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Genetic Polymorphism of Mitochondrial Genome D-loop Region in Bhotia and Buxa Population of Uttarakhand

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Abstract

To study genomic diversity polymorphic DNA Markers are widely used as most of them are selectively neutral, more ubiquitous than polymorphic protein and enzyme markers. Mitochondrial DNA (mtDNA) characterized high mutation rates caused by lack of protective histones, inefficient DNA repair systems and continuous exposure to mutagenic effects of oxygen radicals. In the present study PCR-RFLP techniques were used to reveal polymorphism of mtDNA D-loop region in Bhotia and Buxa tribes of Uttarakhand. The results showed 3 restriction patterns for HaeIII enzyme for Bhotia community and no restriction pattern for Alu I enzyme. In Bhotia and Buxa tribes of Uttarakhand ethnic prejudice is very high and kinship marriages greatly occurred between them. Furthermore, both the tribes lived in a close geographical area and have a little and limited communication with other parts of the country and these conditions confirmed the low level of heteroplasmy and polymorphic patterns for mtDNA D-loop region that observed in this population.

Keywords: Genomic diversity; Bhotia; Buxa; mitochondrial DNA; Alu I; Hae III.

Introduction

Genetic Polymorphism is the variation at the genetic level. It occurs when two or more clearly different phenotypes exist in the same population of a species. It is related to biodiversity, genetic variation and adaptation; it usually functions to retain variety of form in a population living in a varied environment. Genetic polymorphism is a much more specific term and describes frequent variation at a specific locus in a genome. It arise from mutation at a locus followed by the action of evolutionary forces, such as natural selection or drift, which spread the mutant allele through the population in which it arose. Also, we now know that less than 5% of the human genome is coding DNA and so the vast majority of genetic polymorphisms lie in non-coding relatively less significant regions of our DNA.

The first DNA variants were detected in southern blots of genomic DNA digested with restriction enzymes. These restriction fragment length polymorphisms (RFLPs) are due to cleavage or non-cleavage at enzyme recognition sites in DNA, and the variability in the length of the DNA fragment is mainly due to the occurrence of SNPs which create or abolish the short sequences recognized by the specific enzymes used to digest the DNA. Restriction enzyme (RE) analysis detects only a proportion of DNA polymorphisms. On the whole, RE analyzes is more effective in detecting polymorphism, since it provides a direct view of non coding as well as coding DNA sequence. Early estimates suggest there are very roughly one or two simple polymorphic sites in every 1000 human bp but more refined estimates are beginning to emerge from deliberate large scale DNA sequence comparisons.

The human mitochondrial DNA (mtDNA) is a circular double-stranded molecule, and was first

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fully sequenced in 1981 by Anderson et. al. (Hoong & Lek, 2005). Most human cells contain hundreds of mitochondria and thousands of mitochondrial DNA copies. Mitochondrial DNA can be regarded as the smallest chromosome. Mt.DNA have 16,569 base pairs in length that codes for 13 subunits of the oxidative phosphorylation system, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). The DNA sequence of mt.DNA has been determined from a large number of organisms and individuals (including some organisms that are extinct), and the comparison of those DNA sequences allows biologists to elucidate the evolutionary relationships among species and also permits an examination of the relatedness of populations, and so has become important in anthropology. Mitochondrial DNA is present in high copy number in human cells, with high mutation rate, haplogroups, without recombination and maternal inheritance. These specifications make mt.DNA easier to obtain for analyzes, and also make it the molecule of choice for analyzing ancient DNA.

The Displacement loop (D-loop) occurs in the main non-coding area of the mitochondrial DNA molecule, a segment called the control region or D-loop region. The D-loop is the location of mitochondrial transcription promoters and it is the major control site for mt. DNA expression because it contains the leading-strand for origin of replication and major promoters for transcription.

Mitochondrial DNA replication begins in the D-loop resulting in the formation of a displacement loop with a newly synthesized heavy, or H, strand of about 700 nt known as 7S DNA. Both strands of the mt. DNA are completely transcribed from the promoters in the D-loop. In addition to the promoter sequences, there are two small regions known as the hyper variable regions I and II (HVI and HVII).

