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DIVERSITY ANALYSIS OF ANKALESHWAR POULTRY BREED

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INTRODUCTION

India sub-continent is a rich source of diverse live germplasm and only very few countries have such a large number of breeds with wide genetic diversity. These vast genetic resources are comprised of 10 breeds of buffaloes, 30 of cattle, 43 of sheep, 20 of goats, 7 of camel, 8 of horse, 19 of poultry and few types of pigs, yolks and other distributed in various agro climatic zone of the country. Conservation of indigenous animal genetic resources tops the list of prime livestock development activities for India. Among all the animal genetic resources poultry is the class of domesticated fowl (birds) used or for their eggs and meat production. These most typically are the members of the order Galliformes (such as chicken and Turkeys) and Anseriformes (water fowls such as ducks and geese).

Indigenous birds manifest a perceptible supremacy in using low quality feeds and adapted to ensure high environmental temperature and humidity and further display higher resistance to tropical diseases. A large part of indigenous poultry stock, except Aseel or more genetic breeds is generally referred to as non-descript or Desi and by and large cannot be precisely allocated to any documented poultry breed.

India was the pride of place in world poultry history to be the original home of the well known Red Jungle fowl, along with neighboring countries in the east, from which modern layer broiler breeds have descended. The earliest evidence of domestication of fowl is from Mohenjodaro. Aseel or Malay fowl is reported to have given rise to all the present day breeds of poultry. There is substantial evidence to show that these birds moved through Middle East Europe and gave rise to present day European breeds, about 2000 years ago. Some records indicated that Italians knew specialized poultry breeding about 50 B.C.

For the characterization of poultry genetic resources, the RFLP analysis, SSCP (single strand conformational polymorphisms) analysis, LSSP-PCR(low stringency single-primer PCR techniques and hybridizations with sequence-specific oligonucleotide probes can be used to quickly analyse amplified mitochondrial DNA sequences. The introduction of cost-effective direct sequencing methods (e.g. asymmetric PCR, immobilization of templates on magnetic

beads, cycle sequencing) that make laborious cloning steps superfluous, made it possible to gain the full information from an mitochondrial DNA sequence at an affordable price and time.

The mitochondrial genome is a closed circular molecule with a size of about 16.5 kb. Length differences observed in closely related species are predominantly caused by the varying size of the major non-coding part of the mitochondrial genome, the control region. The gene content is identical for all mammals with genes coding for 13 polypeptides, specifying coding involved in electron transport and oxidative phosphorylation. Furthermore, two ribosomal RNAs constituting the RNA part of mitochondrial ribosomes and 22 tRNAs are encoded. The mammalian mitochondria genome is the example of extraordinary compactness with no introns and only occasionally detectable repetitive sequences confined to the non-coding control region. There exist pairs of overlapping genes and transnational stop codons can be created by the post transcriptional polyadenylation. The only major non-coding part, the control region contains the promoters necessary for the transcription of both strains and regulatory sequences involved in the H- strand replication.

Different models of sequence evolution need to be applied that take some of the peculiar properties of mitochondria DNA sequence evolution into account. Especially the evolution of the hypervariable control region is marked by a pronounced transition bias, preferred pyrimidine transitions, skewed nucleotide compositions and position-specific rate differences. Thus depending on the number of populations, sequence length and algorithms used, phylogenetic sequence analysis is computationally intensive.

Finally a statistical resampling technique (Bootstrap analysis) is usually applied to determine how well certain branches of a tree are supported by the data set.

Keeping in view the above information, the present study was conducted using mitochondrial DNA of three indigenous poultry populations, Ankaleshwar, Danki and Kadaknath with the **following objectives**:

1. To design the PCR primer from the mitochondrial DNA sequence available for the D-loop region.
2. To develop the sequence of the D-loop region in three poultry populations using single pass Automated DNA sequences.
3. Alignment of D-loop sequences using various softwares.
4. Comparison of mitochondrial D-loop region for estimating transitions, transversion and indels.
5. Phylogenetic relationship among the three populations using various genetic distances and preparations of phylogenetic trees.

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MATERIALS AND METHODS

ANKLESHWAR

Ankleshwar breed of chicken is quite hardy and adapted to its environment and have been intricately associated with culture of the tribal (Vanvasi) which maintain them. As other indigenous chicken breeds Ankleshwar is also normally referred as Desi chicken and are facing threat owing to poor egg production, slow growth, late sexual maturity, broodiness, small egg and body size. These characteristics make these native birds vulnerable to economic forces. Ankleshwar birds are maintained in backyard system and provide a good source of animal protein for the tribals. They are found in Gujarat.

Ankleshwar birds are distributed in Bharuch and Narmada districts of Gujarat. In Bharuch these birds are mainly found in Ankleshwar, Jambusar, Zagadia, Bharuch, Hansot and Valia talukas, whereas in Narmada these are mainly found in Dediapada, Rajpipla, Tilakwada and Nadod talukas. However, in the breeding tract, the birds are known not by the name Ankleshwar but as 'desi/gowrani/gamthi'.

DANKI:

Danki is a poultry bird, it is used mainly for game purpose (fighting). Danki breeds are distributed in Viziangaram district and adjoining parts of Srikakulam and Visakhapatanam districts of Andhra Pradesh. All villages have local poultry in the native tract. Danki cocks are fought in natural heels without slashers. These birds are also used for meat purpose.

Kadaknath- It is locally known as "Kalamasi" meaning the fowl having black flesh. Jhabua and Dhar districts of Madhya Pradesh and the adjoining districts of Rajasthan and Gujarat spreading over an area of about 800sq. miles is considered to be its home tract. These are mostly reared by tribals, adivasis and rural poor. It is considered to be a sacred bird and offered as sacrifice to Goddess after Diwali.

METHODS PERFORMED

Blood Sample Collection

Three populations of poultry, Ankaleshwar (23 samples), Danki (20samples) and Kadaknath (20 samples) were selected at random from field conditions from the breeding tract for these populations, 0.5ml of whole blood was collected aseptically from wing vein of each bird using EDTA coated vacutainer tubes and transported to laboratory at 0-5^{o}C.

DNA Isolation

1. For isolation of DNA from collected sample, the blood was transferred to autoclaved Oakridge centrifuged tubes.
2. The RBCs were lysed with lysis buffer (Ammonium chloride 155 mM, Potassium bicarbonate 10 mM and EDTA 0.1 mM). In all the blood samples, lysis buffer was added.
3. The samples were then mixed gently and kept in ice for 10 minutes.
4. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4^{o}C in the refrigerated centrifuge.
5. The supernatant was carefully decanted and the pellet was redissolved in lysis buffer and washed three times as described above..
6. The pellet was resuspended in 10 ml digestion buffer (Sodium chloride 75mM, Tris-Cl 1 M, pH 8.0 and EDTA 0.5 mM).
7. Vortexed gently and to it added 20% Sodium lauryl sulphate (200 µl/sample) and Proteinase K (1 mg/sample) and incubated at 57^{o}C overnight in a water bath.
8. After incubation, digested solution was obtained to which equal amount of Tris equilibrated phenol (pH 8.0) was added.
9. This was mixed gently by moving the tubes gently in '8' fashion for 10 minutes and centrifuge at 12,000 rpm for 10 minutes at 25^{o}C.
10. After centrifugation the aqueous phase and organic phase were separated. The DNA remained dissolved in aqueous phase while the protein was retained at the interphase.
11. The aqueous phase was transferred carefully by Pasture pipette to another Oakridge tube without disturbing the interphase.
12. To the aqueous phase, phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by moving the tubes gently in '8' fashions for 10 minutes at 25^{o}C to separate the aqueous phase and organic phase.
13. Again the aqueous phase was carefully pipetted to another tube without disturbing the interphase. The aqueous phase was mixed with chloroform: isoamyl alcohol (24:1).
14. The solution was subjected to centrifugation at 12,000 rpm for 10 minutes at 25^{o}C and aqueous phase was carefully transferred to glass culture tubes.
15. The DNA was precipitated by adding 1/10th volume of Sodium acetate (3M, pH 5.2) and 2.5 volume of aqueous phase of chilled absolute alcohol.
16. The tubes were covered with paraffin and mixed slightly, the DNA get precipitated and is visible as white stringy strands (Sambrook, J., Fritsch E.F. and Maniatis. T, 1989).

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Washing and Storage of Isolated DNA

1. *The DNA was spooled out into eppendrof tubes and washed twice with 70% ethanol to remove the salts.*
2. *After washing, the alcohol was allowed to evaporate and DNA was redissolved in 500 µl Tris EDTA buffer (Tris 10mM, EDTA 10mM, pH 8.0)*
3. *Kept out eppendrof tubes at 65^{o}C for 1 hour. The stock DNA was stored at –20^{o}C.*

Estimation of DNA quantity and purity

DNA quantification can be done using spectrophotometric measurement of UV absorption at wavelengths 230, 260 and 280 nm. Measures of DNA purity can be determined by the A_{260}:A_{280} and A_{260}:A_{230} ratios. These ratios provide indications of protein, and polyphenol and carbohydrate contamination, respectively (Manning, 1991). The DNA should show a clear absorbance peak at 260 nm. The A_{260} value provides a measure of concentration (roughly 1.0 reading at A_{260} is equivalent to 50 mg per ml). A pure DNA solution has an A_{260}:A_{280} ratio of 1.8 ± 0.1.

The concentration of unknown double stranded DNA samples was estimated using the formula:

AGAROSE GEL ELECTROPHORESIS FOR DILUTION OF GENOMIC DNA

The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this region, when an electrical potential is placed on the DNA, it will moves toward the positive pole.

