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COMPARITIVE STUDY OF GENETIC DIFFERENTIATION BETWEEN *Plasmodium falciparum* & *plasmodium vivax* IN NORTHERN INDIA

BY

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MAY2010

Submitted in partial fulfillment of the Degree of Bachelor of Technology

DEPARTMENT OF
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CERTIFICATE

This is to certify that the work entitled, "**Comparative study of the genetic differentiation between *Plasmodium vivax* and *Plasmodium falciparum* in Northern India** " submitted by Tushar Kalia and Gurneet Singh Jolly in partial fulfillment for the award of degree of Bachelor of Technology in 2009 of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Harvinder Singh

ACKNOWLEDGMENT

This is to thank our project guide, Dr. Harvinder Singh and all other biotechnology faculty for providing us able guidance to help us pursue our project in the best of directions.

Our special thanks to Head of bioinformatics and biotechnology department JUIT for providing all necessary equipments in our laboratory and an opportunity for exploring our knowledge through valuable project.

We would also like to acknowledge our PhD scholars for their constant support.

Furthermore our lab assistants were a constant support for providing us with a good atmosphere and equipments for carrying out our project in the best manner and thanks to our classmates for their support and motivation.

Our sincere thanks to PGIMER Chandigarh and Dr. Vikram in Muktsar (Punjab) for providing us with malarial infected blood samples and Dr. Umar Farooq for his tireless support with the experimental processes.

Tushar Kalia

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ABSTARCT

The genetic diversity, evolutionary history of the malaria parasite and how it is genetically distinct in different regions of the genome and populations may open up new avenues to population specific malaria control measures. The two principal human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*, seem to be very different in origin and in phylogenetic resemblance to other species of *Plasmodium*. Further, the genetic differentiation that these two parasites express are quite diverse, so also the percentage of nucleotide composition in their respective genomes. Higher genomic diversity in *Plasmodium vivax* due to high mutations rate was observed in Northern India.

SSRs, by virtue of their special mutational and functional qualities, have a major role in generating the genetic variation underlying adaptive evolution. While microsatellites are considerably less common in the *P. vivax* genome than in the AT-rich *P. falciparum* genome and also tend to be shorter in length, these markers can provide useful tools for assessing population structure and for searching for evidence of recent selection events associated with drug resistance. The study of these genetic mutations has been based on polymerase chain reaction (PCR) as the results are validated by wet lab experiments. The amplification by the SSR based primers confirms the polymorphism in *Plasmodium falciparum* and *P. vivax*.

Strand compositional asymmetry may reflect on differences in replication synthesis of the leading versus lagging strand, on differences between template and coding strand giving clearer view about the genome complexity.

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LIST OF ABBREVIATIONS

RFLP – Restriction Fragment length Polymorphism

RAPD- Random amplified polymorphic DNA

ISSR - Inter-simple sequence repeat

SSR - Simple sequence repeats

AFLP - Amplified fragment length polymorphism

SCAR - Sequence characterized amplified region

CAPS - Cleaved amplified polymorphic site

STS- Sequence tagged site

PCR- Polymerase Chain Reaction

NCBI – National Center For Biotechnology Information

EDTA – Ethylene Diamine Tetra Acetate

TAE – Tris Acetate EDTA

CHAPTER 1
INTRODUCTION

Malaria, a disease of antiquity, has proved to be a formidable deterrent to the cultural and socioeconomic progress of man in the tropical, sub-tropical and monsoon prone zones of the world. History is replete with instances of devastation caused by this disease. Malaria is a vector-borne infectious disease caused by protozoan parasites. It is widespread in tropical and subtropical regions, including parts of the Americas, Asia, and Africa. Malaria is one of the most common infectious diseases and an enormous public health problem. The disease is caused by protozoan parasites of the genus *Plasmodium*. Each year, there are approximately 350–500 million cases of malaria, killing between one and three million people, the majority of whom are young children in sub-Saharan Africa.

1.1 Strains of malaria

Five species of the plasmodium parasite can infect humans; the most serious forms of the disease are caused by *Plasmodium falciparum*. Malaria caused by *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* causes milder disease in humans that is not generally fatal. A fifth species, *Plasmodium knowlesi*, is a zoonosis that causes malaria in macaques but can also infect humans. *P. falciparum* is the most common cause of infection and is responsible for about 80% of all malaria cases, and is also responsible for about 90% of the deaths from malaria. Parasitic *Plasmodium* species also infect birds, reptiles, monkeys, chimpanzees and rodents. (*Fong YL et al, 1971*)

Plasmodium falciparum

Plasmodium falciparum is a protozoan parasite, one of the species of *Plasmodium* that cause malaria in humans. It is transmitted by the female *Anopheles* mosquito. *P. falciparum* is the most dangerous of these infections as *P. falciparum* (or malignant) malaria has the highest rates of complications and mortality. As of 2006 it accounted

for 91% of all 247 million human malarial infections (98% in Africa) and 90% of the deaths.

It is more prevalent in sub-Saharan Africa than in other regions of the world; in most African countries, more than 75% of cases were due to *P.falicparum*, whereas in most other countries with malaria transmission, other Plasmodial species predominate.

Plasmodium vivax

Plasmodium vivax is a protozoal parasite and a human pathogen. The most frequent and widely distributed cause of recurring (tertian) malaria, *P. vivax* is one of four species of malarial parasite that commonly infect humans. It is less virulent than *Plasmodium falciparum*, which is the deadliest of the four, and seldom fatal. *P. vivax* is carried by the female *Anopheles* mosquito, since it is the only sex of the species that bites.

Plasmodium ovale

Plasmodium ovale is a species of parasitic protozoa that causes tertian malaria in humans. It is closely related to *Plasmodium falciparum* and *Plasmodium vivax*, which are responsible for most malaria. It is rare compared to these two parasites, and substantially less dangerous than *P. falciparum*.

Plasmodium malariae

Plasmodium malariae is a parasitic protozoa that causes malaria in humans. It is closely related to *Plasmodium falciparum* and *Plasmodium vivax* which are responsible for most malarial infection. While found worldwide, it is a so-called "benign malaria" and is not nearly as dangerous as that produced by *P. falciparum* or *P. vivax*.

1.2 Malaria in India

Malaria has been known in India from times immemorial. J.A. Sinton, the first Director of the Malaria Institute of India, estimated in 1935 that atleast 100 million people suffered from malaria in India and about one million deaths occurred annually in endemic areas. In 1947, after independence, it was estimated that 75 million people suffered from the disease every year. The mortality, as direct result of disease, was placed at 0.8 million per annum.

There are about 400 species of anopheline mosquitoes throughout the world, but only 60 species are vectors of malaria. In India 9 species out of 45 anopheline species have been incriminated as malaria vectors. The capacity of the vector to transmit malaria results from the interaction between the environment, both natural and man-made and genetically determined characteristics.

In India 60 to 65 % of the infections are due to *P. vivax* and 35 to 40% due to *P. falciparum*. Only few cases of *P. malariae* have been reported from Orissa and Karnataka. (*Shiv Lal et. al, 2000*)

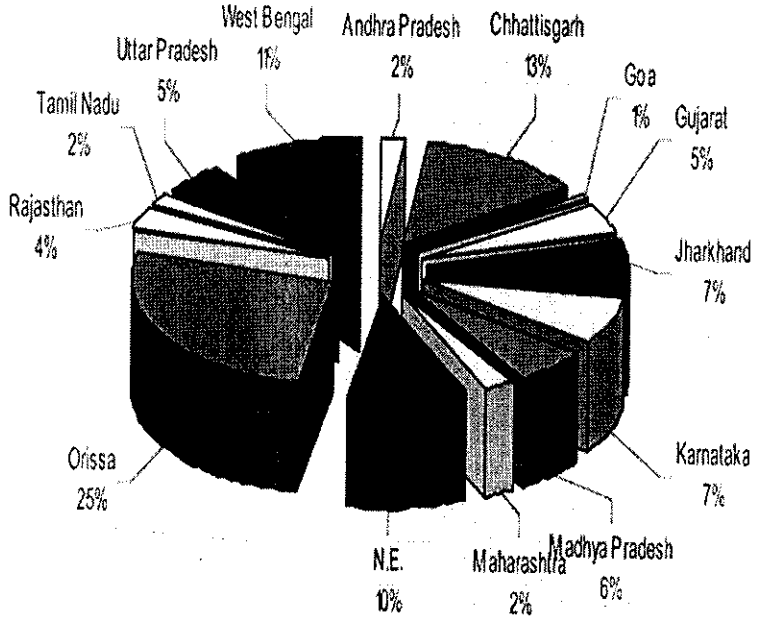

FIG 1.1 :- Statewise distribution of malaria

TABLE 1.1 - Year wise distribution of malaria cases

Year	Total cases	*P. falciparum*	Deaths
1947	75 million	?	8,00,000
1961	49151	?	--
1965	99667	?	--
1976	6.47 million	0.75 million	59
1984	2.18 million	0.65 million	247
1985	1.86 million	0.54 million	213
1986	1.79 million	0.64 million	323
1987	1.66 million	0.62 million	188
1988	1.85 million	0.68 million	209
1989	2.05 million	0.76 million	268
1990	2.02 million	0.75 million	353
1991	2.12 million	0.92 million	421
1992	2.13 million	0.88 million	422
1993	2.21 million	0.85 million	354
1994	2.51 million	0.99 million	1122
1995	2.93 million	1.14 million	1151
1996	3.04 million	1.18 million	1010
1997	2.57 million	0.99 million	874
1998	2.09 million	0.91 million	648
2002	1.84 million	0.87 million	973
2003	1.86 million	0.85 million	1006
2004	1.91 million	0.89 million	949
2005	1.81 million	0.80 million	963
2006	1.78 million	0.84 million	1707
2007	1.5 million	0.74 million	1311
2008	1.52 million	0.75 million	935
2009 (April)	0.27 million	0.16 million	130

1.3 Signs and symptoms

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia. The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three for *P. malariae*. Malaria has been found to cause cognitive impairments, especially in children. It

causes widespread anemia during a period of rapid brain development and also direct brain damage. (*Beare NAV et al, 2006*)

Objectives:

- To study the genetic diversity of *P. vivax* and *P. falciparum* in Northern India.
- In silico analysis of frequecy and distribution of SSRs in *P. vivax* and *P. falciparum* Genome
- Comparative study of genome complexity in both genomes
- Validation of the in-silico predicted SSR markers in *P. vivax* and *P. falciparum*.