Mutation rates in HVI and HVII are especially high on average and there is evidence that the rates vary within the regions as well. Mt.DNA analysis has been focused mainly on the HVI and the correlation between the HVI and HVII regions has not been well established.

This greater polymorphic rate in the D-loop region has been reported in humans and polymorphism patterns of this region are variable in humans.

Tribal Demography of Uttarakhand

The tribal community and their habitats constitute very significant parts of our country. About half of the total tribal population of the world lives in India.

In Uttarakhand, there are five major tribal communities, namely Bhotia, Jaunsari, Raji, Tharu and Buxa. In the Indian context, a tribe is defined as a group with traditional territory, specific name, common language, strong kin relations, association with clan structure, tribal authority and rigid inclination to religion and belief.

The habitation of a particular tribe is confined to a particular tribal location in the Uttarakhand, such as Tharu and Bhoksa are confined to Tarai Bhabar region, while Bhotia (Shauka, Tolcha, Marcha and Jad) are confined to the higher altitudes of Dharchula, Munsyari (Pithoragarh Distt.), Kapkot (Bageshwar Distt.), Joshimath (Chamoli Distt.), Bhatwari and Dunda blocks (Uttarakashi Distt.), and Raji is confined to Kanalichhina and Champawat blocks. Likewise, Jaunsari resides in Chakrata and Kalsi blocks of Dehradun district.

Agriculture forms the basic livelihood of Tharu, Bhoksa and Jaunsari tribes (Pant, 2006). Trade is the main source of livelihood of the Bhotia tribe. It is worth mentioning that as per 1991 census Jaunsari (34.24%) and Tharu (33.3%) contribute more than two third tribal population of the state. Bhotia and Bhoksa tribes have about 15.8 and 15.52%, respectively and only 0.6% tribal population belongs to Raji tribe while 0.54% population is enumerated as unclassified. Traditionally, agriculture, animal grazing and breeding have been the main occupations of the Bhotias. However, with the advent of time, the Bhotias have increasingly engaged in government services and business ventures, whereas Bhoksa or sometimes pronounced Buxa are indigenous people living mainly in the Uttarakhand. They are mostly concentrated in the Dehradun and Nainital in the Kumaon foothills of the outer Himalayas. The Bhoksa are found along the slopes of the lower Himalayas, in a forested region known as the Terai. In Uttarakhand, they are found in Udham Singh Nagar and Dehradun districts.

A molecular marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed. A molecular marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like mini satellites.

Types of Molecular Markers:

Some commonly used types of molecular markers are: AFLP, RAPD, SSLP, SNP, VNTR, STR, RFLP. In molecular biology, restriction fragment length polymorphism, or RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated.

Analysis Technique:

The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe. An RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analyzes.

Limitations

- The process is extremely laborious and time-consuming.
- Radioactive probes pose health and disposal risks.
- A relatively large amount of sample is required to perform the tests.
- The method requires high molecular weight, un-degraded DNA.

Aims and Objectives

The main aim of study was to determine the mitochondrial genome D-loop region polymorphism in Bhotia and Bhoksa populations of Uttarakhand.

The result obtained can be further used to establish the phylogenetic relationship between these communities.

The study was taken up with the following objectives:

1. To collect the blood samples.
2. To extract the DNA from collected blood samples using Phenol-Chloroform method.
3. To quantify the DNA using Biophotometer.
4. To amplify 1024 bp long region of D-loop.
5. To digest the Amplified product with the help of Restriction Enzymes.

Ingman et. al., (2001) Suggested that analyzes of mitochondrial DNA (mt.DNA) sequences has been a potent tool in our understanding of human evolution. However, almost all studies of human evolution based on mt.DNA sequencing have focused on the control region, which constitutes less than 7% of the mitochondrial genome & described a suitable methodology for determining the complete human mitochondrial sequence and the global mt.DNA diversity in humans & also discussed the implications of the results with respect to the different hypotheses for the evolution of modern humans.