Dissolving of Agarose

- *Prepared the 1X TAE buffer by diluting 50X TAE buffer.*
- *Weighed out 0.9 gm of agarose (0.6%) and add 150 ml 1X TAE.*
- *In microwave oven heat the slurry for 3 minutes in one minute incriminates, swirling the solution gently between heating cycles to release trapped air and resuspend any agarose particles caught on the side of the Erlenmeyer flask.*
- *Ethidium bromide was added to the warm solution and cooling the solution about 50^{o}C. [Ethidium bromide is intercalating dye and carcinogenic, handle this gel only while wearing gloves].*

Casting the gel

After cooling the solution it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. The gel was 3-5 mm thick. There were no air bubbles under or between the teeth of comb. In case, if bubbles form, they can be removed by pocking them with the pointed end of pipette tips before the gel has set. After the gel has solidified, the comb was removed carefully by wriggling back and forth gently and then lifting up carefully, not rip the bottom of the well.

Preparing the sample

While the gel was cooling, prepared the DNA sample by adding 5 µl of tracking dye to 1 µl of each DNA sample. The tracking dye contains Bromophenol Blue and Xylene cynol FF and 50% glycerol. Adding tracking dye to the sample increased its density so it falls into the well of the gel and provided a visible marker to monitor the progress of electrophoresis. Also prepared molecular size standard by mixing 5 µl of the 1 kb ladder with 1 µl of tracking dye. Bromophenol Blue migrated through agarose gel approximately 2.2 fold faster than Xylene cynol FF dye, independent to agarose gel concentration.

Loading and running the gel

The gel on the tray, inserted horizontally into the electrophoresis chamber and flooded the top of the gel with fresh running buffer (1X TAE) to cover the gel to depth of about 1 mm. Sucked the solution (DNA sample with dye) into the pipette, placed the tip in the top of the well and gently expelled the solution into the well. The lid and power leads were placed on the apparatus, 40-80 V current is applied, and current flowing was confirmed by observing bubbles coming off from the electrodes. Run the gel until the Bromophenol blue and Xylene cynol FF was migrated the appropriate distance about three-fourth of the way across the gel.

Visualization Of DNA

Bright orange coloured DNA bands were visualized by placing the tray onto High Performance UV Transilluminator.

POLYMERASE CHAIN REACTION AMPLIFICATION OF MITOCHONDRIAL D-LOOP REGION

*Polymerase chain reaction (PCR), is the major scientific development of the last quarter century, sometime referred to as "**molecular photocopying**", that can characterize, analyse, and synthesize any specific piece of DNA or RNA. Polymerase chain reaction is an* in vitro *method for analysing defined sequence of DNA. Kary Mullis, while working at Cetus Corporation, California invented PCR in 1985 for which he was awarded the Nobel Prize in chemistry in 1993.*

There are three major steps in PCR. All of them were carried out in the same vial but at different temperatures and are repeated for 30 cycles. This was done on an automated thermocycler, (ABI Perkin Elmer System/ Gene Ampli PCR 9700,Bio-Rad/ICycler), *which automatically heated and cooled the tubes with the reaction mixture in a very short time.*

Denaturation at 94^{o}C: *During the denaturation, the first part of the process, the double strand melted, open to single stranded DNA, all enzymatic reactions stop (e.g., the extension from a previous cycle).*

Annealing at 56^{o}C: *The primers were jiggling around, caused by the Brownian motion. Ionic bonds were constantly formed and broken between the single stranded primer and the single*

stranded template. The polymerase enzyme attached to the double stranded DNA (template and primer) and started copying the template. The vial is cooled to 56^{o}C. At this temperature, the primers bind or "anneal" to the end of the DNA strands. Touch down PCR was used by lowering the annealing temperature from 56˚C to 54˚C

Extension at 72^{o}C: *This was the ideal working temperature for the polymerase. Primers that were on positions with no exact match get loose (because of the higher temperature) and didn't give an extension of the fragment.*

Selection of Primers

The primers were designed using Primer III software to amplify ------bases of D- loop region of chicken mitochondrial DNA.

Taq DNA Polymerase

Taq DNA polymerase is obtained from the thermophilic archaea family bacteria Thermus aquaticus. It possesses a 5'→ 3' polymerase activity and a double strand specific 5'→ 3' exonuclease activity.

PCR Buffer

PCR Buffer (Sigma) contains Tris–Cl (100 mM, pH 8.3 at 25^{o}C), KCl (500 mM), $MgCl_2$ (15 mM), gelatin (0.01%). It supports the activity of Taq polymerase.

Magnesium Chloride concentration

Magnesium concentration is a crucial factor affecting the performance of Taq DNA polymerase. Reaction components, including template DNA, chelating agents present in the sample (e.g., EDTA), dNTPs can affect the amount of free magnesium. In the absence of adequate free magnesium, Taq DNA polymerase is inactive. Conversely, excess free magnesium reduces enzyme fidelity and may increase the level of nonspecific amplification. The optimal $MgCl_2$ concentration is use for each reaction.

PRE-PCR PREPARATION

The PCR was performed under standard condition as described by Kaul et al., 2001. The genomic DNA was diluted so as to contain about 50 ng DNA/µl.

Cocktail Preparation

Table 1: The cocktail for PCR one reaction (15 µl) consisted of:

	Volume	Concentration
10X PCR Buffer	1.5 µl	-
DNTPs	0.2 µl	100 mM
Primer (Forward)	1.0 µl	4 pmol
Primer (Reverse)	1.0 µl	4 pmol
Taq Polymerase	0.2 µl	0.4 Unit

Milli Q water	10.1 µl	-
Total	15 µl	

PCR programme

1.0 µl of the template DNA was directly added into the cocktail in each lane of the PCR plate. The PCR was carried out in Applied Bio-system and Bio-Rad thermocycler.

PCR Program used for amplification

Initial Denaturation 95˚C- 5 minutes

{94-45"

[56^0-5 cycles

55^0-5 cycles

54^0-10 cycles]-45"

72˚C- 60"}

Final extension 72˚ C-10 minutes

Hold at 4 ̊C -ά

AGAROSE GEL ELECTROPHORESIS OF PCR AMPLIFIED DNA

After completion of the PCR programme, the products were checked on 2% agarose gel for the amplification. Before loading into the well, gel-loading dye (xylene cynol FF, bromophenol blue in glycerol) was added to the sample and the samples were run under constant voltage conditions (150 V) till the two dyes were separated. Amplified product appeared as sharp orange color bands under UV Transilluminator due to the intercalation of ethidium bromide.

CYCLE SEQUENCING

DNA sequencing is a powerful technique in molecular biology. It was first devised in 1975. DNA sequence allows analysis of genes at the nucleotide level. Furthermore a molecular biologist can utilize sequencing to identify the site of mutation. There are only a few examples illustrating the way in which DNA sequencing has revolutionized molecular biology. In this work we used automated DNA sequencer model 3100 ABI PRISM DNA Sequencer. Cycle sequencing has emerged as a powerful alternative to conventional isothermal method.

Cycle sequencing is a simple method in which successive rounds of denaturation, annealing, and extension in a thermal cycler result in linear amplification of extension products. The

products are then injected into a capillary. All current ABI PRISM DNA sequencing kits use cycle sequencing protocols (Marra.M.Weinstok1996).

Template:

Template purity and the optimum concentration are crucial in obtaining the good quality of DNA sequence. PCR product as well as single stranded DNA can be used as a template for sequencing.

Template purification:

PCR product should be clear of dimers and non specific amplifications. It should be free from excess reaction component like dNTPs, Taq polymerase etc. For this digestion was done.

Exo-SAP Digestion of PCR product:-

Make a master mix of Exonuclease I and shrimp alkaline phosphate as per the following table:

Component	**Final Concentration (U/ul)**
Exo.1 (20 U/ul)	0.5
SAP (1 U/ul	0.5
PCR buffer 10 x	1 ul
Milli Q water	Make up the
Total volume	final volume 10 ul

Add 1 ul of the master mix to 10 ul of PCR product (50 to 100 ng) and set up the following incubation protocol in the thermal cycler

Hold = 37˚C for 120 minutes
Hold = 85˚C for 15 minutes
Hold= 4˚C for infinity.
The purified PCR product can be used for sequencing

Cycle sequencing reaction/Ready reaction process: -

Reaction is set as per recommendation of AB1 using 8 ul of ready reaction mix in addition to template and primer.

Ready reaction mix:

ABI PRISM™ Ready reaction mix /Dideoxy terminator premix (1000 ul) Applied Biosystems (ABI) consists of:
1.58 mM of ddATP, 94.74 mM ddTTP, 0 .42 uM ddGTP, 47.37 uM ddCTP, 98.95 uM dITP, 15.79 uM dATP, 15.79 uM dCTP, 15.79 uM dTTP, 168.42 mM Tris-HCl (pH-9), 4.21 mM (NH4)2 S04, 42.1 mM MgCl2, and 0.42 U/ul ampli Taq DNA polymerase.

Pre-sequencing composition:

PCR Product	50-100 ng
RR Mix	8 ul
5 x Sequencing buffer	4 ul
Primer (Forward/Reverse)	4 pmol
Milli Q Water	Make up the volume 20 ul.

Mix the content briefly and keep it in a thermal cycler set at following reaction conditions

Initial Denaturation- 96ºC for 1 minute
Denaturation-96ºC for 10 seconds
Annealing- 50ºC for 5 seconds
Extension- 60ºC for 4 minutes
30 cycles
Hold at 4ºC -ά

For short PCR product, number of cycles can be reduced (e.g., 20 cycles for a 300 bp or smaller fragment).
If the Tm of a primer is >60ºC , the annealing step can be eliminated
If the Tm of a primer is <50ºC, increase the annealing time to 30 seconds or decrease the annealing temperature.
For templates with high GC content (>70%), heat the tubes at 98ºC for 5 minutes before cycling to help denature the template.