CHAPTER 2

REVIEW LITERATURE

2.1 Types of DNA markers

Techniques for generating DNA markers have undergone rapid change in the last 30 years. The use of a specific marker technique is determined by the crop species, technical expertise, lab equipment, and research funding. Molecular markers, unlike morphological markers are stable and have been found to be very useful in population studies. (*Aitkin et al., 1994; Lakshmi et al., 1997*)

The most commonly-used types of DNA markers, table 2.1 include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeats (SSR) and amplified length fragment polymorphism (AFLP). Many variations also exist on these methods and new marker techniques are constantly appearing. PCR-based markers are generally preferred due to their technical simplicity for generation and are relatively cheap. SSRs are PCR-based markers and extremely versatile, since they are used for basic and applied research.

2.2 Properties desirable for ideal DNA markers

- Highly polymorphic nature
- Codominant inheritance (determination of homozygous and heterozygous states of diploid organisms)
- Frequent occurrence in genome
- Selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices)
- Easy access (availability)
- Easy and fast assay
- High reproducibility
- Cost

Table 2.1 - Characteristics of commonly-used types of DNA markers

Marker type	Feature of method	Lab procedure	Reliability	Cost
RFLP	Restriction digestion of genomic DNA and detection with cDNA or genomic DNA probes (via Southern blotting)	Complex	High	High
RAPD	Random amplified markers usually generated by 10-mer arbitrary primers	Simple	Low	Low
ISSR	Primers designed from simple sequence repeat motif with 'anchors'	Simple	Medium	Low
SSR	Primers designed from DNA sequences flanking SSR repeats.	Simple	High	Low
AFLP	Genomic DNA restriction digestion followed by adapter ligation and PCR.	Complex	High	High
SCAR or STS	represent 'second generation' markers derived from specific DNA sequences of markers (e.g. RFLPs, RAPDs or AFLPs)	Simple	High	Low
CAPS	Presence/absence of a restriction enzyme site in PCR amplicons.	Moderate	High	Medium

In case of PCR based markers, the primers of known sequence and length are used to amplify genomic and cDNA sequences which are visualised by gel electrophoresis technique. In our study, we have used a PCR based marker – SSRs (Simple Sequence Repeats).

2.3 SSR's AS MOLECULAR MARKERS

The repeating sequences of 1-6 base pairs of DNA have been termed as Simple Sequence Repeats (SSRs) (*Jacob et al., 1991*), microsatellite (*Litt and Luty, 1989*) or Short Tandem Repeat (SSR) (*Edwards et al. 1991*).

It is believed that when DNA is being replicated, errors occur in the process and extra sets of these repeated sequences are added to the strand. Over time, these repeated sequences vary in length between one cultivar and another. These markers often present high levels of inter- and intra-specific polymorphism, particularly when tandem repeats number ten or greater. The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri-, and tetranucleotide repeats respectively), and can be repeated 10 to 100 times.

In areas of the genome with high rates of mutation there is a wider range in the number of repeats found within individuals of a population.

Example of allelic variation in SSRs:

Allele A: CACACACA (4 repeats of the CA sequence)
Allele B: CACACACACACA (6 repeats of the CA sequence)

These variations in length are easy to trace in the lab and allow us to track genotypic variation in breeding programs. Also, it can be used for population, kinship studies as well as for studies of gene duplication or deletion.

Advantages of SSRs-

- Co-dominant (more informative when dealing with heterozygotes)
- Highly variable (important for species with narrow gene pools)
- Widely used
- Excellent for use in marker assisted selection, fingerprinting and marker assisted backcrossing.

Disadvantages of SSRs-

- They typically require polyacrylamide gel electrophoresis and generally give information only about a single locus per assay, although multiplexing of several markers is possible.
- SSR markers also require a substantial investment of time and money to develop.

2.4 Genetic diversity and evolutionary history of *Plasmodium falciparum* and *Plasmodium vivax*

Understanding the genetic diversity and evolutionary history of the malaria parasite and how it is genetically distinct in different regions of the genome and populations, may open up new avenues to population-specific malaria control measures. The two principal human malaria parasites, *P.falciparum* and *P.vivax*, seem to be very different in origin and in phylogenetic resemblance to other species of Plasmodium. Whereas African populations of *P. falciparum* are highly diverse at the DNA level among other populations, high genetic diversity was found in Asian *P.vivax*; clearly depicting that *P.falciparum* haspossibly originated in Africa and *P.vivax* in Asia. (*Coluzzi et al, 1999*) (*Hartl et al, 2004*)

So far two basic approaches have been followed in population genetic studies of malaria parasites: (i) to understand the parasite population genetic structure and intrahost dynamics, including the stability of strains and complexity of infections, and

(ii) studies assessing the diversity of specific genes encoding vaccine antigens and those associated with drug resistance. (*Cui et al, 2003*)

2.5 Evolutionary history of genes and populations of *P. falciparum*

The recent upsurge of malaria owing to a coincidence of mutually reinforcing factors might echo the first expansion of malaria, which is thought to have taken place about 10,000 years ago. (*Livingstone et al, 1958*) The events that could have conjoined to create this earlier expansion include: (i) climate change in Africa after the last glaciation leading to optimal warm and humid conditions in the equatorial regions about 10,000 years ago, (ii) increase in human population density and (iii) proliferation and rapid diversification of the highly anthropophilic Anopheles mosquito vectors which efficiently transmit the malaria parasites. (*Hey et al, 1999*)

2.6 Evolutionary history genes and populations of *P. vivax*

Until the middle of the 1900s, *P. vivax* was the most globally widespread and arguably the most prevalent of the four malaria parasite species that infect humans. The ability of this parasite to complete its sporogonic cycle at a minimum lower temperature of 16°C, compared with 21°C for *P. falciparum*, has substantially contributed to its success in establishing stable foci of transmission in the temperate zones. (*Mendis et al, 2001*) Although responsible for less mortality than *P. falciparum, P. vivax* causes considerable morbidity. *P. vivax* confounds control measures owing to the presence of dormant stages in the liver, which lead to relapses of the infection, weeks after the initial episode. (*Baird et al, 2004*)

P. vivax seems to have extraordinary phenotypic diversity, especially in its relapse patterns, and is found in a broad range of ecotypes, from Russia to the tropical regions of Asia, the Pacific, and South and Central America. The relatively stable GC-rich genome of *P. vivax* (which has a GC content double that of *P. falciparum*) has few di-nucleotide microsatellite markers, and the (TA) and (CA) motifs commonly seen in *P. falciparum* and other organisms such as yeasts, are rare in *P. vivax*. (*Carlton, J. M. et al, 1999*) (*Gardner, M. J. et al, 2002*) (Jane M. Carlton *et al, 2008)*

2.7 Genome statistics

TABLE 2.2- Genome Statistics of *P.falciparum* and *P.vivax*

FALCIPARUM	VIVAX
Genome size (Mb) :-23.3	Genome size (Mb) :-26.8
No. of chromosomes :-14	No. of chromosomes :-14
GC content :- 19.4 %	GC content :- 42.3 %
AT content :- 80 %	AT content :- 55 %

CHAPTER 3

MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

TABLE 3.1 – List of collected blood samples infected with *P.vivax* and *P.falciparum* from Punjab region

SERIAL NO.	TYPE	PLACE
1	Plasmodium vivax	Muktsar (Punjab)
2	Plasmodium vivax	Muktsar (Punjab)
3	Plasmodium falciparum	Samana (Punjab)
4	Plasmodium vivax	Samana (Punjab)
5	Plasmodium vivax	Samana (Punjab)
6	Plasmodium falciparum	Samana (Punjab)
7	Plasmodium vivax	Chandigarh
8	Plasmodium vivax	Chandigarh

3.2 MICROSATTELITE SEARCH

An EST database was developed sequence. Protein functions were predicted by BlastX similarity searches against the protein database in the GenBank, and annotated in terms of the associated biological processes, cellular components, and molecular functions using the Gene Ontology vocabulary. The Perl script MIcroSAtelitte (MISA) was used to identify SSRs in the *P.vivax* and *P.falciparum* sequences. (*Liang et al, 2009*) The parameters for the SSR search were defined as follows.

The size of motifs was two to six nucleotides, and the minimum repeat unit was defined as six for di-nucleotides and four for tri-, tetra-, penta-, and hexa-nucleotides.

1) Download the sequences of all chromosomes of *Plasmodium falciparum* and *Plasmodium vivax* from NCBI.
2) Compilation of whole genome is done using perl.
3) Microsatellites are identified using MISA (Microsatellite identification tool)
4) 2 files were obtained in .misa and .statistics format.

3.3 PRIMER DESIGNING:

Twenty Four primers were designed based on the gene sequence taken from the different chromosomes of *Plasmodium vivax* and *Plasmodium falciparum*. The gene sequences were downloaded from NCBI. For our study and primer designing, we have considered the microsatellites based primers.

Procedure

1. The genome sequences for the two plasmodium species were downloaded from NCBI.
2. SSR's were identified using MISA and sequences with corresponding SSR's were located.
3. Primer3 was used for designing, flanking regions along the SSR's were designed.
4. We selected 2 primer pairs, one for SSR dimer and one for SSR trimer. The selection criteria according to priority was-

   i). Maximum number of repetitions of SSR lying between the forward and reverse primer.

   ii). Maximum product length.

   iii). Melting temperature(Tm) above 50^0 C and should be approximately same for the primer pair.

   iv). GC content more than 35%.

v). Also, the primer length was taken into consideration(18-25bp).