Kumar et. al., (2003) studied status of Austro-Asiatic groups in the peopling of India on the available prehistoric, linguistic and biological evidences. It has been suggested that the communities affiliated to the Austro-Asiatic linguistic family are perhaps the first to settle in India and the paleo-anthropological evidences suggest the earliest settlement probably around 60,000 years. Recent speculations, based on both traditional genetic markers and DNA markers, seem to corroborate the aforesaid view. The results of the analyses of anthropometric and genetic marker data indicate that the Austro-Asiatic groups, particularly the Mundari speakers with certain exceptions, show greater homogeneity among them when compared to the other linguistic groups, although certain groups show as outliers (Kumar et. al.,2003). However, traditional genetic markers show lower within population heterozygosis compared to Dravidian and other Indian populations. This is contrary to what has been claimed in case of certain DNA markers.

Jin-young et. al.,(2006) analyzed that molecular nature of mitochondrial DNA (mt.DNA) could be power full marker of anthropological studies of modern population and surveyed mtDNA polymorphism in the HVSI of noncoding D-loop region and its upstream region from 430 unrelated Korean population by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing analyzes.

Bhasin., (2009) concluded that the variation in the frequencies of genetic markers and mean values of morphological traits distribution in the

Himalayan may due to contact between various population group of western and central Himalayas with population group of Central Asia, that of Eastern Himalayas with the Northern Mongoloid populations. In the Central India admixture with the population group of West India can be assumed. In South India, different patterns of allele/haplotype frequencies and mean value is generally observed among the tribal population groups, for which one of the main causes might be seen in small population sizes. Inbreeding is prevalence among certain communities like Muslim, Parsis etc. and in most of the different population group particularly from South India, which might have also resulted in the marked variation in the distribution of frequencies and mean values among the Indian population are due to racial elements present among them in varying degrees, migrations and admixture from time to time and other factors of evolutionary changes like mating patterns genetic drift, mutation and selection under different environments.

Dada et. al., (2012) revealed that a genetic sketch of six population groups of Rajasthan based on 12 autosomal loci . The Study was conducted to gain an insight in to inter and intra-population affinities or variations among the six populating groups of Rajasthan. DNA samples from 221 unrelated individuals belonging to six endogamous population group of Rajasthan, including both tribal (Bhil, Damaria, Garasia, Mina and Sharia) and caste population (Rajput) were screened for 12 DNA (seven Alu in- del and five RFLP) markers. All the loci were found to be polymorphic in all the studied population. The Gst values which determines the group of Rajasthan are genetically identical to other Indian population, reflecting a common genetic unity among the Indian populations.

Materials and Methodology

4.1 Reagents Used - The list of reagents that were used in the project are summarized as follows:

0.5M Tris-HCl:-

6.005gm Tris in 100ml double distilled water. (pH – 7.5)

0.5M KCl:-

3.7275gm of Kcl in 100ml of double distilled water.

0.5M MgCl2:-

4.7605gm MgCl2 in 100ml double distilled water.

0.5M EDTA:-

18.612gm EDTA in 100ml double distilled water. (pH- 8.0).

10 % SDS:-

10gm SDS in 100ml double distilled water.

1M NaCl:-

5.844gm NaCl in 100ml double distilled water.

TKM1(2X) for 100ml:-

*4ml of 0.5M Tris-HCl.

*4ml of 0.5M KCl.

*4ml of 0.5M MgCl2.

*0.8ml of 0.5M EDTA.

*87.2ml of MQ water (volume make up to 100ml)

TKM1 (1X) Low salt buffer:-

*100ml of TKM1 (2X)

100 ml double distilled water mixed with 100 ml TKM1 (2X) and make 200 ml of TKM1 (1X) buffer.

TKM2 (1X) High salt buffer:-

*50ml of TKM1 (2X)

*40ml of 1M NaCl

*10ml of double distilled water, make up 100ml of TKM2(1X) Solution

5M Sodium perchlorate:-

*70.23gm sodium perchlorate in 100ml double distilled water

TE Buffer:-

*4ml Tris-Hcl (0.5M)

*0.4ml EDTA (0.5M), make up to 200ml

NSS (Normal Saline Solution)

*9gm NaCl in 100ml double distilled water.