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Purification of the Sequencing product:

After the sequencing reaction, the products are purified by the following protocol

1. Added 2ul of 125mM EDTA to stop the reaction and mixed well
2. Added 2ul of 3 M Sodium acetate pH 4.6 to each reaction well. Ensure the proper mixing of the contents.
3. Added 50 ul of 95% ethanol to each well and incubate at room temperature for 15 minutes.
4. Spin at a speed of 1650g for 45 minutes at room temperature.
5. Invert the plate on paper towel and give a short spin at 180g for removing supernatant.
6. Added 200ul of 75% ethanol and spin at 1650 g for 5 minutes.
7. Invert the plate slowly on paper towel and spin at 180g for 1 minute.

Denaturation:

1. Add 10 ul of Hi Di Formamide and denature at 95°C for 5 minutes and chilled on ice (snap chilling) immediately for 5 minutes.

2. The plate can be now loaded in automated DNA sequencer.

ABI 3100 Avant- Automated DNA Sequencer:

- Provided with 4 capillaries with different array sizes (22cm, 36cm, 50cm and 80cm). The large surface area of a capillary allows heat generated during electrophoresis to be dissipated efficiently, allowing high voltage electrophoresis. The result is rapid, high-resolution separation of DNA fragments.
- Polymer POP-6 can be used for sequencing (Performance Optimized Polymer 6, medium used to separate DNA fragments).
- Plate record to be loaded with appropriate model number and sequencing chemistry.
- 96 well plate to be linked and start the run.

Data collection and Extraction:

- After the completion of the electrophoresis (run) chromatogram drawn by data collection software is used to extract the sequence data.

- The sequences with chromatogram can be visualized, edited for vector sequences and further saved by ABI PRISM ® DNA sequencing Analysis Software.

Statistical Analysis: The following parameters were estimated from the mitochondrial d-loop DNA sequence data.

i) The general information about the polymorphism, the number of sites with alignment gaps, the number of morphophism site, the number of polymorphic sites segregating for two, three or four nucleotides were estimated.
ii) Sites with alignment gaps
iii) Haplotype diversity and its sampling variance
iv) Nucleotide diversity, II, the average number of nucleotide differences per site between two sequences (Nei, 1987; Nei and Miller, 1990).
v) The estimates of nucleotide diversity (Jukes and Cantor), II (JC), the average number of nucleotide substations per site between two sequences (Lynch and Crease, 1990).
vi) Theta per sites i.e. the watterson estimator, The Eta (n) is the total number of mutations and S the number of sites which are segregating.
vii) The average number of nucleotide differences, K (Tajima 1983) alongwith stochastic and sampling variances.
viii) The Linkage disequilibrium (Kelly 199; Rozas et al., 2001) was also calculated.
ix) The DNA Divergence between the two population and was calculated on the basis of average number of nucleotide differences (Tajima, 1983).
x) The genetic diversity analysi for each individual population was also estimated alongwith the total data for the three populations.
xi) The genetic differentiation analysis based on Haplotype (Hudson et al., 1922) and testing their significance using X2 (Hudson, 1992 and Nei, 1982) and permutations test (Hudson et al., 1992a).
xii) The gene flow analysis was carried out using both haplotype data information and nucleotide sequence data information.
xiii) The linkage disequilibrium between nucleotide variants as per the methods and procedures given by Lewutin and Kajima 1964' Hill and Robertson, 1968.
xiv) The distribution of observed pairwise nucleotide site differences (mismatch distribution) was estimated in a stable population i.e. population with constant population size (watterson, 195; Slatkin and Hudson 1991, Rogers and Harpendeng, 1992).

xv) The statistical tests Dad F proposed by Fu and Li (1993) for testing the selective neutrality of the various mutations was also carried out.

xvi) The various genetic distances were calculated and the dendrogram were prepared using UPGMA and Neighbour joining algorithm.

RESULTS AND DISCUSSIONS

We utilized three indigenous poultry breeds for amplification of the D-loop region of mitochondria. The two primer sequences utilized for the amplification of the region were

Primer Forward: CTCGCCGTATCAGATGGATT

Primer Reverse : AGTGGTAGAAGGCGGGTTTT

Alignment of Sequences

The sequences of varying length were obtained for the 63 sequences (23 of Ankleshwar, and 20 each of Danki and Kadaknath) utilized in the study. The variation of the length is due to varying output of ABI automated DNA sequencer. We utilized 80cm array for the purpose. The alignment of the output sequence was done using the Clustal W software. The procedure followed was alignment of the two sequences followed by alignment of multiple sequences. After the alignment, a stretch of 650 bases were taken for further study and analysis.

Molecular Diversity indices

The molecular diversity indices were calculated for all the three populations. the values are given in tabular form-

Table: 2

Parameter	Ankleshwar	Danki	Kadaknath
No. of observed transition	2	2	3
No. of observed transversions	4	0	0
No. of substitution	6	2	3
No. of observed indels	1	1	1
No. of nucleotide sites	651	651	651

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Table: 3

Nucleotide composition	Ankleshwar	Danki	Kadaknath
C	25.69	25.74	25.73
T	37.05	37.05	37.06
A	21.90	21.83	21.98
G	15.36	15.37	15.23
No. of Haplotypes	7	4	6

As is evident from the table that transversions occurred only in ankaleshwar population while the transitions Were present in the population. the nucleotide composition in the three population was not significantly different from one another but significantly different from the normal ratio of 25% of nucleotide each.

The data obtained on the haplotypes for the three breeds of poultry was analysed for estimation of the pairwise difference among the haplotypes within the species the values obtained for each breed has been given in the three tables above for ankaleshwar, danki and kadaknath respectively.

Variation of Sequence Data

The 650 base sequence data presented a site with gaps and missing data making a total of 651 base sequences. The total number of invariable sites (monomorphic) were 640 while 10 sites were variable (polymorphic). This represented 10 mutations out of which 6 were singleton variable sites while 4 were parsimony informative sites. The singleton variable sites were at position 70, 143, 294, 344, 455 and 540 in the 651 base sequence of the D-loop region. The parsimony information sites (two variants) were at site positions 16,21,236 and 342. The D-loop region does not exhibit any protein coding region of the mitochondrial DNA. The G+C content of the 650 sites was 0.410.

Haplotype Distribution

The total number of haplotypes were found to be 10 when the alignment gaps were not considered while the number of haplotypes increased to 12 when the haplotypes were considered in the analysis. The value of haplotype diversity was estimated to be 0.589 with variants of 0.00423 when the gaps were not included in the analysis. When the gaps were included the haplotypic diversity was 0.7732.

The 12 haplotypes obtained alongwith the total number of the birds possessing the haplotype are :

Table: 4

Haplotype Numbers	**Number of birds**	**Distribution of birds among populations**
Hap-1	7	4 (Ankleshwar), 3 (Kadaknath)
Hap-2	1	1 (Ankleshwar)

Hap-3	2	2 (Ankleshwar)
Hap-4	1	1 (Ankleshwar)
Hap-5	26	11 (Ankleshwar), 11 (Danki), 4 (Kadaknath)
Hap-6	1	1 (Ankleshwar)
Hap-7	13	3 (Ankleshwar), 7 (Danki), 3 (Kadaknath)
Hap-8	1	1 (Danki)
Hap-9	1	1 (Danki)
Hap-10	6	6 (Kadaknath)
Hap-11	1	1 (Kadaknath)
Hap-12	3	3 (Kadaknath)

Relative frequencies:

Haplotypes	**Ankaleshwar(23)**	**Danki(20)**	**Kadaknath(20)**
Hap_1	**0.174**	**0**	**0.15**
Hap_2	**0.0435**	**0.00**	**0.00**
Hap_3	**0.087**	**0**	**0**
Hap_4	**0.0435**	**0**	**0**
Hap_5	**0.478**	**0.55**	**0.2**
Hap_6	**0.435**	**0**	**0**
Hap_7	**0.13**	**0.35**	**0.15**
Hap_8	**0**	**0.05**	**0**
Hap_9	**0**	**0.05**	**0**
Hap_10	**0**	**0**	**0.3**
Hap_11	**0**	**0**	**0.05**
Hap_12	**0**	**0**	**0.15**

Testing Nucleotide Loci for Linkage Disequilibrium

In case of ankaleshwar the 7 nucleotide sites were tested for linkage disequilibrium. The exact test was performed using a Markov chain with 100000 steps with a dememorisation of 1000 steps . the 7 sites used along with their significance at 0.05 are given below .

Table: 5

	15	20	69	235	293	408	539
15	*	-	-	-	-	-	-
20	-	*	-	-	-	-	-
69	-	-	*	-	+	-	+
235	-	-	-	*	-	-	-

293	-	-	+	-	*	-	+
408	-	-	-	-	-	*	-
539	-	-	+	-	+	-	*

+ depicts the significant linkage disequilibrium.

In case of danki these were 4 polymorphic nucleotide loci. Two loci pair 142-408 and 408-454 were in significant linkage disequilibrium.

Table: 6

	142	408	454
142	*	+	-
408	+	*	+
454	-	+	*

In case of kadaknath there were 4 nucleotide loci, which were polymorphic. Locus 235-341 were found to be in significant linkage disequilibrium.

Table: 7

	235	341	343	408
235	*	+	-	-
341	+	*	-	-
343	-	-	*	-
408	-	-	-	*

Statistical Test for Detection of Selection

Natural selection is one of the most important factor in the differentiation among the population. However, in case of mitochondrial DNA it is difficult to observe the change in the nucleotides as this may be a result of selection or genetic drift. Any deviation from the neutrality points to the presence of some sort of selection operating.