5. All the 24 pairs of prmers that have been designed were custom synthesised from G-Biosciences and used for PCR amplification.

PRIMERS *FALCIPARUM*

Primer 1

Chromosome-1

Length-643292

Region- 419723-420412

Sequence size-690

Product size-401

SSR type-AT

	GC %	Tm	Primer length
F -	45.45	59.69	22
R -	45	57.95	20

PRIMERS VIVAX

Primer 1

Chromosome-13

Length-2031768

Region- 899363-899984

Sequence size-622

Product size-208

SSR type-AC

	GC %	Tm	Primer length
F –	55	60.12	20
R -	55	59.93	20

TABLE 3.2: List of novel primers designed from the flanking regions of SSR present in *P.falciparum* and *P.vivax* genome sequences.

S.No	PRIMERS FALCIPARUM						
		SEQUENCE	LENGTH	GC %	Tm	PRODUCT SIZE	SSR
1	Forward Primer	AACAAAGGAGTTGCAGGTAAGG	22	45.45	59.69	401	AT
	Reverse Primer	ACAAAAGCTCGTGATGGAAG	20	45	57.95		
2	Forward Primer	TACCACCACCACCATCATCA	20	50	60.66	106	TAT
	Reverse Primer	GTGCATGCATCCCAAAATAA	20	40	59.38		
3	Forward Primer	CGACGAAGAAGATGAATCCAA	21	42.86	60.2	338	GAA
	Reverse Primer	TTCGTTTTCCAAGAACAGCTC	21	42.86	59.48		
4	Forward Primer	CCTCAGTGCTTTATGCTGCT	20	50	58.28	299	ATA
	Reverse Primer	TTTTACACACCCGTCCATCTT	21	42.86	59.35		
5	Forward Primer	AAAGAGGAAGGAGTAAAAGATGAGG	25	40	59.71	453	TGA
	Reverse Primer	GGGCGATGTGCAATGTAACT	20	50	60.92		
6	Forward Primer	GGAGAAGACGAAAGGGAAAGA	21	47.62	59.81	434	AC
	Reverse Primer	ACAAGGAAAGCTGCAGAGGA	20	50	60.13		
7	Forward Primer	AAGAACACGTATCAACGGATCA	22	40.91	59.5	302	GA
	Reverse Primer	TCTTTCCCTTTCGTCTTCTCC	21	47.62	59.81		
8	Forward Primer	GGAATCTGCATCGTCCTCTC	20	55	59.77	386	AT
	Reverse Primer	TTGCACAGGATGGAGAAACA	20	45	60.24		

9	**Forward Primer**	GTCCCCGTATGAAATATGGTTT	22	40.91	59	381	TAG
	Reverse Primer	CTTCATTTGTGCTTAGTGTTGG	22	40.91	57.51		
10	**Forward Primer**	CGGAAAATTGTCTGGTAGCAT	21	42.86	59.1	250	TA
	Reverse Primer	TCCCGTTTGAAGGAGTGTTA	20	45	58.2		
11	**Forward Primer**	TCCTAAGGCAGTCGAACAATTT	22	40.91	60.13	163	AAT
	Reverse Primer	AGCAGATGGATCATTGCTACTAC	23	43.48	57.56		
12	**Forward Primer**	TCGACAACGAACTAATGGACTT	22	40.91	58.77	448	ATT
	Reverse Primer	GCCGTGCTCGATATGGTATT	20	50	59.95		
	PRIMERS VIVAX						
1	**Forward Primer**	CCGTCCTGCCTACCAAGTTA	20	55	60.12	208	AC
	Reverse Primer	AGTCGCGCCTCTACGTGTAT	20	55	59.93		
2	**Forward Primer**	ACTTTTTGTTTGGGGGAGGT	20	45	59.71	247	AT
	Reverse Primer	CACCCGTACGTTTTGTGTGT	20	50	59.38		
3	**Forward Primer**	GTGGCTGTCACTGGGAATTT	20	50	59.97	374	GA
	Reverse Primer	CTTAGCAGCGGTAGGAGTGG	20	60	60.03		
4	**Forward Primer**	GGGGAGGAATCCTAAAGCTG	20	55	60.03	360	TG
	Reverse Primer	CTAGCACAGGGCCATTCATT	20	50	60.1		
5	**Forward Primer**	GGAATTGTAAAGGGGTGCTTC	21	47.62	59.83	395	TA
	Reverse Primer	GCGAATGCCATACACATCAC	20	50	59.96		

6	**Forward Primer**	GCGGCACAGTTCAAAGGTAT	20	50	60.14	286	GT
	Reverse Primer	TCAAGCAATTTGTGCTTTGC	20	40	60		
7	**Forward Primer**	GCCTTTGTGACCATTTCGTT	20	45	59.98	193	GTT
	Reverse Primer	GTAAGGTAAAACGCGGGTGA	20	50	60		
8	**Forward Primer**	AATCTCTCGTACGGGTGTGC	20	55	60.14	268	CTC
	Reverse Primer	CGTAGCAAATTCACCCGTTT	20	45	60		
9	**Forward Primer**	GCTTTTGGTCTTCTCGGATG	20	50	59.81	323	CCG
	Reverse Primer	GTACCGCGAGAGGAAGAGTG	20	60	60.01		
10	**Forward Primer**	CCCAAATGGGTAACGGAAAT	20	45	60.77	277	GTA
	Reverse Primer	CAAAGGAACATGCTCGATGA	20	45	59.8		
11	**Forward Primer**	TTTGTAGCCCCTCGTTTGAC	20	50	60.11	345	AGT
	Reverse Primer	CAATCATGCAAAGGGTAGGG	20	50	60.32		
12	**Forward Primer**	TTAAGGGGAGGAATCGGAAC	20	50	60.26	283	GCA
	Reverse Primer	GGGCTCAAGTCTTCGTCATC	20	55	59.81		

3.4 ISOLATION OF DNA

1) *Rapid DNA extraction method*

DNA was extracted from blood according to the method described by *Foley et al (1992)*

- 50-100 µl of whole blood was added to 5mM sodium phosphate (pH 8.0)
- After mixing well by vortexing, the tubes were centrifuged in micro centrifuge at 10,000 rpm for 10 minutes at 4°C.
- The supernatant was discarded.
- To the cell pellet, 500 µl of ice cold 5mM sodium phosphate (pH 8.0) was added.
- It was mixed by vortexing and spun at 10,000 rpm for 10 minutes at 4°C.
- This procedure was repeated 3 times.
- To the final cell pellet, 50 µl of sterile glass distilled water was added and mixed well by vortexing.
- The suspension was then boiled for 10 minutes in a water bath, and cooled to room temperature.
- It was centrifuged at 10,000 RPM for 10 minutes.
- 40 µl supernatant was transferred in a fresh tube and 10 µl supernatant was used as a DNA template for PCR amplification.

Sodium phosphate buffer

- 28.4 gm Disodium hydrogen phosphate in 200 ml distilled water.
- 31.2 gm Sodium dihydrogen phosphate in 200 ml distilled water.
- After we have made the above solutions we add 47.35 ml of disodium hydrogen phosphate and 2.65 ml of sodium dihydrogen phosphate to get the sodium phosphate buffer (pH 8.0)

2) *Isolation of DNA from parasite infected blood*

DNA was extracted from blood according to the method described by (*Am et al., 2005*)

- Blood (0.5–1 mL) was withdrawn from each patient and 500 µL of lysis buffer (1.6 M sucrose, 5% [v/v] Triton X-100, 25 mM $MgCl_2$, 60 mM Tris-HCl, pH 7.5) was added to the blood.
- The mixture was centrifuged at 13,000 rpm for one minute in an Eppendorf microcentrifuge.
- The supernatant was discarded and the excess liquid was air-dried.
- The pellet was resuspended in 5x filtered proteinase K buffer (0.375 M NaCl, 0.12 M EDTA, pH 8), proteinase K, and 20% sodium dodecyl sulfate, and adjusted up to a volume of 400 µL with distilled water.
- The mixture was incubated at 55°C with constant shaking for at least 30 minutes.
- One volume of phenol-chloroform was then added and mixed by vortexing to remove protein contaminants. The sample was then centrifuged at 10,000 rpm for five minutes at room temperature.
- The aqueous phase was transferred to a new vial, precipitated with cold ethanol, and incubated at –70°C for one hour.
- The vial was centrifuged at 13,000 rpm for 15 minutes at 4°C.
- The DNA was pelleted by centrifugation at 13,000 rpm for five minutes and the pellet was washed with 70% ethanol. The supernatant was discarded and the DNA pellet was washed again with 70% ethanol.
- The sample was centrifuged at 13,000 rpm for five minutes and the DNA pellet was dried.

3.5 QUANTIFICATION OF PURIFIED DNA

1) ***Preparation of 0.8% agarose gel***-

a) Stock solution of TAE buffer (5M) was diluted to a concentration of 1M.

2) It is loaded onto the casting tray and allowed to solidify with the combs in it.

3) Stock of uncut λ DNA with concentration 200ng/µl was converted into 3 different concentrations- 50ng/µl, 100ng/µl and 150ng/µl.

4) 1µl of 6X loading dye (bromophenol blue) is mixed with 2µl of purified DNA on parafilm.

5) Loading of uncut λ DNA and the samples was done on 0.8% agarose gel.

TABLE 3.3 – Quantification of parasite DNA

λDNA	0.5µl	1µl	1.5µl	
dH2O	1.5µl	1µl	0.5µl	
total	2µl	2µl	2µl	2µl
dye	1µl	1µl	1µl	1µl

6) The intensity of the bands obtained from λ DNA in the three different concentrations were compared with the bands of different samples in order to know the concentration of DNA in samples.

7) The gel was allowed to run for 1hr.

8) After 1hr, the gel was stained in EtBr solution for 10min so that the DNA shows fluorescence in UV.

9) The gel is washed to remove excess stain.

