B X Alp B	5	13	6	..	..	..
Alp B_C X Alp B	1	..	3	2	..	..
Alp A_B X Alp A_B	2	13	3	..	..	..
Alp B X Alp A	1	3	..	..	..	..
Alp A_B X Alp A_D	1	1	..	..	2	..
Alp D X Alp A_B	1	..	..	..	2	1

TABLE III

CHI-SQUARE ANALYSIS OF PARENTAL CROSSES

Parent Phenotypes	df*	X^2	P
Alp A_B X Alp B_C	3	1.0	> .70
Alp A_B X Alp B	1	1.89	> .10
Alp B_C X Alp B	1	0	> .99
Alp A_B X Alp A_B	2	5.65	> .05
Alp B X Alp A	3	1.0	> .70
Alp A_B X Alp A_D	1	0.08	> .70
Alp D X Alp A_B	1	0	> .99

*Degrees of freedom.

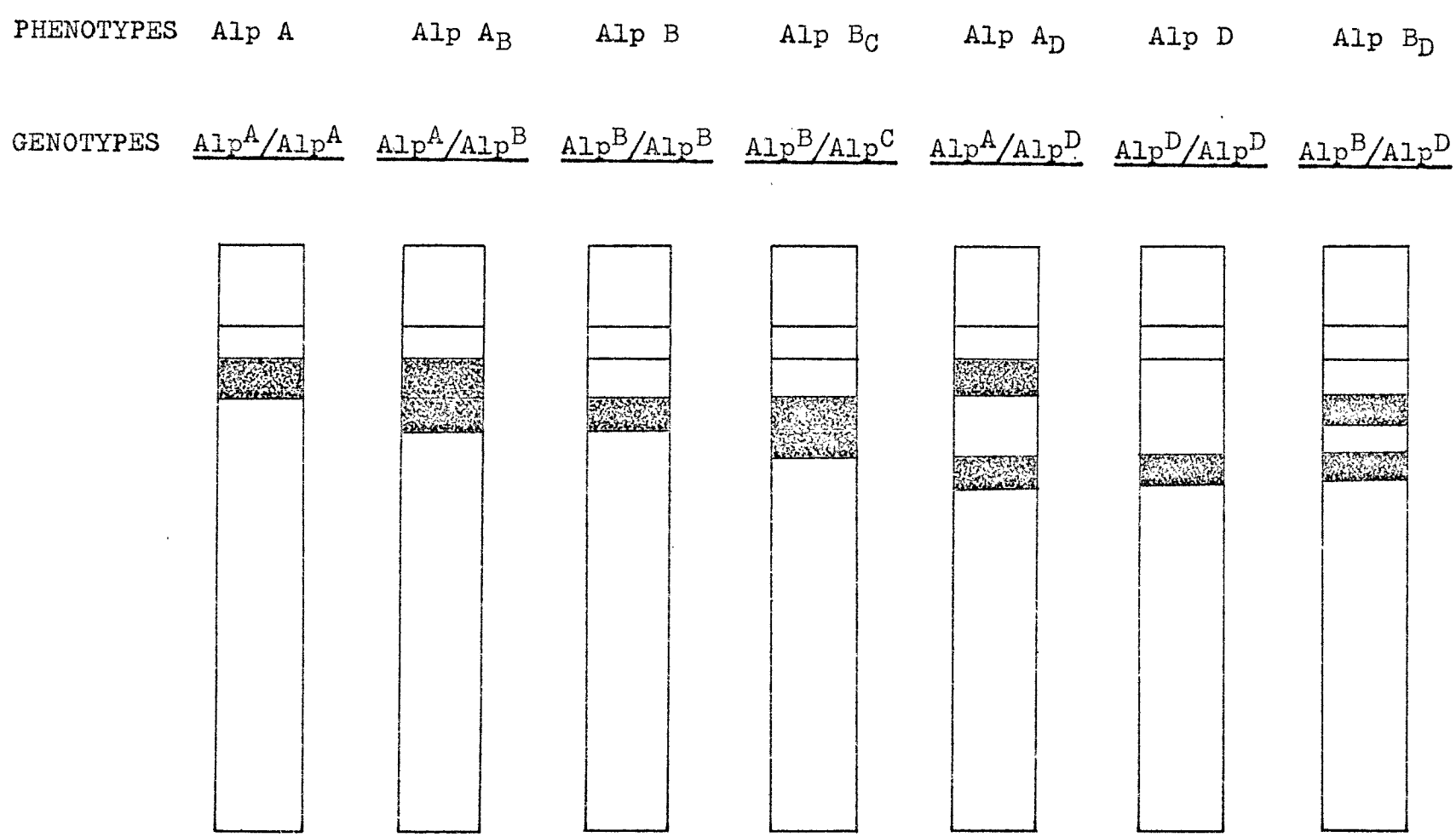

Fig. 1--A schematic representation of the banding patterns exhibited by the alkaline phosphatase of the pigeon (Columba livia).