Tajima (1989) gave a test conceptually similar to Wattersons homozygosity test. The test examines the relationship between the number of segregating sites and nucleotide diversity. The mean and variance of D are approximately 0 and 1 but the distribution of D is quite different from the normal distribution. Similarly Fu and Li's D statistics (1993) have been proposed. The values obtained for the analysis were FL-D test statistics were -1.09115 while the statistical significance revealed no significant deviation (P> 0.10). The Fu and Li F test statistics revealed a value – 1.30076 for the three population and the values were statistically non significant (P>0.10). The results reveal there is no selection in the individuals of the three poultry populations.

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Ankaleshwar

There were seven haplotypes in Ankleshwar chicken. The inter haplotypic distance matrix using pair-wise differences with no Gamma correction are presented below. The neighbor joining (NJ) tree based on inter haplotypic distances is given in figure .

Table: 8

	Hap_1	Hap_2	Hap_3	Hap_4	Hap_5	Hap_6	Hap_7
Hap_1	-	0.9992	1.4120	1.4120	0.9992	1.4120	1.4120
Hap_2	1.0000	-	1.7281	1.7281	1.4120	1.7281	1.7281
Hap_3	2.0000	3.0000	-	1.9938	0.9992	1.9992	1.4120
Hap_4	2.0000	3.0000	4.0000	-	1.7281	1.4120	1.9938
Hap_5	1.0000	2.0000	1.0000	3.0000	-	1.7281	0.9992
Hap_6	2.0000	3.0000	4.0000	2.0000	3.0000	-	1.9992
Hap_7	2.0000	3.0000	2.0000	4.0000	1.0000	4.0000	-

Inter-haplotypic distance matrix (s.d. above diagonal)

Danki

There were four haplotypes in Danki chicken. The inter haplotypic distance matrix using pairwise differences with no Gamma correction are presented below. The neighbor joining (NJ) tree based on inter haplotypic distances is given in figure .

Table: 9

	Hap_5	Hap_7	Hap_8	Hap_9
Hap_5	-	0.9992	1.4120	1.4120
Hap_7	1.0000	-	0.9992	0.9992
Hap_8	2.0000	1.0000	-	1.4120
Hap_9	2.0000	1.0000	2.0000	-

Inter-haplotypic distance matrix (s.d. above diagonal)

Kadaknath

There were six haplotypes in Kadaknath chicken. The inter haplotypic distance matrix using pairwise differences with no Gamma correction are presented below. The neighbor joining (NJ) tree based on inter haplotypic distances is given in figure .

Table: 10

	Hap01	Hap05	Hap07	Hap10	Hap11	Hap12
Hap01	-	0.9992	1.4120	0.9992	1.4120	0.9992
Hap05	1.000	-	0.9992	1.4120	1.7281	1.4120

Hap07	2.000	1.000	-	1.7281	1.9938	0.9992
Hap10	1.000	2.000	3.000	-	0.9992	1.4120
Hap11	2.000	3.000	4.000	1.000	-	1.7281
Hap12	1.000	2.000	1.000	2.000	3.000	-

Inter-haplotypic distance matrix (s.d. above diagonal)

Pair-wise Divergence between Populations

The number of fixed differences between populations nucleotide sites at which all of the sequences in one population are different from all of the sequences in the record position (Hey 1991) were found to be zero in all the three combination pairs of the populations. The number of polymorphic sites in Ankleshwar was 6 while in Kadaknath there were three polymorphic sites. The average number of nucleotide differences were 1.036 (k) and 1.058 (k) for Ankleshwar and Kadaknath populations. The number of polymorphic sites in Danki were 2 and the average number of nucleotide differences were 0.2 (k). The values of the nucleotide diversity (pi) (Nei 1987) and nucleotide diversity with Jukes and Cartor (Nei 1987 and Lynch and Crease 1990) their variance is given in Table:

Table: 11

	Ankleshwar	Danki	Kadaknath
No. of sequences	23	20	20
No. of Polymorphic sites	6	2	3
Average number of nucleotide differences (k)	1.036	0.200	1.058
Nucleotide Diversity with Jukes and Cantor.	0.00160	0.00031	0.00163

Further, on analysis of the data we found that the mutations that were polymorphic in Ankleshwar but monomorphic in Kadaknath were 5 while the mutations that were polymorphic in Kadaknath but monomorphic in Ankleshwar were 2. The mutation that were polymorphic in Danki but monomorphic in Kadaknath were 2 while the mutations polymorphic in Kadaknath but monomorphic in population and Danki were 3 in number. The mutations that were polymorphic in Ankleshwar but monomorphic in Danki were 6 in number. The mutation that were polymorphic in Danki but monomorphic in population were two in number. However, the total number of shared mutations was zero between the three population pairs. The average number of nucleotide differences between populations of Ankleshwar and Danki was 0.709 with average number of nucleotide substitutions per site between these populations Dxy was 0.00109. The number of net nucleotide substitutions per site between Ankleshwar and Danki Da was 0.00014. The values

between populations of Danki and Kadaknath were 1.150 (average number of nucleotide differences); 0.00177 (Dxy) and 0.00080 (Da). The values between Ankleshwar and Kadaknath were 1.263; 0.00194 (Dxy) and 0.00033 (Da).

Gene Flow and Genetic Differentiation

The analysis of the three poultry populations was carried out considering the alignment gaps as the fifth state. The haplotype diversity was estimated to be 0.74308, 0.60 and 0.84211 for Ankleshwar, Danki and Kadaknath while the value was 0.77317 for the total data set consisting of 63 sequences. The haplotype based statistics (Hudson et al., 1992) was utilized. The Hs value were obtained as 0.7291 while the H_{st} was found to be 0.056691. The X^2 value obtained by using permutation was 0.0034 which was significant at 0.01% level of significance at 22df. The nucleotide sequence based statistics was calculated as Ks, 1.6474 with K_{st} value as 0.14694. The permutation test revealed it to be very highly significant (P< 0.001). Thus the population are greatly differentiated among themselves. The number of migrants based on haplotype data information was N_m= 4.20 while a value of N_m=1.18 was obtained using sequence data information. The effective number of migrants Nm based on the F_{st} estimator has been found to be 0.95.

Various Parameters of Genetic Differentiation among Populations

Various genetic parameters among three breed pairs were estimated to find out the genetic differentiation among various breeds. The F_{ST} values indicated maximum differentiation between Danki and Kadaknath while Ankleshwar and Kadaknath were closest. The $Gamma_{ST}$ was estimated assuming that all the nucleotides have the same probability of undergoing transitions/transversions.

Table: 12

Populations		H_S	K_S	K_{XY}	G_{ST}	Gamma S_T	F_{ST}
Ankleshwar	Danki	0.677	1.016	1.172	0.014	0.102	0.149
Ankleshwar	Kadaknath	0.789	1.378	1.615	0.040	0.100	0.142
Danki	Kadaknath	0.721	1.111	1.630	0.075	0.214	0.319

Phylogenetic Tree Building

Several statistical methods exist that can be used for reconstruction of the phylogenetic trees from the molecular sequence data. The major methods are

i) Distance methods
ii) Parsimony methods and
iii) The Likelihood methods.

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Now it is customary to consider the reconstruction of phylogenetic tree as statistical inference of a true phylogenetic tree which is unknown. The inference involves the estimation of the topology and estimation of branch lengths for a given topology. The four methods utilized for the purpose are given below.

UPGMA

The simplest method is un-weighted pair group method using the arithmetic averages (UPGMA). This is also called a phenogram. When we utilized the nucleotide sequence data this model provided reasonably good trees compared to the other distance methods. The UPGMA was utilized to reconstruct the tree from the individual nucleotide sequences. The distance matrix produced was 63 x 63 matrix has not been presented and only the tree has been depicted.

The tree using the nucleotide number of differences among the sequences of the three populations was carried out. The mean diversity values were obtained to be 0.7645. The mean diversity of the entire data was 0.9565 with mean inter-population diversity of 0.192. The overall coefficient of differentiation was found to be 0.2007.

Minimum Evolution Tree

The minimum evolution tree was generated and has been depicted in The use of minimum evolution tree is that it examines all possible topologies and then estimates the smallest genetic distance. It is a very time consuming algorithm and using our data it took 7 hours for Pentium IV and 300 MHz Computer with 2GB RAM for the construction of this tree. The process starts with the construction of a Neighbour joining tree and then a set of topologies close to the NJ tree is examined to find a tree with small distance values and a tree is constructed (Temporary Minimum Evolution tree) and the process is repeated till no further distance value is obtained.

Neighbor Joining Tree

The Neighbor joining tree as given by Saitou and Nei (1987) was used to develop the tree using the algorithm, which is a simplified version of minimum evolution tree. The neighbour joining tree was constructed using the sequences of the three breeds/populations.