10) It is visualized in GelDoc.

50x TAE Composition :

In 1000 ml

Tris(hydroxymethyl) aminomethane	242.28 g (2 M)
Acetic acid (glacial)	57.1 ml
0.5M EDTA (pH8.0)	100ml(50 mM)

1X-

10ml of 50X TAE buffer and 490 ml distilled water.

0.8 % agarose

0.48 gm agarose in 60 ml 1X TAE buffer

3.6 PCR AMPLIFICATION

The primers were dissolved with 200µl of autoclaved distilled water by vortexing for 30 sec. The primer solutions were stored at 4 ℃ overnight and then stored at -20 ℃ till their usage. The stock solution of primer was diluted to a concentration of 50 pM for PCR amplification. 24 primer pairs were used for the study of genetic diversity among *Plasmodium vivax* and *Plasmodium falciparum*. Amplification reactions were performed in volumes of 15 µl containing 9.3µl autoclaved distilled, 1µl forward and reverse primers (0.83pM), 0.5µl dNTPs (0.2mM), 0.8µl $MgCl_2$ (1mM), 1µl PCR Buffer (1.3X), 0.4 µl Sigma Taq polymerase (0.05 units) and 1 µl of DNA. DNA amplification was performed using a Gene Cycler.

The amplification was done using Touchdown PCR. The first cycle consisted of denaturation of template DNA at 94 ^{0}C for 5 min. The denaturation was reduced to 1 min for the next 20 cycles during which the primer annealing was done at a fall of 0.5 ^{0}C for 1 min. Then, primer extension was done at 72 ^{0}C for 1 min. Now, when the template at an appropriate temperature has been found, the denaturation of template DNA is done at 94 ^{0}C for 1 min, primer annealing at Tm ± 2.5^{0}C for 1.50 min and the primer extension at 72 ^{0}C for 2 min. was done for 42 cycles. Then, the final extension was done at 72 ^{0}C for 7 min and storage of the PCR product at 4 ^{0}C for infinity. The amplification products were resolved in 2% agarose gel (1X TAE buffer) followed by ethidium bromide staining and visualization in UV light for photography which have been performed three times and were found reproducible. (*Sambrook et al.,1989*)

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TABLE 3.4 – PCR CYCLE

SERIAL NO.	STAGE	STEP	TEMPERATURE	TIME
1.	STAGE 1 *1 CYCLE		95° C	4 mins
2.	STAGE 2 *30 CYCLES	STEP 1	95° C	30 sec
		STEP 2	GRADIENT	30 sec
		STEP 3	72° C	2 mins
3.	STAGE 3 *1 CYCLE		72° C	10 mins
4.	STAGE 4		4° C	∞

3.7 ASYMMETRY IN COMPOSITION OF GENOME OF *P. VIVAX* AND *P. FALCIPARUM*

DNA sequences used in this study include complete genomes of *P. vivax* and *P. falciparum*. The C-richness is assessed with a 1000-kb window but is subject to many fluctuations with a 10-kb sliding window. In seeking to explain the strand compositional asymmetry in *P. vivax* and *P. falciparum* we examined intergenic regions and codon site in genes where mutational substitutions are expected to be more pronounced. A caveat concerning intergenic regions: these contain regulatory signal sequences (e.g., promoters, cis-acting elements, Shine-Dalgarno sequences, and others) that may be biased. The asymmetries are assessed in a given strand via (C-G) / (C+G) counts in a sliding window of 10 kb length and 1 kb displacement; (C-G) / (C+G) = (nC-nG) / (nC+nG) where nC (nG) is the number of C (G) nucleotides in the window at hand. A corresponding asymmetry in (A-T) / (A+T) counts is weak or nonexistent. (*Mrazek J et al, 1998*)

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 DNA ISOLATION

Samples were collected from hospitals of Muktsar, Samana and Chandigarh. Standard DNA isolation protocol was followed. Further modifications were done for better results. At the DNA samples had on average a concentration of 50-100ng/ul, which was used for further analysis (Fig 4.1).

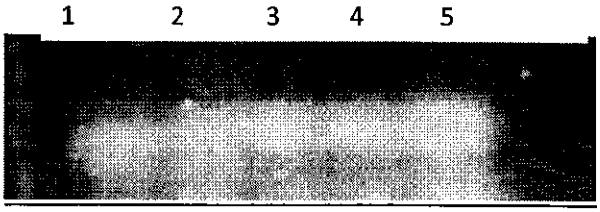

FIG 4.1:- DNA isolation from diseased blood samples

4.2 WHOLE GENOME SSR MINING

The significant change in the number of SSR's in *P.vivax* and *P.falciparum* gives us an idea about the mutation trends and the evolution of the organism over the periods. While microsatellites are considerably less common in the *P. vivax* genome than in the AT-rich *P. falciparum* genome and also tend to be shorter in length, these markers can provide useful tools for assessing population structure and for searching for evidence of recent selection events associated with drug resistance.

Plasmodium falciparum

Defining of microsatellites (unit size/minimum number of repeats): (2/10) (3/5)

Total number of sequences examined: 14

Total size of examined sequences (bp):22860235

Total number of identified SSRs: **29168**

Number of SSR containing sequences: 14

Number of sequences containing more than 1 SSR:14

Plasmodium vivax

Defining of microsatellites (unit size/minimum number of repeats): (2/10) (3/5)

Total number of sequences examined: 14

Total size of examined sequences (bp): 22621071

Total number of identified SSRs: **582**

Number of SSR containing sequences: 14

Number of sequences containing more than 1 SSR: 14

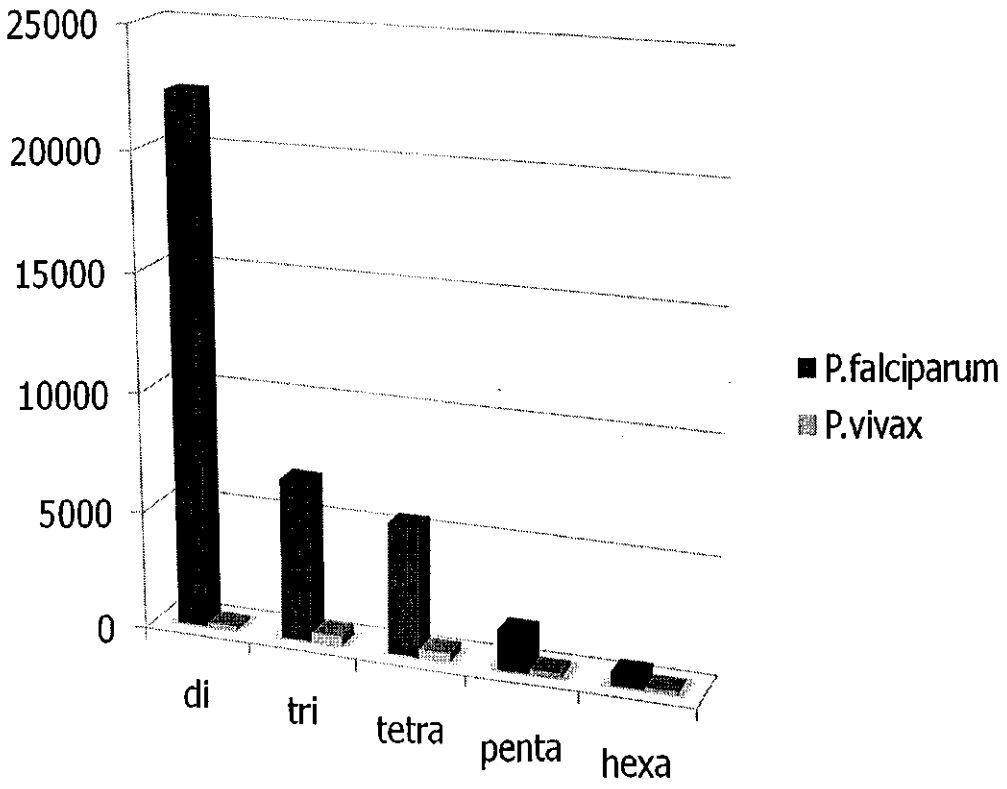

FIG 4.2:- Graph showing no. of SSRs in *P.falciparum* and *P.vivax*

TABLE 4.1 – Frequency of SSRS in *P.Falciparum*

Repeats	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	TOTAL
AC	2	3	0	0	0	0	0	0	0	5
AG	2	0	0	0	0	0	0	0	0	2
AT	1804	11692	1381	20	1	0	0	0	0	14898
CA	3	0	0	0	0	0	0	0	0	3
GA	0	1	0	0	0	0	0	0	0	1
TA	1051	5907	470	7	0	0	0	0	1	7436
TC	0	1	0	0	0	0	0	0	0	1
TG	1	3	0	0	0	0	0	0	0	4
AAC	60	6	1	0	0	0	0	0	0	67
AAG	28	0	0	0	0	0	0	0	0	28
AAT	1174	108	0	1	0	0	0	0	0	1283
ACA	16	2	0	0	0	0	0	0	0	18
ACC	5	0	0	0	0	0	0	0	0	5
ACG	1	0	0	0	0	0	0	0	0	1
ACT	16	1	0	0	0	0	0	0	0	17
AGA	20	0	0	0	0	0	0	0	0	20
AGG	2	0	0	0	0	0	0	0	0	2
AGT	11	0	0	0	0	0	0	0	0	11
ATA	1039	90	5	0	0	0	0	0	0	1134
ATC	77	1	0	0	0	0	0	0	0	78
ATG	80	2	0	0	0	0	0	0	0	82
ATT	884	79	3	0	0	0	0	0	0	966
CAA	18	2	0	0	0	0	0	0	0	20
CAT	32	1	0	0	0	0	0	0	0	33