CHAPTER IV

DISCUSSION

When the alkaline phosphatase banding patterns of the adult racing pigeons were compared with those of their offspring, a genetic basis was suggested. Since the family lines were polymorphic, it was believed that the banding patterns were inherited as simple Mendelian traits functioning as codominant alleles.

The number and intensity of alkaline phosphatase bands vary according to the techniques employed for their separation and even to the methods of extraction from tissues. Baker and Pellegrino (1954) found that alkaline phosphatase migrated in association with the second alpha-globulin fraction when human serum was subjected to paper electrophoresis. However, Taleisnik, Paglini and Zeitune (1955), using the same technique, showed that the serum alkaline phosphatase of rats could be separated into two fractions. Rosenberg (1959) with the aid of starch-block electrophoresis also indicated that the major zone of the alkaline phosphatase activity migrated with the second alpha-globulin fraction.

Haije and de Jong (1963) applied agar-gel electrophoresis to the study of isoenzyme patterns of alkaline phosphatases and showed three main bands of activity. Estborn (1963), using the starch-gel technique, concluded that the serum alkaline phosphatase migrated separately from the main serum protein fractions and that the association with the alpha-globulins reported by investigators using starch grains was coincidental. Hodson, Latner, and Raine (1962) supported this conclusion as a result of their observation that the position of alkaline phosphatase bands could be varied by using a discontinuous buffer system.

The degree of resolution obtained by different investigators varies considerably. Boyer (1961) reported the occurrence of sixteen bands of alkaline phosphatase activity in human serum. However, Wilkinson (1964) classified the bands into six zones of activity. Few comparative electrophoretic studies of the alkaline phosphatases of different species have been reported, but Paul and Fottrell (1957) found wide variation in mobilities of the liver isoenzymes in starch-gel. In the rat and mouse two fast-moving bands were reported, whereas in the guinea pig, frog, and pigeon a single, slower band was found.

Evidence of a genetic polymorphism involving human serum alkaline phosphatase has been observed by Boyer (1961), who grouped the multiple bands into five zones. Beckman and Johnson (1964) observed an electrophoretic variation in the larval alkaline phosphatase of Drosophila melanogaster which was separated into two zones. Escherichia coli is known to exhibit four and possibly five distinct zones after electrophoresis (Levinthal et al., 1962).

In this investigation seven banding patterns were observed. If the polymorphism were actually a four allele system, ten patterns could possibly exist. The limited population and absence of the C homozygote from the matings made it impossible to find all ten phenotypes. For more conclusive evidence of a co-dominant method of inheritance, the C homozygote and more A homozygotes should be found. The A homozygotes should be mated, and samples from their offspring electrophoresed. Then A homozygotes would be mated to the B, C, and D homozygotes, and serum from their offspring tested.

In conclusion, none of the observed banding patterns differed from those expected. A Chi-square analysis showed that only a theory of codominance could be accepted. Therefore, the evidence indicates that the

electrophoretic patterns of serum alkaline phosphatase in the pigeon are inherited as codominant genes.

APPENDIX

PHENOTYPES AND THE CORRESPONDING BAND NUMBERS OF THE PIGEONS USED IN THIS INVESTIGATION

Phenotype	Band Number	Phenotype	Band Number
Alp A	1105	Alp A_B	745
			746
Alp A_B	22		753
	66		967
	82		1215
	168		1413
	523		1469
	607		1475
	608		1736
	615		2408
	661		
	662	Alp B	5
	663		531
	664		605
	665		606
	683		609
	686		610
	687		612
	690		631
	703		633
	705		634
	706		638
	707		685
	708		722
	709		731
	712		754
	713		986
	714		1835
	717		2744
	718		
	729	Alp B_C	11
	730		611
	743		641

APPENDIX--Continued

Phenotype	Band Number	Phenotype	Band Number
Alp B_C	684 704	Alp A_D	727 1855
Alp A_D	613 614 670	Alp B_D	671
		Alp D	17

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