Maximum Parsimony Tree

We also constructed a maximum parsimony tree. The maximum parsimony tree does not have many assumptions that are required for nucleotide substitutions in the distance and maximum likelihood methods. The maximum parsimony methods of tree construction were utilized since any mathematical model currently is a crude approximation to reality, it is expected that the model free maximum parsimony methods may give more reliable trees than any other method when the extent of sequence divergence is low (Miyamoto and Craeraft, 1991). The maximum parsimony tree was prepared and depicted as

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Most natural populations of organisms contain a large amount of genetic variation. The genetic polymorphism at a locus is generated by mutation such as nucleotide substitution insertions/deletion, gene conversions etc. The main concern in this study was to study the maternal lineage and maintenance of genetic polymorphism and to study the mechanism of evolution at the population levels. We analysed the nucleotide sequence data of the D-loop region and tried to infer the genealogical parameters of the three poultry populations. It is presumed that the haplotypes that are similar among the populations are likely to have similar maternal lineage followed by the other lineages which are very similar/have undergone few mutational changes. DNA sequences are much more informative compared to protein/electrophoretic variations of protein. The nucleotide substitutions can also be easily deciphered. The extent of DNA polymorphism may be measured in several different ways. (Nei 1987), but the most common measures are the number of segregating sites per nucleotide site and the nucleotide diversity. In the present three populations we found transitions to be more frequent compared to transversions. The maximum number of transversions were observed in Ankleshwar poultry (4) with Danki and Kadaknath did not reveal that transversions and only transitions were recorded. Generally the more ancient the population is the longer it had to mutate and accumulate the mutations. So that the ancient populations would be more diversified genetically and the haplotypes present in them would be more shared by other populations. (Torroni et al., 1993; Ward et al., 1993). The result of the present study revealed maximum number of haplotypes to be 7 in Ankleshwar followed by Kadaknath breed of poultry. All the 7 haplotypes of Ankleshwar were shared by Kadaknath as well as Danki populations. Thus, Ankleshwar seems to be the most ancient population from such contention. The Danki breed of poultry has only four haplotypes of which two are unique to the population while the two haplotyps are shared among the three breeds. The major reason for this being that Danki is a game bird and most likely there should be a lot of selection pressure on the Danki population to prepare specific type of cocks for game purposes and this has reduced the number of the haplotypes present in the population. The value obtained for the number of haplotypes for Kadaknath poultry were 6. Three haplotypes were unique to this population while the two were shared by both Danki and Kadaknth while one was shared with Ankleshwar. In points towards a selection pressure in Kadaknath poultry also but Ankleshwar poultry remains the most ancient one in terms of haplotypes. Kadaknath also known as Kalamasi due to the black colour of their body parts including the skin and internal organs is known for its delicacy and has a priority over the normal birds of the region. The black colour is attributed to a quantitative trait with a single major gene effect called Fm (Fibromelanosis) which is dominant in its character but has several (multifactorial) modifiers. This trait gives protection to skin against UV radiation improved radiation from skin, increased packed cell volume and plasma protein. Thus the number of haplotypes in a breed./population point towards the selection pressure as well.

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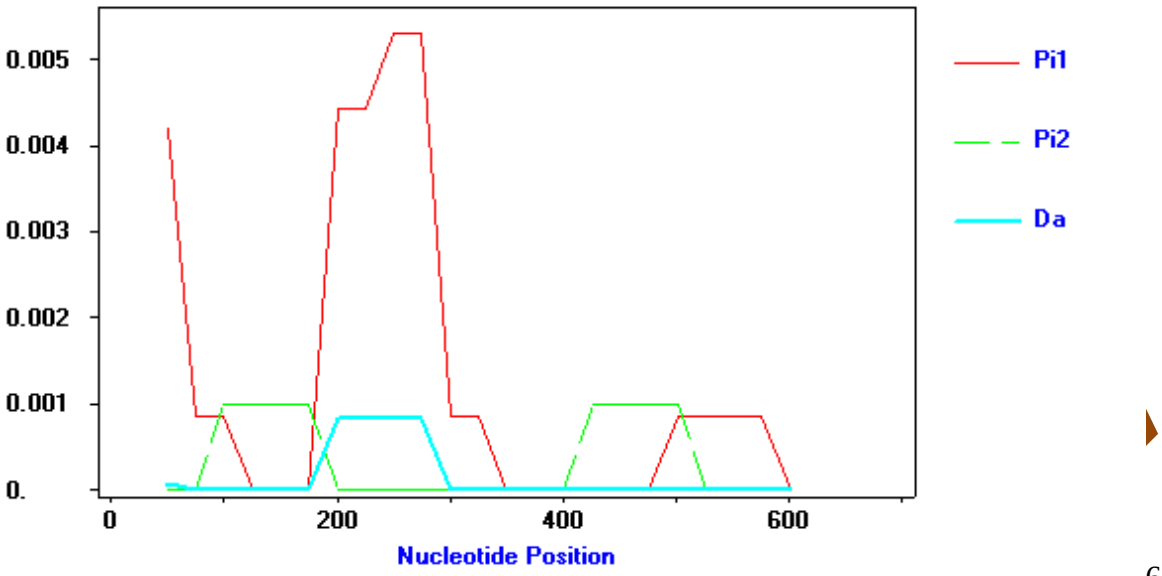
Genetic differentiation of three native chicken breeds:-

Ohno(1997) deduced that a set of full or maternal half sister should have inherited identical mitochondrial genome from their mother but each sisters female descendants invariably establish an independent lineage which over a period of time would accumulate its own characteristics mutations to become a distinct sublineage. On a whole Ankleshwar had 7 lineages Hap 1 to Hap 7 forming seven distinctive characters. The Danki population exhibited 4 haplotypes Hap 5, Hap7, Hap8 and Hap9 while the six Kadaknath haplotypes have been shown as Hap1, Hap05, Hap07, Hap10, Hap11 and Hap12. The minimum spanning tree of the three breeds/populations of indigenous poultry have been depicted in the form of trees.

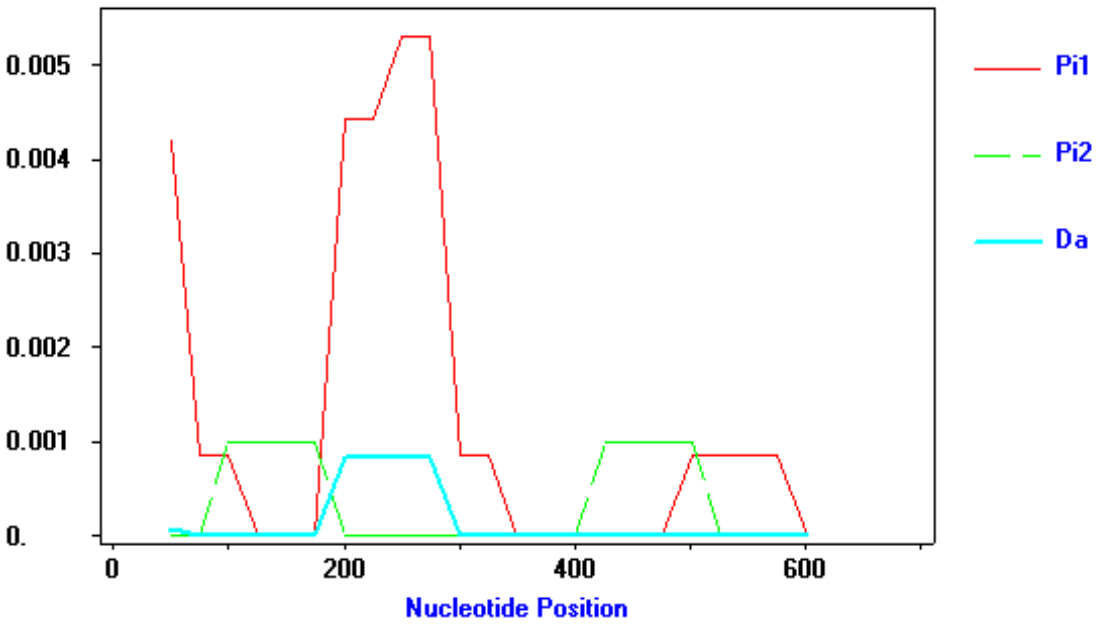
All of the above suggested that these three breeds shared the lineages and that their genetic background were complex. Mating might have occurred between the lineages or some of them might have differentiated during the process of evolution. The Haplotype 5 and Haplotype7 are present in all the three breeds/population under study and these haplotypes may be the evolutionary lineages. The results suggest that these breeds belonged to the same linage or that they shared the common maternal ancestor regardless of external features and ecological types of these breeds. Zlm (1958) suggest that mutation was the major reason that more than 30 subspecies or variants of domestic chicken were found by gradual mutations. In the present study we have a normal egg layer breed (Ankleshwar), a specific pigmented breed (Kadaknath),and a Figher bird (Danki). Masson (1987) speculated that the Chicken was first used as recreational breed such as Game Cock than for various religious purposes and eventually as a source of meat and egg. Thus it is certain that the chicken breeds might have originated from common ancestors. In so far as original ancestor of the native fowl are concerned there are several studies which include the biochemical genetics and nuclear DNA studies (Hashguchi et al 1993; Chang et al., 1996; Mohd-Azmi et al.,2000). All of them concluded that the domestic chicken from different areas or countries were genetically very close to their indigenous red jungle fowl (G.gallus).

The neighbour joining tree revealed that Kadaknath and Ankleshwar to have common lineages while Danki, Kadaknath and Ankleshwar sharing common lineages. Similar results were also obtained in UPGMA tree and Maximum likelihood tree generation. The minimum spanning tree in Ankleshwar given Haplotype 1 as the haplotype on the root from where the mutation resulted in haplotype 4, Hap 6, Hap 2 and Hap 5. Hap 5 resulted in Hap 3 and Hap 7 in ANkleshwar poultry. The minimum spanning tree in Danki represents Haps to be closest to the root from where three other lineages emerged due to mutations in the D-loop region. In Kadaknath the six haplotypes resulted in minimum spanning tree with Hap1 andHap 12, Hap 10 and Hap 11 being close to one another and join the root which is joined on other side to Hap 5 and Hap 7 which are close to one another.