CCA	1	0	0	0	0	0	0	0	0	1
CTA	11	0	0	0	0	0	0	0	0	11
CTT	14	0	0	0	0	0	0	0	0	14
GAA	62	0	0	0	0	0	0	0	0	62
GAC	1	0	0	0	0	0	0	0	0	1
GAG	9	0	0	0	0	0	0	0	0	9
GAT	89	1	0	0	0	0	0	0	0	90
GGA	1	0	0	0	0	0	0	0	0	1
GGT	2	0	0	0	0	0	0	0	0	2
GTA	15	1	0	0	0	0	0	0	0	16
GTC	1	0	0	0	0	0	0	0	0	1
GTG	18	0	0	0	0	0	0	0	0	18
GTT	8	5	0	0	0	0	0	0	0	13
TAA	539	44	1	0	0	0	0	0	0	584
TAC	42	1	1	0	0	0	0	0	0	44
TAG	31	0	0	1	0	0	0	0	0	32
TAT	1044	79	2	0	0	0	0	0	0	1125
TCA	55	4	0	0	0	0	0	0	0	59
TCC	1	1	0	0	0	0	0	0	0	2
TCG	3	0	0	0	0	0	0	0	0	3
TCT	34	3	0	0	0	0	0	0	0	37
TGA	26	0	0	0	0	0	0	0	0	26
TGG	1	0	0	0	0	0	0	0	0	1
TGT	28	5	0	0	0	0	0	0	0	33
TTA	701	66	0	0	0	0	0	0	0	767
TTC	55	1	0	0	0	0	0	0	0	56
TTG	41	3	1	0	0	0	0	0	0	45

Frequency of classified repeat types (considering sequence complementary)

	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	Total
AC/GT	6	6	0	0	0	0	0	0	0	12
AG/CT	2	2	0	0	0	0	0	0	0	4
AT/AT	2855	17599	1851	27	1	0	0	0	1	22334
AAC/GTT	171	23	2	0	0	0	0	0	0	196
AAG/CTT	213	4	0	0	0	0	0	0	0	217
AAT/ATT	5381	466	11	1	0	0	0	0	0	5859
ACC/GGT	27	0	0	0	0	0	0	0	0	27
ACG/CTG	2	0	0	0	0		0	0	0	2
ACT/ATG	264	5	1	0	0	0	0	0	0	270
AGC/CGT	4	0	0	0	0	0	0	0	0	4
AGG/CCT	13	1	0	0	0	0	0	0	0	14
AGT/ATC	221	7	0	1	0	0	0	0	0	229

TABLE 4.2 – Frequency of SSRS in *P.vivax*

Repeats	0-10	11-20	21-30	31-40	41-50	51-60	61-70	total
AC	0	3	0	0	0	0	0	3
AT	16	57	12	0	0	0	0	85
CA	1	2	0	0	0	0	0	3
CT	0	2	0	0	0	0	0	2
GA	0	0	1	0	0	0	0	1
GT	3	2	0	0	0	0	0	5
TA	18	38	7	0	0	0	0	63

TC	1	1	0	0	0	0	0	2
TG	0	2	0	0	0	0	0	2
AAC	0	0	1	0	0	0	0	1
AAG	13	0	0	0	0	0	0	13
AAT	9	0	2	0	0	0	0	11
ACC	4	0	0	0	0	0	0	4
ACT	1	0	0	0	0	0	0	1
AGA	16	1	1	0	0	0	0	18
AGC	8	0	0	0	0	0	0	8
AGG	1	0	0	0	0	0	0	1
AGT	2	0	0	0	0	0	1	3
ATA	10	0	0	0	0	0	0	10
ATC	3	0	0	0	0	0	0	3
ATG	7	0	0	0	0	0	0	7
ATT	7	0	0	0	0	0	0	7
CAA	1	1	0	0	0	0	0	2
CAC	2	0	0	0	0	0	0	2
CAG	8	0	0	0	0	0	0	8
CAT	8	0	0	0	0	0	0	8
CCA	2	0	0	0	0	0	0	2
CCG	3	0	0	0	0	0	0	3
CCT	4	1	0	0	0	0	0	5
CGA	1	0	0	0	0	0	0	1
CGG	1	0	0	0	0	0	0	1
CGT	2	0	0	0	0	0	0	2
CTA	3	0	0	0	0	0	0	3

CTC	11	2	0	0	0	0	0	13
CTG	6	0	0	0	0	0	0	6
CTT	26	1	0	0	0	0	0	27
GAA	25	2	1	1	0	0	0	29
GAC	5	0	0	0	0	0	0	5
GAG	23	0	1	0	0	0	0	24
GAT	14	0	0	0	0	0	0	14
GCA	4	0	0	0	0	0	0	4
GCC	1	0	0	0	0	0	0	1
GCT	7	0	0	0	0	0	0	7
GGA	7	0	0	0	0	0	0	7
GGC	1	0	0	0	0	0	0	1
GGT	3	0	0	0	0	0	0	3
GTA	1	1	0	0	0	0	0	2
GTC	1	0	0	0	0	0	0	1
GTG	4	0	0	0	0	0	0	4
GTT	3	1	0	0	0	0	0	4
TAA	2	0	0	0	0	0	0	2
TAC	3	0	0	0	0	0	0	3
TAG	1	0	0	0	0	0	0	1
TAT	7	0	0	0	0	0	0	7
TCA	10	0	0	0	0	0	0	10
TCC	28	2	0	0	0	0	0	30
TCT	18	1	0	0	0	0	0	19
TGA	5	1	0	0	0	0	0	6
TGC	8	0	0	0	0	0	0	8

TGG	5	0	0	0	0	0	0	5
TGT	4	1	0	0	0	0	0	5
TTA	7	1	0	0	0	0	0	8
TTC	31	2	0	0	0	0	0	33
TTG	3	0	0	0	0	0	0	3

Frequency of classified repeat types (considering sequence complementary)

Repeats	0-10	11-20	21-30	31-40	41-50	51-60	61-70	total
AC/GT	4	9	0	0	0	0	0	13
AG/CT	1	3	1	0	0	0	0	5
AT/AT	34	95	19	0	0	0	0	148
AAC/GTT	11	3	1	0	0	0	0	15
AAG/CTT	129	7	2	1	0	0	0	139
AAT/ATT	42	1	2	0	0	0	0	45
ACC/GGT	20	0	0	0	0	0	0	20
ACG/CTG	27	0	0	0	0	0	0	27
ACT/ATG	33	1	0	0	0	0	0	34
AGC/CGT	23	0	0	0	0	0	0	23
AGG/CCT	74	5	1	0	0	0	0	80
AGT/ATC	25	1	0	0	0	0	1	27
CCG/CGG	6	0	0	0	0	0	0	6

Di and tri nucleotide microsatellites from *P. vivax* show comparable variation to *P. falciparum* microsatellites with similar repeat array length and structure. The number of SSRs were much higher incase of *P. Falciparum* than *P. Vivax* (Table 4.1 and 4.2).

SSR instability can be indirectly advantageous by supplying abundant quantitative genetic variation with minimal genetic load, while variations in repetition purity and motif length enable site-specific adjustment of both mutation rate and mutation effect.

4.3 POLYMORPHISM SURVEY

Amplification of specific DNA fragments from whole genome sequence was carried out at annealing temperature determined with specific primers.

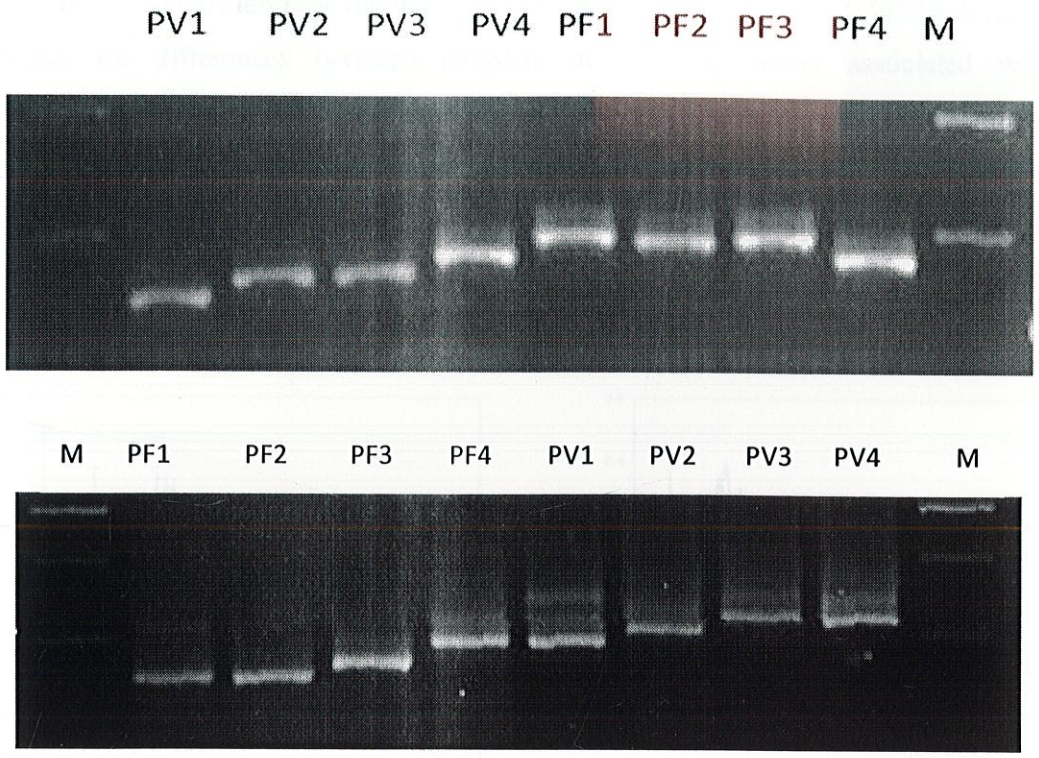

Fig 4.3 - Amplification of DNA with different primers; M-100bp ladder, PV-*P.vivax* and PF-*P.falciparum*

The technique (using gene specific SSR primers) has been successfully used in our genetic diversity studies and it was found suitable for our use with *P. vivax and P. falciparum* populations because of its ability to generate reproducibly polymorphic markers. 24 primer pairs (12 each for *P. vivax and P. falciparum*) were testsed on 8 samples, of these 24 primer pairs 17 (8 for *P. vivax and* 11 for *P. falciparum*) gave single consistent amplification. In the case of *P.vivax* 8 SSR markers generated a total of 18 alleles with an average of 1.5 alleles per marker, whereas in *P.falciparum* 11

SSR markers generated a total of 23 alleles with an average of 1.9 alleles per marker. For both parasites the number of alleles was in the range of 1-4, with primer 1 generating highest number of allele 4 (Fig.4.3). The amplification clearly shows polymorphism in the two *plasmodium* species.