4.2 Functions of Reagents:

- *NaCl:* It removes polysaccharides as they inhibit PCR reactions. Polysaccharides interfere with several biological enzymes such as Polymerases, Ligases and Restriction Endonucleases. NaCl facilitated the removal of polysaccharides by increasing their solubility in ethanol so they did not precipitate with the DNA effectively decreasing co-precipitation of the polysaccharides and DNA.
- *Tris:* Tris interacts with lipopolysaccharides present on the outer membrane which helps in making membrane permeable. This effect is enhanced with the addition of EDTA.

- *Ethylene Diamine Tetra-acetic Acid (EDTA):* It is a chelating agent. It traps magnesium ions Mg2+ and prevents DNAase activity. Further inactivates nucleases, by binding to metal ions required by these enzymes and prevents degradation of DNA.
- *Ascorbic acid:* It helps in the removal of polyphenols in DNA extraction.
- *β-mercaptoethanol:* It prevents oxidation of secondary metabolites. It is used as an antioxidant, especially for polyphenolics which become insoluble when oxidized and tend to precipitate with the DNA. It is often included in DNA extraction buffers to prevent oxidation of polyphenols present.
- *Chloroform:* It is used to remove soluble proteins from the sample.
- *Isoamylalcohol (IAA):* It separates phenol from the solution. The ratio of chloroform and isoamyl alcohol depends on phenolic compounds and proteins.
- *Ethanol (96%, 70%):* Used for washing the pellet and also for the removal of contamination.
- *Tris EDTA(TE) Buffer:* It maintains the molecular structure at 8.2 and keeps in suspended form."TE" is derived from its components: Tris, a common pH buffer and EDTA, a molecule chelating like Mg2+.The purpose of TE buffer is to protect DNA or RNA from degradation.
- *Isopropanol*: It's main role is to precipitate the DNA by engaging with water molecules as not giving chance for the DNA to get dissolve in the water.
- *Potassium Chloride:* Salt would attract the phosphate ends of DNA, therefore it pulls it way from other substance in the sample (separation of DNA from surroundings).
- *Tris Borate EDTA (TBE):* TBE usually use like TAE in electrophoresis. The difference in the size of molecular DNA that we want to separate. We can use TBE for the small size than TAE.
- *Lysozyme:* Lysozyme is an enzyme that is used during DNA extraction to degrade the cell wall.
- *Sodium Dodecyl Sulphate (SDS):* SDS is an anionic detergent which disrupts cell membrane and destabilizes all hydrophobic interaction holding macromolecules in their native form.
- *Sucrose:* It provides osmotic shock to the blood cells (leukocytes). By adding sucrose to the solution it absorbs (osmosis) glucose from the cell, hence cell is shrieked and ruptured, so nucleus is available for extraction.

Methodology

Sample Collection; Twenty blood Samples were randomly collected from the Bhotia and Bhoksa community. Samples were obtained from Nimbuwala and Shimla by-pass region of Dehradun. Blood samples were collected through disposable syringes in spray dried K2 EDTA vacationer. 5 ml amount of blood was collected from each individual along with their consent.

Equipments: PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify (Wallace, D.C.1994). At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

The PCR reaction requires the following components:

1. DNA template - the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

2. DNA polymerase – an enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is Taq DNA polymerase (from *Thermis aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is widely used bacause of its higher fidelity when copying DNA.

3. Primers - short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

4. Nucleotides (dNTPs or deoxynucleotide triphosphates) - single units of the bases A, T, G, and C, which are essential "building blocks" for new DNA strands.

RESULTS

5.1 DNA Quantification Result

The Purity of extracted DNA samples varies from1.61 to 1.88. The concentration and purity of these samples are below in table 5.1.

The purity value of highly pure DNA samples lies around 1.8.