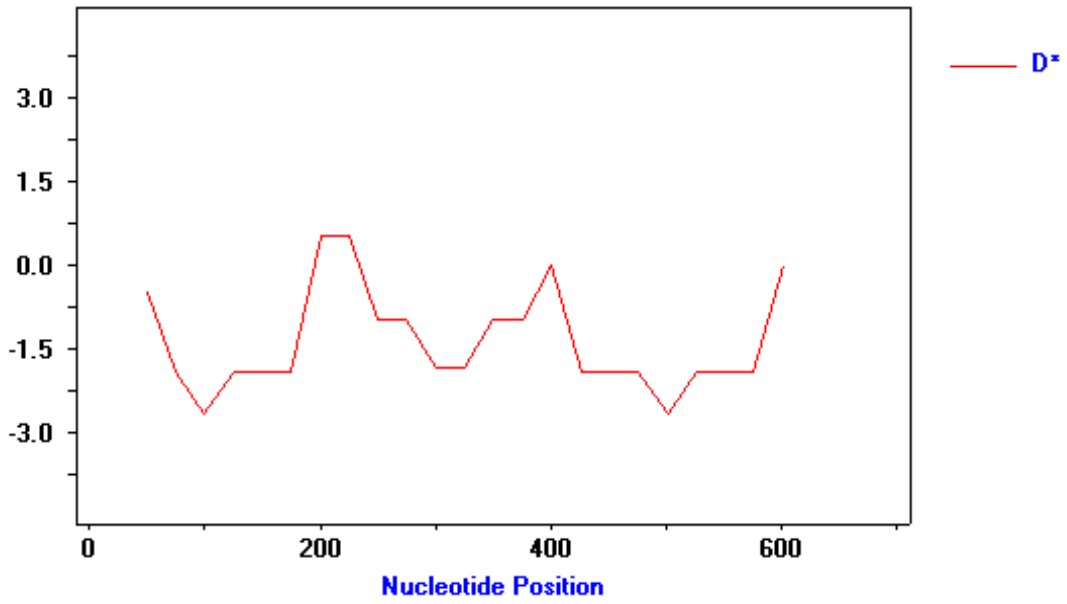
6

Table: 13: Graph predicting



5

Table:14 Graph predicting



7

Table:15 Graph predicting

8

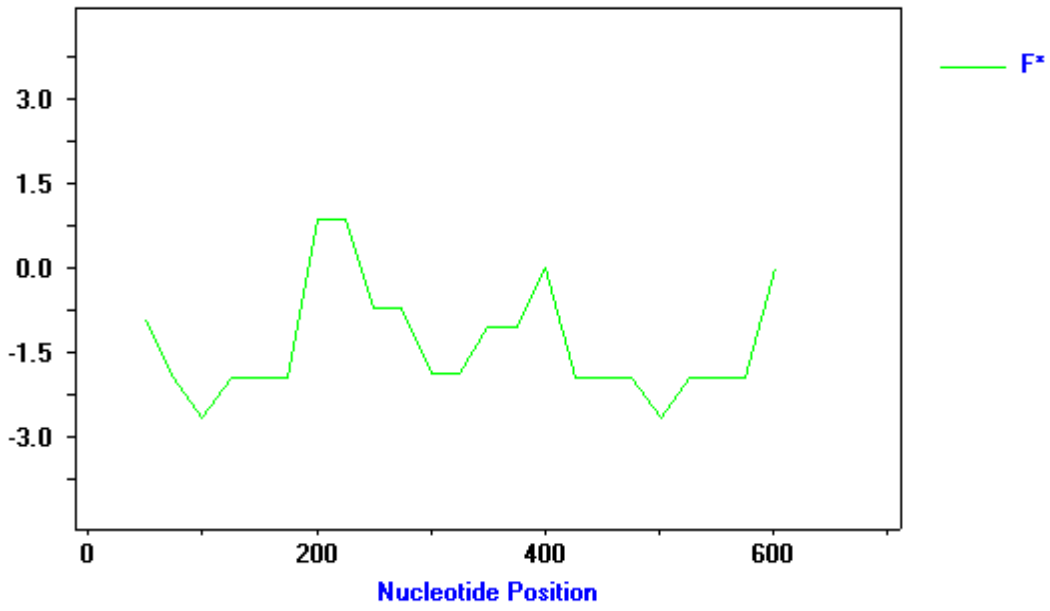


Table:16 Graph predicting

9

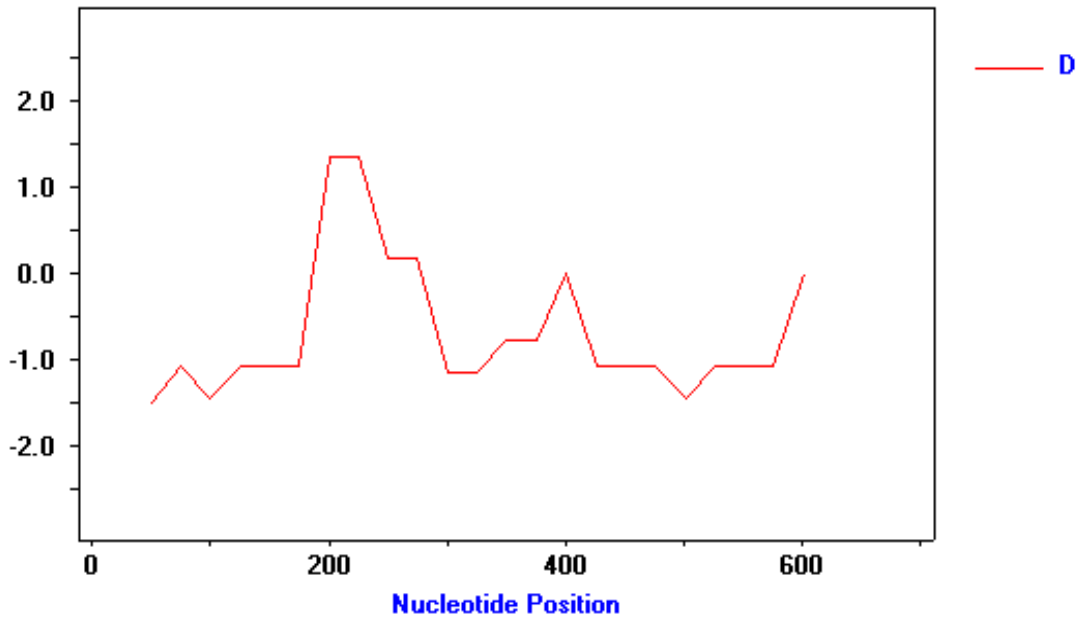
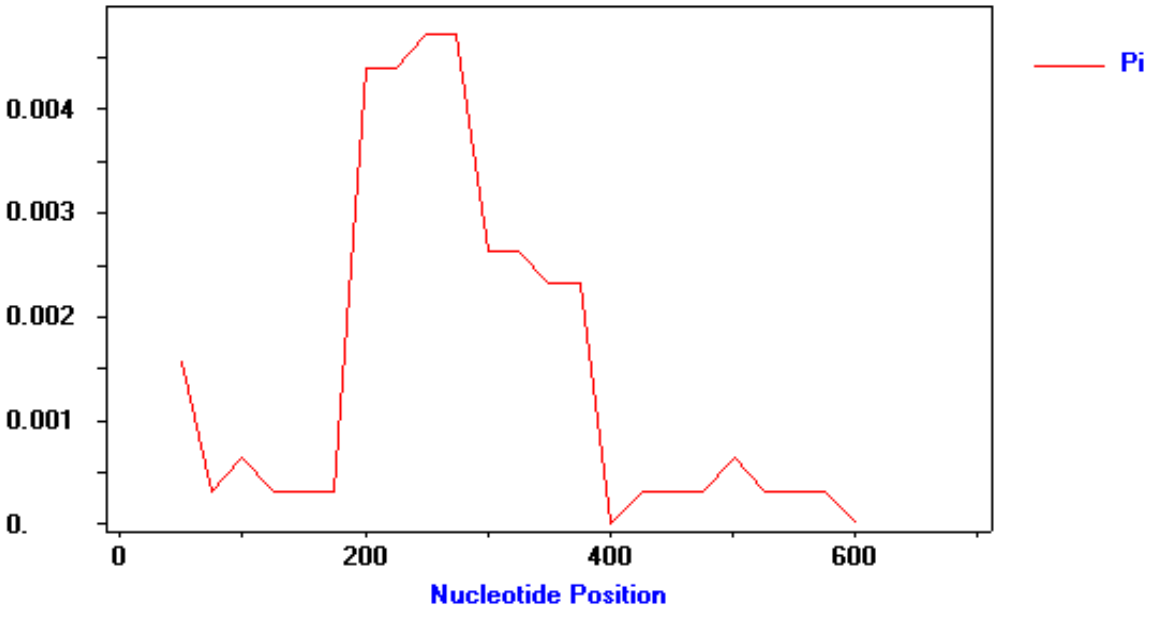
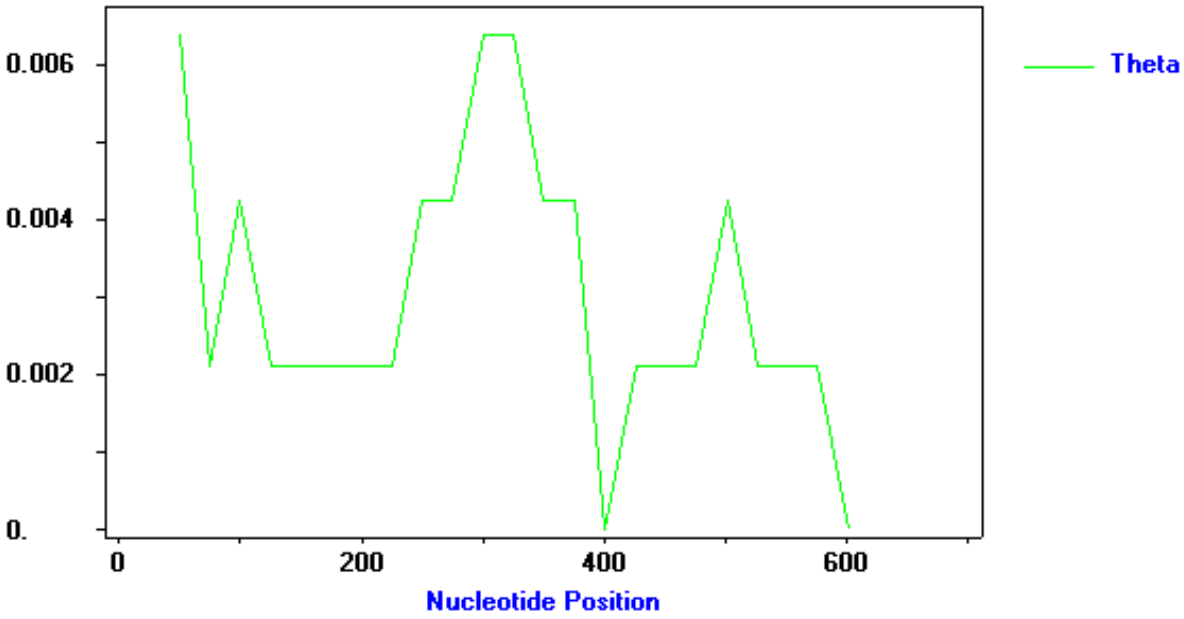