4.4 ASYMMETRY IN COMPOSITION OF GENOME OF *P. VIVAX* AND *P. FALCIPARUM*

Sliding window (C-G) / (C+G) counts are displayed in the following graphs for various chromosomes of *P. vivax* and *P. falciparum*. Strand compositional asymmetry may reflect on differences in replication synthesis of the leading versus lagging strand, on differences between template and coding strand associated with transcription-coupled repair mechanisms, on differences in gene density between the two strands, on differences in residue and codon biases in relation to gene function, expression level, or operon organization, or on differences in single or context-dependent base mutational rates.

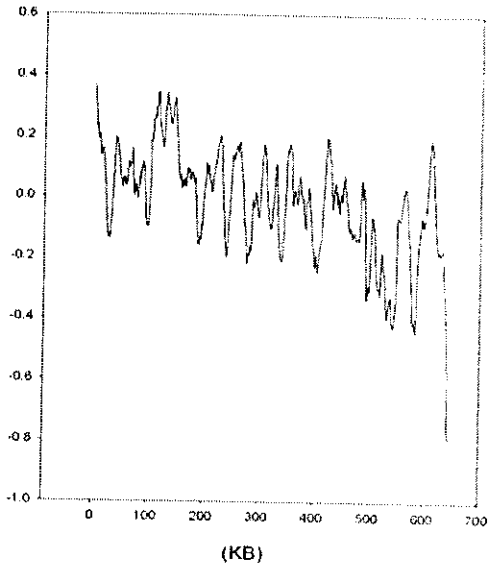

Fig 4.4 - *P.falciparum* chromosome 1

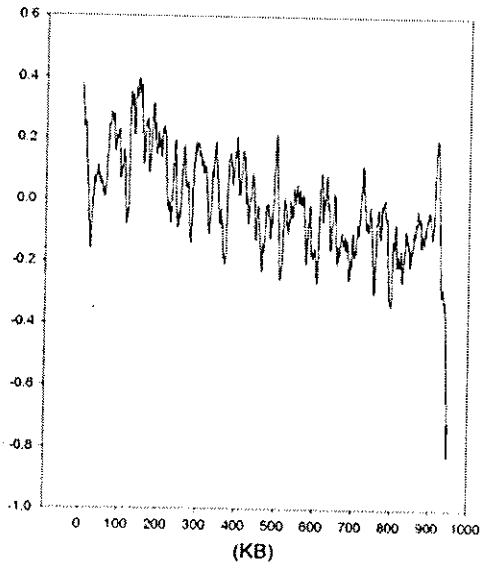

Fig 4.5 - *P.falciparum* chromosome 2

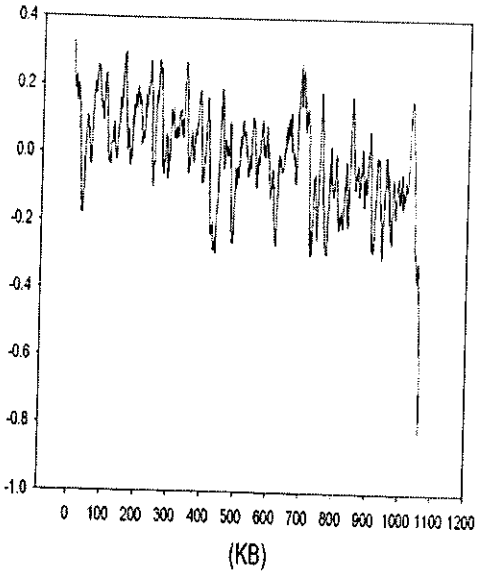

Fig 4.6 - *P.falciparum* chromosome 3

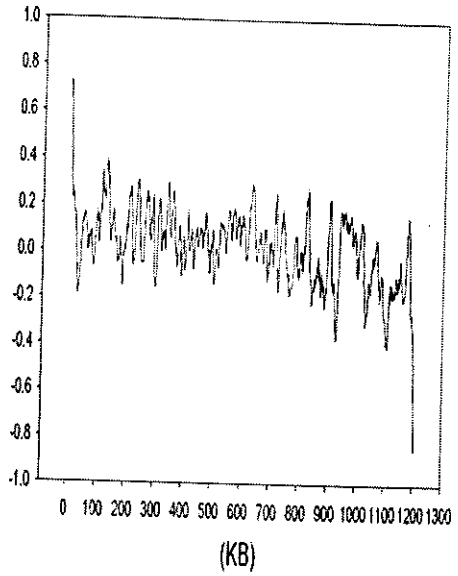

Fig 4.7 - *P.falciparum* chromosome 4

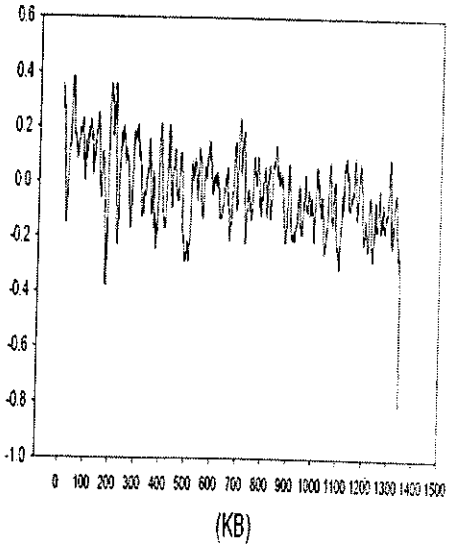

Fig 4.8 - *P.falciparum* chromosome 5

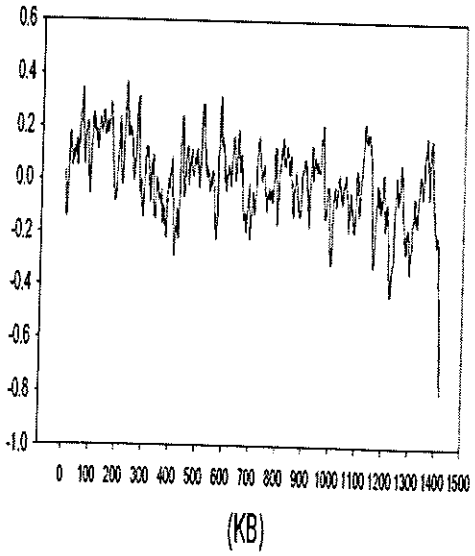

Fig 4.9 - *P.falciparum* chromosome 6

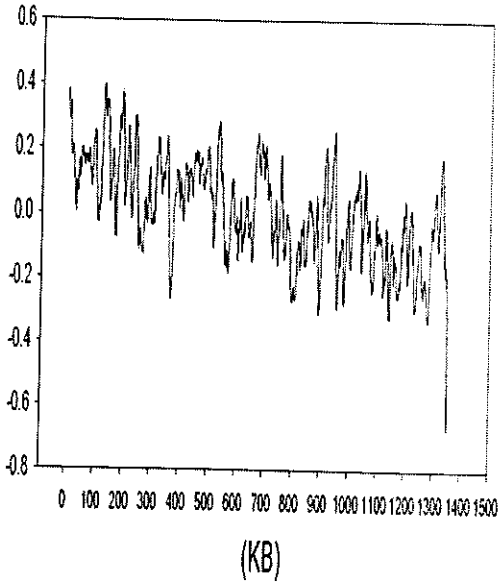

Fig 4.10 - *P.falciparum* chromosome 7

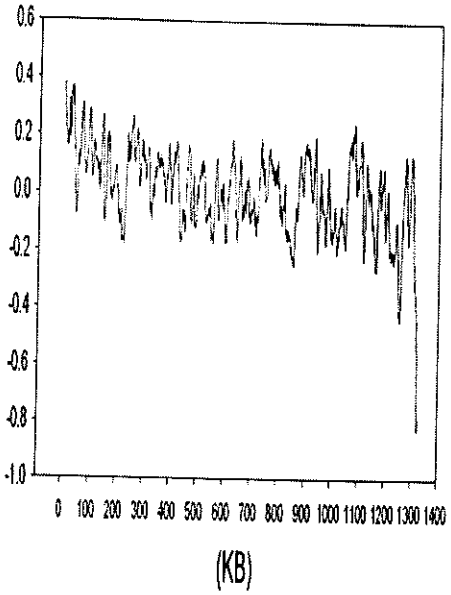

Fig 4.11 - *P.falciparum* chromosome 8