Table 5.1: O.D. of DNA samples of Bhutia and Bhoksa community

S.No.	Sample	A_{260}	Purity	Concentration(µg/ml)
1.	BHO01	0.977	1.73	1221.4
2.	BHO02	0.508	1.89	635.5
3.	BHO03	0.816	1.88	1019.6
4.	BHO04	0.795	1.70	993.9
5.	BHO05	0.456	1.93	570.0
6.	BHO06	0.337	2.36	420.9
7.	BHO07	0.177	1.83	221.6
8.	BHO08	0.585	1.87	730.9
9.	BHO09	0.941	1.21	1176.0
10.	BHO10	0.358	1.89	448.0
11.	BHK01	0.076	1.68	95.0
12.	BHK02	0.303	1.81	378.5
13.	BHK03	0.034	1.61	240.7
14.	BHK04	0.193	2.21	42.8
15.	BHK05	0.474	1.88	592.0
16.	BHK06	1.33	1.019	24.0
17.	BHK07	1.91	0.540	675.1
18.	BHK08	1.633	1.22	2040.6
19.	BHK09	0.014	1.50	17.0
20.	BHK10	0.024	1.18	59.2

PCR Amplification results:

The 1024 bp long DNA sequence of D-Loop region of human mitochondrial genome was amplified by PCR.

Fig. 8: Amplification of target DNA sample of Bhotia community

Lane 1-Marker 100 bp

Lane 2-11 Identification no. BHU01-10

Lane 12 Negative control which showed no amplification

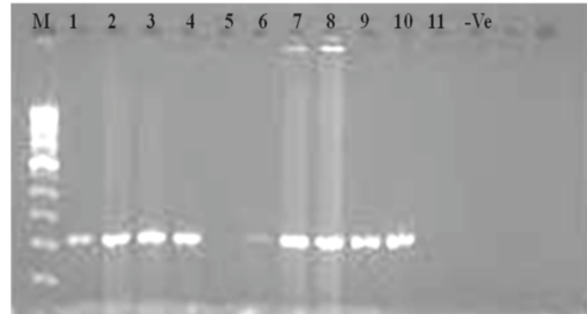

Fig.9: Amplification of target DNA sample of Bhoksa community

Lane 1-Ladder 250 bp

Lane 2-12 Identification no. BHK01-10

Lane 13 Negative control which showed no amplification

5.3 Restriction Digestion

The Amplified PCR product was digested with restriction enzymes namely HaeIII & AluI.

Recognition Sites and Cleveage Sites of the Restriction enzymes are as follows.

Hae III- 5′ G G ↓ C C 3

3′ C C ↑ G G 5′

Alu I - 5′ A G ↓ C T 3′

3′ T C ↑ G A 3′

Fig.10: Digestion results of PCR products of mt.DNA D-loop region with HaeIII Enzyme in Bhotia tribe. (Lane M) is ladder having stretches of DNA of 100 bp, lane 1 is 384 and 500, lane 3,4,5,6 is 444 & 490, lane 7,,8,9,10 is 480 & 544

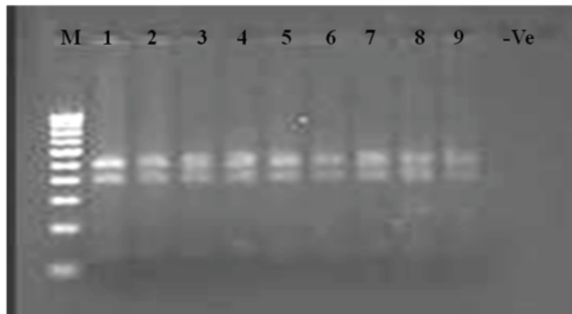

Fig. 11: Digestion results of PCR products of mt.DNA D-loop region with AluI Enzyme in Bhotia tribe. (Lane M) is ladder having stretches of DNA of 100 bp, lane 2-10 is 400 and 500 bp. No Polymorphism was observed.

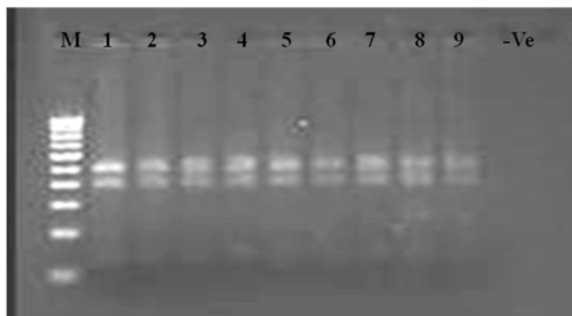

Fig. 12. No Polymorphism was observed in Digestion of PCR products of mt.DNA D-loop region with AluI enzyme in Bhoksa tribe. All the bands are of same size.