Table 17: Graph predicting



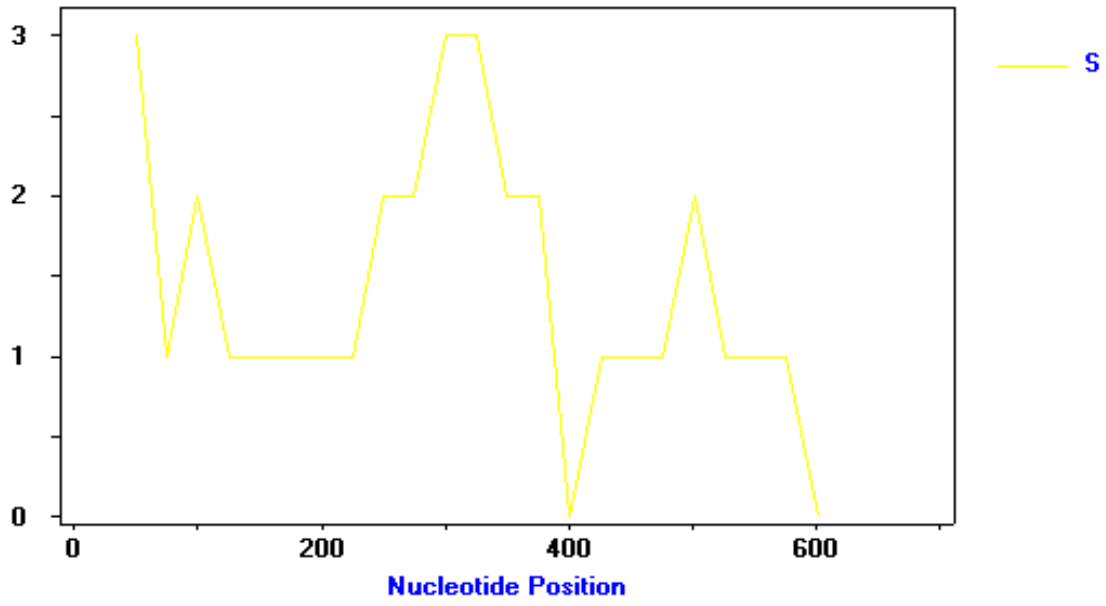
1

Table:18 Graph predicting



2

Table 19: Graph predicting



3

Table 20: Graph predicting

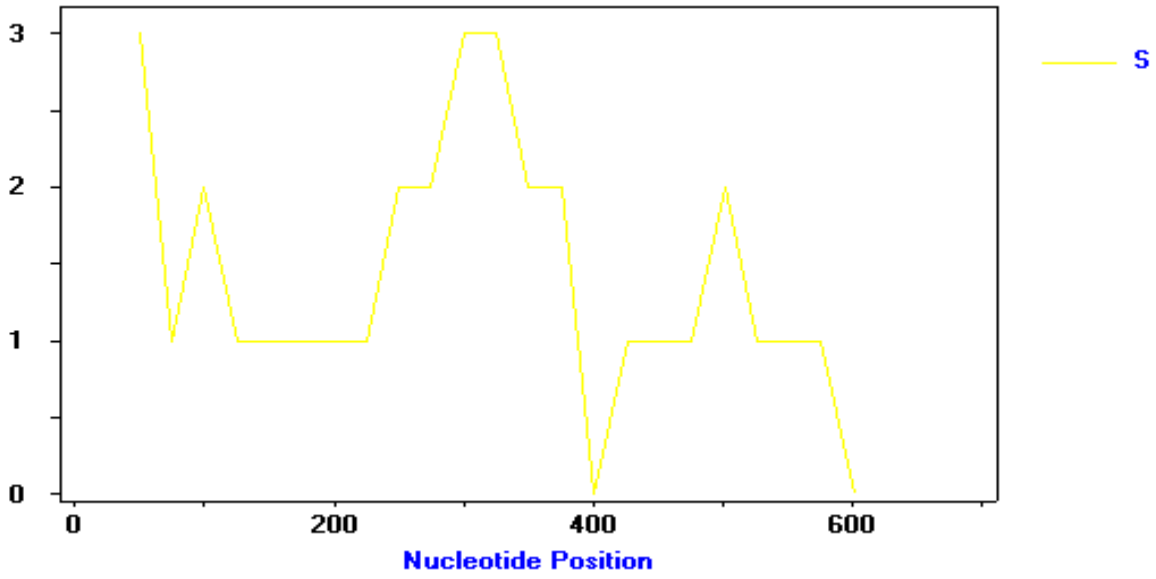


Table 21: Graph predicting

REFERENCES

♦ AKISHINONOMIYA FUMIHITO, TETSUO MIYAKE et. al. 1996; Monophyletic origin and unique dispersal patterns of domestic fowls. Evolution, Volume 93, pages 6792-6795, June 1996.

♦ AKISHINONOMIYA, F., MIYAKE, T., SUMI, S., TAKADA, M., OHNO, S. AND KONDO, N.1994; Proc. Natl. Acad. Sci. U.S.A. 91, pages 12505-12509.

♦ ANDERSON S, BANKIER AT, BARRELL BG, DE BRÜIJN MHL, COULSON AR, DROUIN J, EPERON IC, NIERLICH DP, ROE BA, SANGER F, SCHREIER PH, SMITH AJH, STADEN R, YOUNG IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290: pages 457–465

♦ ANTONIS ROKAS et. al. 2003; Animal Mitochondrial DNA recombination revisited, Trends in Ecology and Evolution, Volume 18, No. 8, pages 411-417.

♦ BROWN WM (1983). Evolution of animal mitochondrial DNA. In: Nei M, Koehn RK (eds) Evolution of Genes and Proteins. Sunderland, Mass: Sinauer, pages 62–88 .

♦ BROWN WM, GEORGE M, WILSON AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76: pages 1967–1971

♦ BROWN WM, PRAGER EM, WANG A, WILSON AC (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J Mol Evol 18: pages 225–239

♦ C. VILA et. al., 1999; Mitochondrial DNA phylogeography and population history of the Grey Wolf *Canis lupus*, Molecular Ecology (1999)8,pages 2089-2103.

♦ DENARO M, BLANC H, JOHNSON MJ, CHEN KH, WILMSEN E, CAVALLI-SFORZA LL, WALLACE DC (1981) ethnic variation in *HpaI* endonuclease patterns of human mitochodrial DNA. Proc Natl Acad Sci USA 78: pages 5768–5772

♦ DESJARDINS, P.and R MORARIES .(1990). Sequence and gene organization of the chicken Mitochondrial genome : A novel gene order in higher vertebrates.J Mol Biol 20:599-634.

♦ EWENS, W. J. (1972). The sampling theory of selectively neutral alleles. Theor. Pop. Biol. 3: 87-112.

♦ F. M. ELDATH , H.C. HINES (1996)Detection of nucleotide variations in the D-Loop region of Bovine mitochondrial DNA using polymerase chain reaction based methodology, Animal genetics ; vol,27 pages 333-336.

♦ FINNILA S, LEHTONEN MS, MAJAMAA K.et al,(2001) Phylogenetic network for European mtDNA. Am J Hum Genet. 2001; 68: pages 1475-84.

♦ FOS M, DOMINGUEZ MA, LATORRE A, MOYA A (1990) Mitochondrial DNA evolution in experimental populations of *Drosophila subobscura*. Proc Natl Acad Sci USA 87: pages 4198–4201

♦ FU, Y,D. NIU and H. RUAN (2001) Studies of genetic diversity of native chicken breeds in Zhejiang province of china . Acta Genetica Sinica .28:606-613.

♦ FU, Y.-X. (1995). Statistical properties of segregating sites. Theor. Pop. Biol. 48: 172-197.

♦ FU, Y.-X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915-925.

♦ FU, Y.-X. and W.-H. LI. (1993). Statistical tests of neutrality of mutations. Genetics 133: 693-709.

♦ GILBERT, D. (1996). A biological sequence editor and analysis program. Indiana University.

♦ HUDSON, R. R. (1990). Gene genealogies and the coalescent process. Oxf. Surv. Evol. Biol. 7: 1-44.