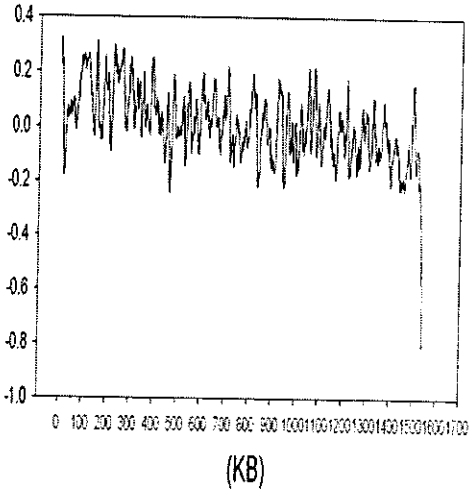

Fig 4.12 - *P.falciparum* chromosome 9

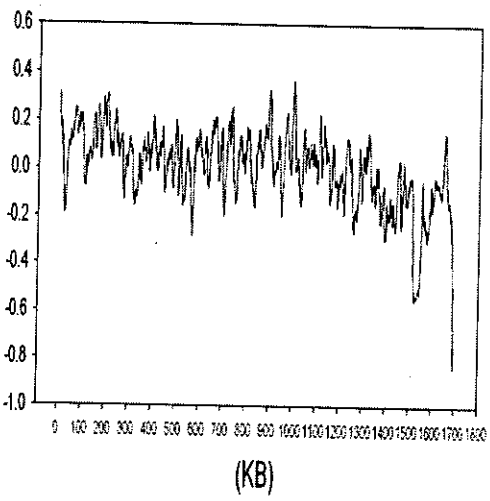

Fig 4.13 - *P.falciparum* chromosome 10

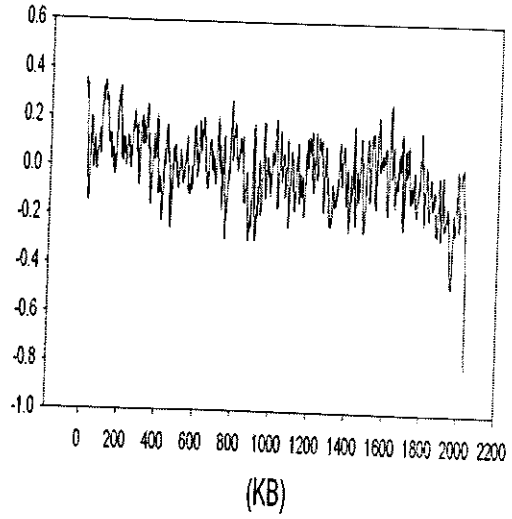

Fig 4.14 - *P.falciparum* chromosome 11

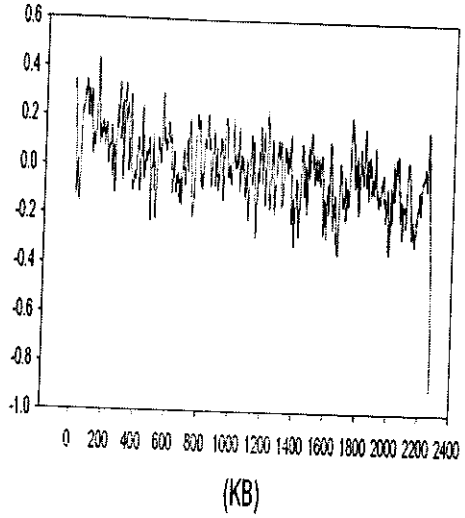

Fig 4.15 - *P.falciparum* chromosome 12

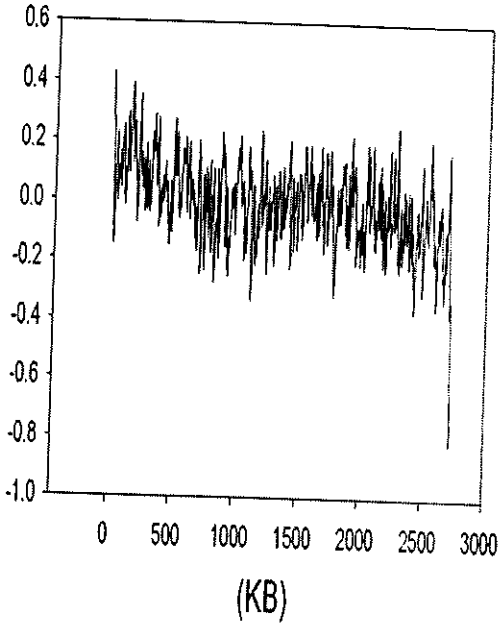

Fig 4.16 - *P.falciparum* chromosome 13

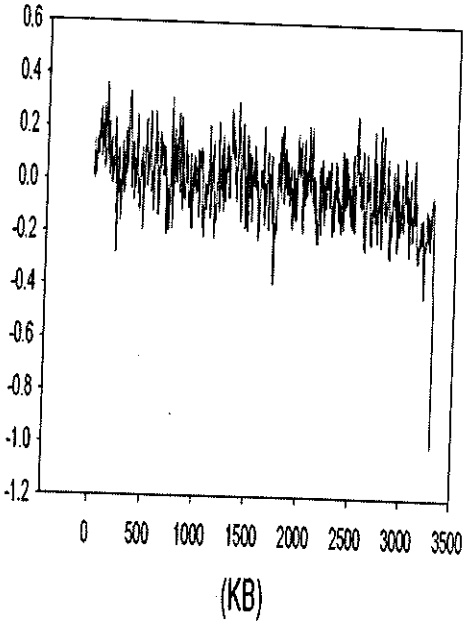

Fig 4.17 - *P.falciparum* chromosome 14

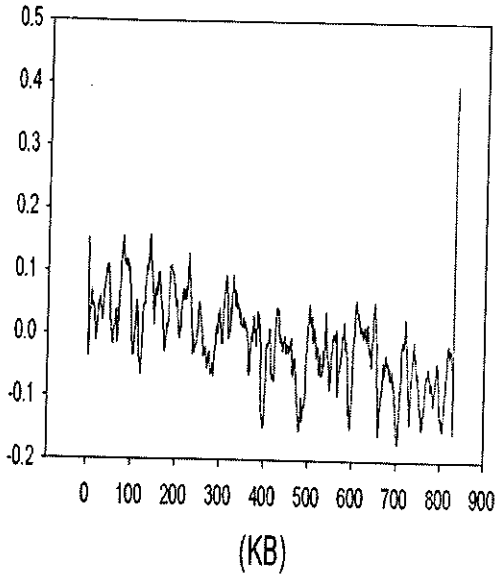

Fig 4.18 - *P.vivax* chromosome 1

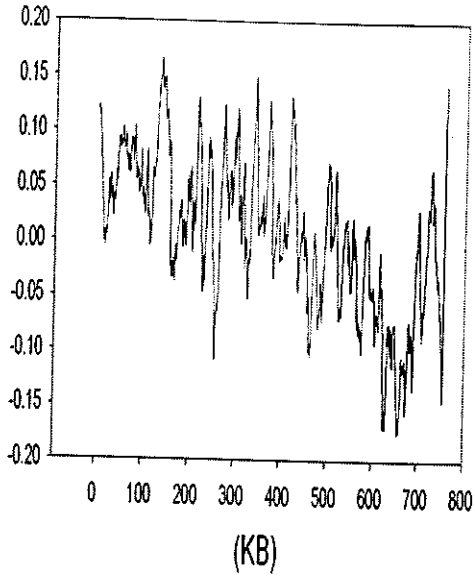

Fig 4.19 - *P.vivax* chromosome 2

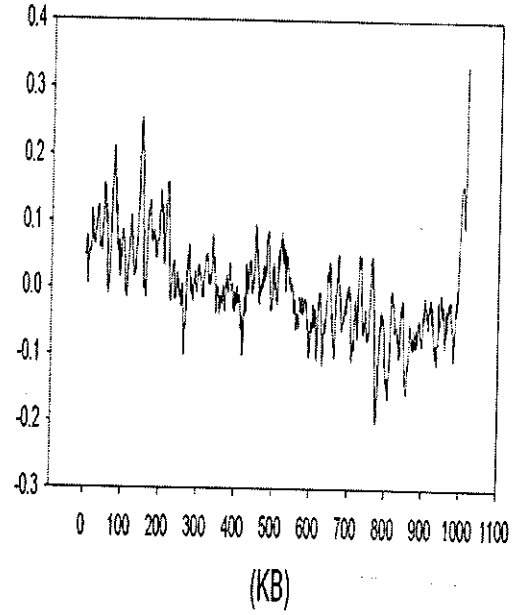

Fig 4.20 - *P.vivax* chromosome 3

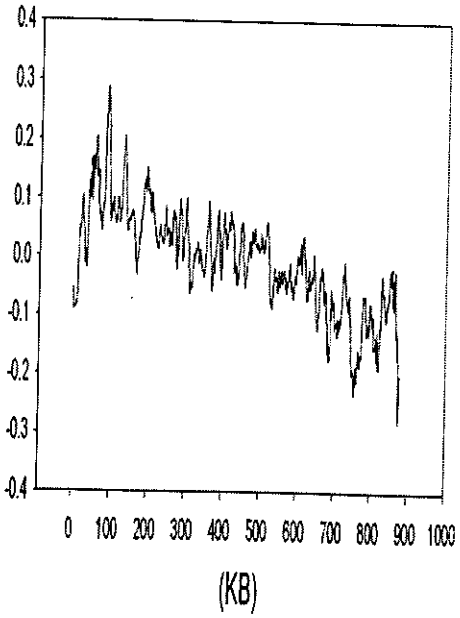

Fig 4.21 - *P.vivax* chromosome 4

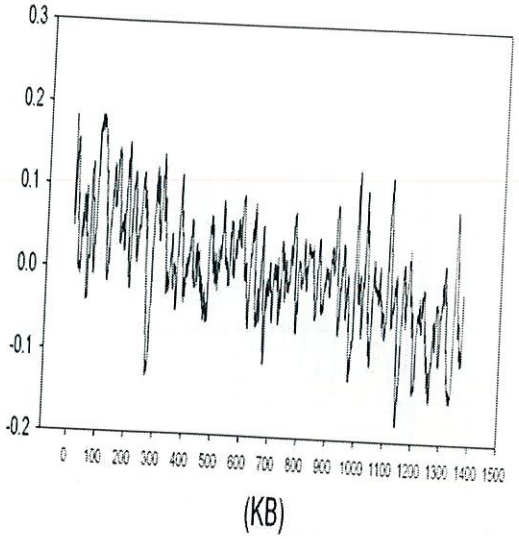

Fig 4.22 - *P.vivax* chromosome 5

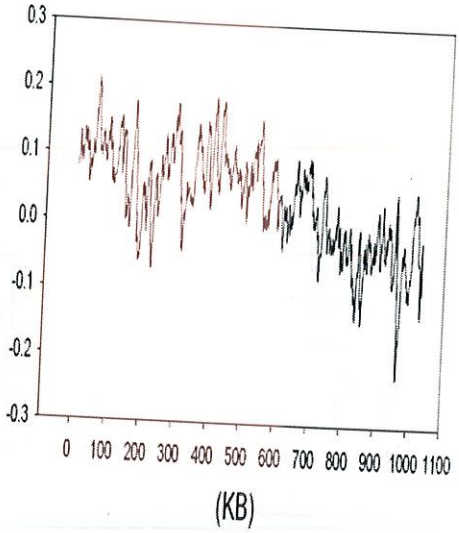

Fig 4.23 - *P.vivax* chromosome 6

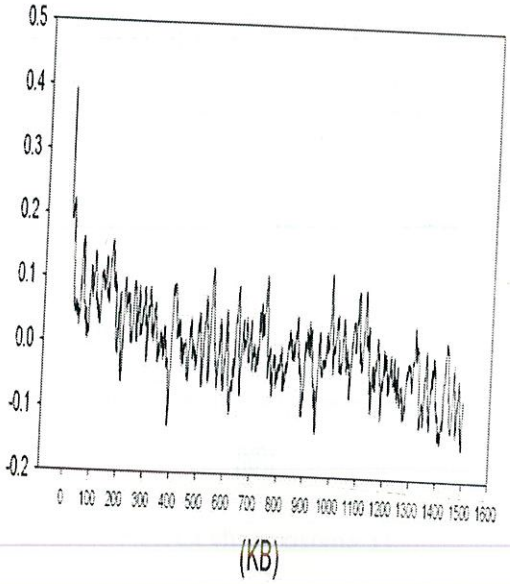

Fig 4.24 - *P.vivax* chromosome 7

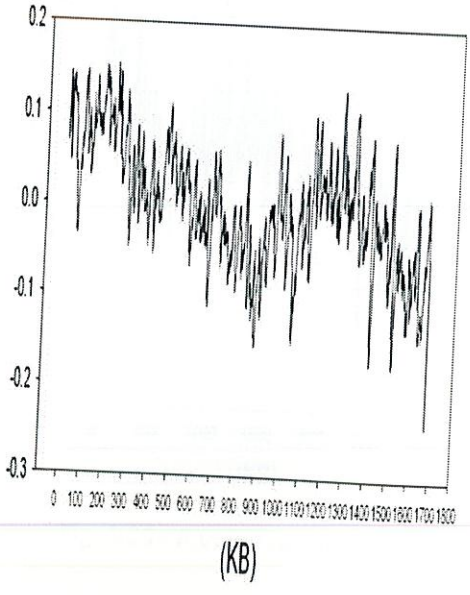

Fig 4.25 - *P.vivax* chromosome 8

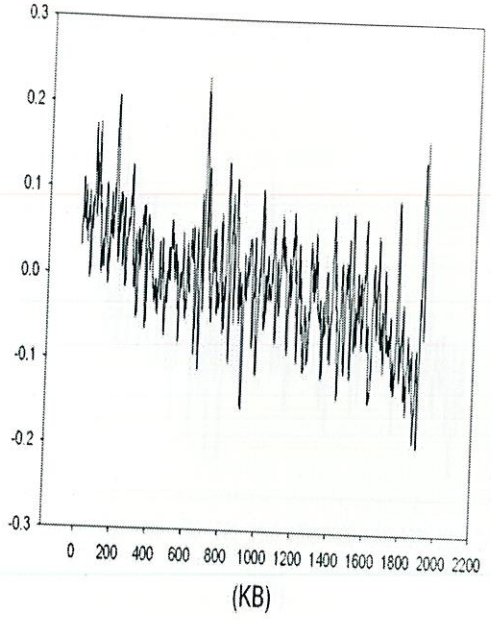

Fig 4.26 - *P.vivax* chromosome 9

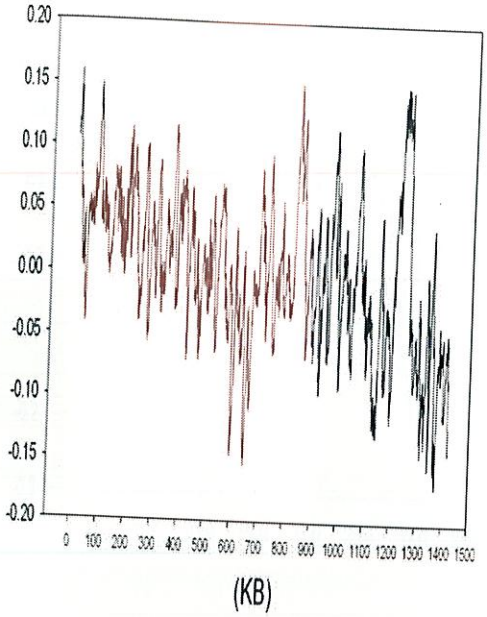

Fig 4.27 - *P.vivax* chromosome 10

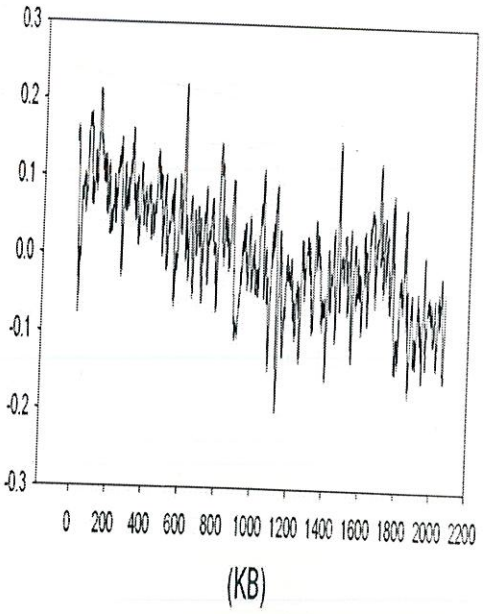

Fig 4.28 - *P.vivax* chromosome 11