Discussion

The properties of mt.DNA D-loop that make it valuable for both evolutionary and human identification studies include the high copy number, cytoplasmic inheritance, and rapid rate of evolution (Miyazono et. al., 2002; Pereira et. al., 2010).

The mutation rate of mt.DNA is several orders of magnitude higher than that of nuclear genes, with an estimated rate of 1.7 times 10-8 substitutions per site per year for the whole genome excluding the control region (Ingman et. al., 2000).

Tracing migrations of Homo sapiens with the help of mt.DNA is based on the observation that the occurrence of certain haplotypes is often associated with certain world regions and the assumption that this is a result of an accumulation of various mutations in different maternal lineages that occurred as people migrated and inhabited new regions (Tiedemann et. al., 2000).

Many other methods of studying mt.DNA recently applied, such as RFLP analysis and direct sequencing; enable more precise calculations to be made. A number of techniques, developed in the last few decades have greatly contributed to the methodology used, with the most pronounced ones, such as PCR based methods that allowed the copying of even minute amount of the sequence of interest (Hoong & Lek, 2005).

In the present study PCR-RFLP techniques were used to reveal polymorphism of mt.DNA D-loop region in Bhotia and Bhoksa tribes of Uttarakhand. The results were showed 3 restriction patterns for HaeIII enzyme for Bhotia community and no restriction pattern for Alu I enzyme. The results showed low heteroplasmy and polymorphic patterns for mt.DNA D-loop region after digestion with these enzymes in these populations.

In Bhotia and Bhoksa tribes of Uttarakhand ethnic prejudice is very high and kinship marriages greatly occurred between them. Furthermore, Bhotia and Bhoksa tribes live in a closed geographical area and have a little and limited communication with other parts of the country and these conditions confirmed the low level of heteroplasmy and polymorphic patterns for mt. DNA D-loop region that observed in this population. These specifications could be used in human population studies and forensic investigations of Bhotia and Bhoksa tribes of Uttarakhand.

The above findings indicated that D-loop region of human mt.DNA variability, as detected by the number of different RFLP morphs, is useful for detection of maternal inheritance.

Our results are consistent with the facts that low levels of variability in mt. DNA D-loop region in Bhotia and Bhoksa tribes are found. Characteristics and conditions such as ethnic prejudice, existence of traditional rural area and lack of the appropriate roads with other parts of the state that cause high level of kinship marriages and low migration in Bhotia and Bhoksa population and promote human population studies and forensic investigations in future.

Summary

In this study, the blood samples of 20 unrelated individuals of two different communities i.e. of Bhutia and Bhoksa inhabiting different regions of Uttarakhand were collected in dried K2 EDTA vacutainers. The DNA was extracted from the collected blood sample by using phenol: chloroform: iso amyl alcohol method and stored at -20oC.

Unlike nuclear DNA, which is inherited from both parents and in which genes are rearranged in the process of recombination, there is usually no change in mitochondrial DNA from parent to offspring. Because of this and because the mutation rate of human mitochondrial DNA is higher than that of nuclear DNA, mt.DNA is a powerful tool for tracking ancestry through females (matrilineage) and have been used to track the ancestry of many species back hundreds of generations. The low effective population size and rapid mutation rate(in humans) and absence of recombination makes mt.DNA useful for assessing genetic relationship of individuals or groups within a species and also for identifying and quantifying the phylogeny among different species, provided they are not too distantly related.

The extracted DNA was quantified to determine the purity and the concentration of the DNA samples. The amplification of the 1024 bp long sequence of D-loop region of mt.DNA was carried out using the primers (Forward primer- 5′ CACCATTAGCACCCA 3′; Reverse Primer- 5′ CTGTTATTTGTGCATACCGCCA 3′).

The amplified product was digested with two restriction enzymes namely HaeIII and Alu I. The restriction pattern was observed using agarose gel electrophoresis. The gel pattern of the digested DNA showed high level of variability in Bhotia while no polymorphism was found in Bhoksa community. The sample digested with HaeIII revealed high level variability in Bhotia community (i.e. □ 40% variability in Bhotia and none in Bhoksa) as compared to Alu I that shows no variability in both the communities.

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