♦ HUDSON, R. R. (2000). A new statistic for detecting genetic differentiation. Genetics 155: 2011-2014.

♦ HUDSON, R. R. (2002). Generating samples under a Wright–Fisher neutral model of genetic variation. Bioinformatics 18: 337-338

♦ HUDSON, R. R. and N. L. KAPLAN. (1985). Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics 111: 147-164.

♦ JARZEMBOWSKI T., NAUMIUK L. AND CIECHANOWSKI M. 2004; control region variability of the mitochondrial DNA of Pipistellus nathusii (*Chiroptera, Vaspertilionidae*): first results of a population genetics study. Mammalia 68 (4): pages 421-425.

♦ JOHNSON MJ, WALLACE DC, FERRIS SD, RATAZZI MC, CAVALLI-SFORZA LL (1983) Radiation of human mitochondrial DNA types analyzed by restriction endonuclease cleavage patterns. J Mol Evol 19: pages 255–271

♦ JORDE LB, BAMSHAD MJ, WATKINS WS, ZENGER R, FRALEY AE, KRAKOWIAK PA, CARPENTER KD, SOODYALL H, JENKINS T, ROGERS AR (1995) Origins and affinities of modern humans: A comparison of mitochondrial and nuclear genetic data. Am J Hum Genet 57: pages 523–538

♦ JOSE A. CASTRO et. al., 1998; Mitochondrial DNA: A tool for populational genetics studies, International Microbiology (1998) 1: pages 327-332.

♦ JUAN C, OROMI P, HEWITT GM (1995) Mitochondrial DNA phylogeny and sequencial colonization of Canary Islands by darkling beetles of the genus *Pimelia* (Tenebrionidae). Proc R Soc Lond B 261: pages 173–180

♦ JUKES, T. H. and C. R. CANTOR. (1969). Evolution of protein molecules, pp. 21-132. In H. N. Munro (ed.), Mammalian Protein Metabolism. Academic Press, New York.

♦ KUMAR ,S . , K . TAMURA and M.NEI .(1993) MEGA : Molecular evolutionary genetics analysis , version 1.02. The Pennsylvania State University .

♦ L . PEGORARO et al, (1996) sequence comparison of Mitochondrial tRNA genes and origin of light strand replication in Bos tourus and Nellore (Bos indicus) breeds Animal genetics ; vol,27 ; pages 91-94.

♦ LANGLEY, C. H., Y. N. TOBARI and K. KOJIMA. (1974). Linkage disequilibrium in natural populations of Drosophila melanogaster. Genetics 78:921-936.

♦ LATORRE A, HERNANDEZ C, MARTINEZ D, CASTRO JA, RAMON M, MOYA A (1992) Population structure and mitochondrial DNA gene flow in Old World populations

♦ MOHD-AZMI , M. L., A. S. ALI and W.K.KHENG .(2000). DNA finger printing of RED JUNGLE FOWL , village chicken and broiler . Asian-Aust .J.Anim .sci 13: 1040-1043.

♦ MORTON, B. R. (1993). Chloroplast DNA codon use: Evidence for selection at the psb A locus based on tRNA availability. J. Mol. Evol. 37: 273-280.

♦ NEI, M. (1982). Evolution of human races at the gene level, pp. 167-181. In B. Bonne-Tamir, T. Cohen, and R. M. Goodman (eds.), Human genetics, part A: The unfolding genome. Alan R. Liss, New York.

♦ NEI, M. (1987). Molecular Evolutionary Genetics. Columbia Univ. Press, New York.

♦ NEI, M. and J. C. MILLER. (1990). A simple method for estimating average number of nucleotide substitutions within and between populations from restriction data. Genetics 125: 873-879.

♦ NEI, M. and T. GOJOBORI. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3: 418-426.

♦ NEI, M. and W.-H. LI. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269-5273.

♦ NISHIDA,T HAREGAWA et al .(1994). Polymorphic sequence in the D-Loop region of equine Mitochondrial DNA Animal genetics; vol 25; pages 215-221.

♦ OHNO, S. (1997). The one ancestor per generation rule and three other rule of mitochondrial inheritation.Proc .Natl . Acad .science USA . 94:8033-8035.pages 134-40.

♦ PERICIC M, LAUC LB, KLARIC IM, ROOTSI S, JANICIJEVIC B, RUDAN I, et al(2005). High-resolution phylogenetic analysis of Southeastern Europe (SEE) traces major episodes of paternal gene flow among Slavic populations. Mol Biol Evol. In press 2005.

♦ PETER FORSTER et. al., 2001; Phylogenetic Star Contraction Applied to Asian and Papuan mtDNA Evolution, Mol. Biol. Evol. 18(10), pages 1864-1881.

♦ PICORNELL A, MIGUEL A, CASTRO JA, RAMON M, ARYA R, CRAWFORD MH (1996) Genetic variation in the population of Ibiza (Spain): Genetic structure, geography, and language. Hum Biol 68: pages 899–913

♦ POLONI ES, SEMINO O, PASSARINO G, SANTACHIARA- BENERECETTI AS, DUPANLOUP I, LANGANEY A, EXCOFFIER et al,(1997) L.Human genetic affinities for Y-chromosome p49a,f/TaqI haplotypes show strong correspondence with linguistics. Am J Hum Genet. 1997;61: pages 1015-35.

♦ REBECCA L. CANN et. al., 1987; Mitochondrial DNA and Human Evolution, Nature 325, pages 31-36.

♦ RICHARDS M, CORTE-REAL H, FORSTER P, MACAULAY V, WILKINSON-HERBOTS H, DEMAINE A,(1996) et al. Paleolithic and neolithic lineages in the European mitochondrial gene pool. Am J Hum Genet. 1996;59: pages 185-203.

♦ RICHARDS M, MACAULAY V, HICKEY E, VEGA E, SYKES B, GUIDA V, et al(2000). Tracing European founder lineages in the Near Eastern mtDNA pool. Am J Hum Genet. 2000;67: pages 1251-76.

♦ RICHARDS MB, MACAULAY VA, BANDELT HJ, SYKES BC.(1998) Phylogeography of mitochondrial DNA in western Europe. Ann Hum Genet. 1998;62(Pt 3): pages 241- 60.

♦ RITTE U, NEUFELD E, PRAGER EM, GROSS M, HAKIM I, KHATIB A, BONNÉ-TAMIR B (1993) Mitochondrial DNA affinity of several Jewish communities. Hum Biol 65: pages 359–385

♦ ROGERS, A. R. and H. HARPENDING. (1992). Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9: 552-569.

♦ ROOTSI S, MAGRI C, KIVISILD T, BENUZZI G, HELP H, BERMISHEVA M, et al(2004). Phylogeography of Y-chromosome haplogroup I reveals distinct domains of prehistoric gene flow in Europe. Am J Hum Genet. 2004;75: pages 128-37.

♦ ROSSER ZH, ZERJAL T, HURLES ME, ADOJAAN M, ALAVANTIC D, AMORIM A, et al(2000). Y-chromosomal diversity in Europe is clinal and influenced primarily by geography, rather than by language. Am J Hum Genet. 2000;67: pages 1526-43.

♦ SOODYALL H, JENKINS T (1993) Mitochondrial DNA polymorphisms in Negroid populations from Namibia: New light on the origins of the Dama, Herero, and Ambo. Ann Hum Biol 20: pages 477–485

♦ STUART B. PIERTNEY et. al., 1999; matrilineal Genetic structure and female- mediated gene flow in Red Grouse, (*Lagopus lagopus scoticus):* An analysis using Mt DNA, Editor J. Neigel.

♦ TAJIMA, F. (1983). Evolutionary relationship of DNA sequences in finite populations. Genetics 105: 437-460.

♦ TAJIMA, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585-595.

♦ TAJIMA, F. (1989). The effect of change in population size on DNA polymorphism. Genetics 123: 597-601.

♦ The software can be obtained by anonymous ftp: iubio.bio.indiana.edu/molbio/seqpup

♦ TILFORD CA, KURODA-KAWAGUCHI T, SKALETSKY H, ROZEN S, BROWN LG, ROSENBERG M, et al(2001). A physical map of the human Y chromosome. Nature. 2001;409: pages 943-5.

♦ TOLK HV, PERICIC M, BARAC L, KLARIC IM, JANICIJEVIC B, RUDAN I, et al(2000). MtDNA haplogroups in the populations of Croatian Adriatic Islands. Coll Antropol. 2000;24: pages 267-80.

♦ TORRONI , A .,T. G. SHURR and M. F. CABELL (1993).Asian affinities and continental radiation of four founding Native American mitochondrial DNA . Am. J. HUM .GENET .53:563-590.

♦ TORRONI A, BANDELT HJ, D'URBANO L, LAHERMO P, MORAL P, SELLITTO D, et al.(1998) mtDNA analysis reveals a major late Paleolithic population expansion from southwestern to northeastern Europe. Am J Hum Genet. 1998;62: pages 1137-52.

♦ UNDERHILL PA, JIN L, LIN AA, MEHDI SQ, JENKINS T, VOLLRATH D, et al(1997). Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res. 1997;7: pages 996-1005.

♦ WATSON JD, GILMAN M, WITKOWSKI J, ZOLLER M (1992) Recombinant DNA. New York: WH Freeman and CO.WATTERSON, G. A. (1975). On the number of segregating sites in genetical models without recombination. Theor. Pop. Biol. 7: 256-276.

♦ WEIR, B. S. (1996). Genetic Data Analysis II. Sinauer Associates, Inc. Sunderland.

♦ Z. G. LIU et. al. 2004; Genetic variability of Mt- DNA sequences in Chinese native chicken breeds, Asian Aust. J. Animal. Sci., Volume 17: pages 903-909.