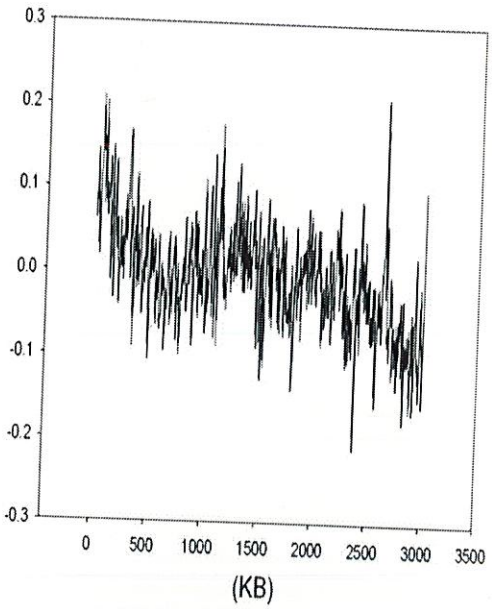

Fig 4.29 - *P.vivax* chromosome 12

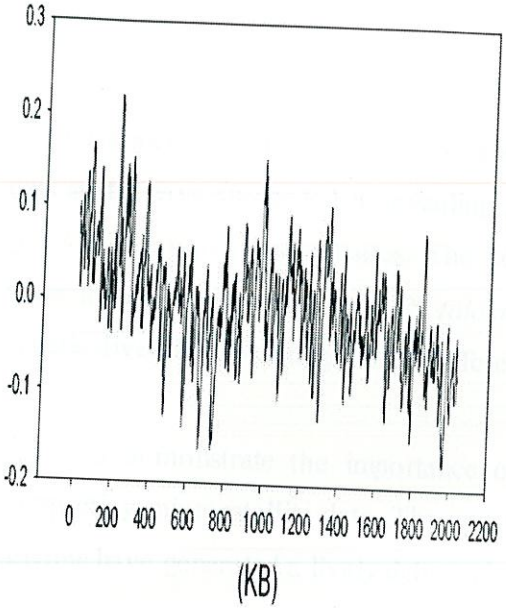

Fig 4.30 - *P.vivax* chromosome 13

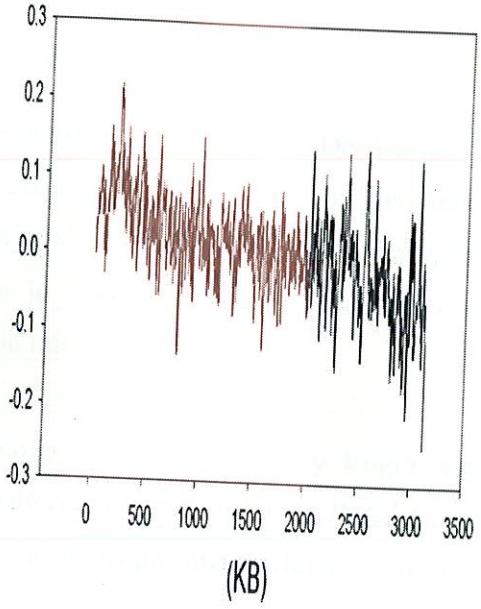

Fig 4.31 - *P.vivax* chromosome 14

CONCLUSION

The advancements in genomics and parallel statistical analyses of the DNA sequence data so far have succeeded in revealing new evolutionary information on the parasite genes and populations statistics. The current study was aimed to study the genetic diversity of the *P. vivax* and *P. falciparum* in Northern India. The mutation and genetic diversity was based on the difference in microsatellite number.

The data demonstrate the importance of accounting for repeat array length when interpreting microsatellite data. The unusual patterns of variation in the *P. falciparum* genome have generated a lively debate about parasite origins and evolutionary history.

P. vivax microsatellite sequences show comparable levels of variation to those seen in *P. falciparum* when repeat array length is taken into account. Polymorphism is seen in the two plasmodium species with amplification by the primers based on SSR's.

Comparative study of genome complexity in both genomes showed the strand compositional asymmetry with (C-G) / (C+G) counts.

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