CHARACTERISATION OF Y-STR IN SEXUAL ASSAULT VICTIMS AND COLLECTION OF ALLELE FREQUENCIES AND HAPLOTYPES IN PUNJAB.

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IN THE NAME OF ALLAH, MOST GRACIOUS, MOST MERCIFUL.
PEACE AND BLESSINGS OF ALLAH BE UPON MY LORD MUHAMMAD AND HIS PROGENY.

CONTENTS

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LIST OF ABBREVIATIONS.

DNA	Deoxyribo nucleic acid.
DYS	D= DNA, Y=Y chromosome, S=single copy sequence
STR	Short tandem repeat
VNTR	Variable number of tandem repeat
NCVS	National crime victimization study
DOJ	Department of justice
IPSA	Intimate partner sexual assault
SWGDAM	Scientific Working Group on DNA Analysis Methods.
ISGF	International Society of Forensic Genetics.
R & c	reverse and complement
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucelotide Polymorphism
Ng	nano gram
RAPD	Random Amplification of Polymorphic loci
Taq	Thermal aquaticus
Mt DNA	mitochondrial DNA
CODIS	Combined DNA Index System
µl	miro litre
µg	nicrogram
ml	milliliter
bp	base pair
T_m	melting temperature
Freq	Frequency
n	number
SD	Standard deviation
GD	Gene Diversity
HD	Haplotype diversity
HT	Haplotype diversity

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ABSTRACT:

Sexual assault is a violent crime and a significant problem faced by any developing society. Identification of spermatozoa is the biological evidence most often sought in specimens from sexual assault victims. Absence of spermatozoa usually terminates biological investigations, and the victim's testimony is contested. DNA with high discrimination potential and sensitivity obtained in an unbiased manner would be highly advantageous in sexual assault cases with negative results.

Cervico-vaginal, oral and anal swabs were collected from 930 sexual assault cases, within a period from **July 2006 to December 2007**. In a total of 930 cases, 300 cases (32.25%) were found cytologically negative. Y-Chromosomes were detected in 108/300 (36%) cases, a fairly high percentage indeed for victims whose testimony would have been contested otherwise. Y-chromosome was also evidenced in the population of victims examined after 72 hours of sexual assault. Spermatozoa are rarely detected at such intervals, so medical men are reluctant to take samples, but our studies show that swabs ought to be taken from victims for Y-Chromosome DNA typing even after long lapses of time between sexual assaults and medical examination. Y- STRs are also very useful in detecting the male DNA fraction in male/ female DNA mixture where amount of female DNA is present in a far excess amount. It can also provide evidence in old and wasted samples.

Eleven Y-STR(short tandem repeats), namely DYS 19, DYS385 a,b, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS438, DYS439, residing on the Y-chromosome and amelogenin recognized by an international body "The Scientific Working Group on DNA Analysis Methods" (SWGDAM) were chosen and amplified simultaneously using polymerase chain reaction (PCR) by the Y-PLEX^{TM} 12 system, a

sensitive, valid, reliable, and robust multiplex system. Amelogenin provided results for gender identification and served as internal control for PCR. It did not adversely affect the amplification of Y-STRs in mixture samples containing male and female DNA.

After assurance of quality standards in accordance with the instructions laid down by DNA advisory Board (DAB), population data from Punjab was collected and comparison made with studies from other global population groups in addition to 12 ethnic groups from Pakistan. It showed greater genetic similarity with Africa, West Asia, and Europe but not with China & Japan. Arlequin version 3.0, a standard software for haplotyping analysis was used, the value of haplotype diversity is 0.9766 +/- 0.0074, gene diversity 0.67619 with standard deviation 0.10293. The value of average diversity over loci is 0.672759 +/- .354019. The mean number of pair wise differences are 6.727587 +/- 3.196717.

Therefore, after adopting a proper strategy of designing and optimising multiplex system for Y-STRs, we can successfully resolve the matters of individual identification, determination of perpetrators of violent sexual crime, of unestablished paternity and paternal lineage.

INTRODUCTION.

The remarkable advances in DNA technologies over the past two decades have enhanced the ability of crime investigation laboratories to identify individuals uniquely, as individual identification is desirable in a number of situations including the determination of perpetrators of violent crime such as murder and sexual assault.[1]

SEXUAL ASSAULT.

Sexual assault is generally considered to be one of the most serious offences that affects millions of people every year and can be difficult to prosecute. It causes tremendous physical, mental and emotional trauma to the victim. Among all forms of sexual assault the most heinous and that arouses the most passionate response is the sexual abuse of women. Its victims are at an increased risk of being abused again.[2,3,4]. Sexual violence perpetrators are also at increased risk of perpetrating again.[5]

Statistics shows a persistent increase in sex assault cases with poor conviction rates. However, these statistics vary due to differences in how it is defined and how data is collected. Sexual violence data usually comes from police, clinical settings, nongovernmental organizations, and survey research. Available data greatly underestimates the true magnitude of the problem. Rape is one of the most underreported crimes. Reporting rates for rape vary across different studies. Victims in many cases, decide not to report these offences for the fear of embarrassment, a sense of deep shame and considering her future life. This knowledge, along with vast multitude of emotional sequel of rape and self perceived inferior legal status of involved females results in a high percentage of unreported cases. The National Violence Against Women Survey (NVAWS) found that only 1 in 5 adult women (19%) reported their rapes to police.[6]

Estimates of rapes reported to the police from the National Crime Victimisation Study (NCVS), conducted by the Department of Justice (DOJ), vary widely from year to year, from 39% in 2002 to 54% in 2003.[7] The difference between the NCVS and NVAWS rates is most likely due to different survey methods used in these two studies, so their differing results should not be directly compared.

A man is said to commit rape, if he has sexual intercourse with a woman [8] against her will [9] without her consent. [10] With her consent, if that has been obtained by putting her or any other person in whom she is interested in fear of death, or of hurt [11] with her consent, when at the time of giving such a consent by reasons of unsoundness of mind or intoxication or by administration of stupefying agent, she is unable to understand the nature and consequences of that to which she gives consent. [12] With or without her consent, when she is under the age of sixteen years.

"Will" and "consent" are different. Every act performed against the will of a person is carried out without her consent, but an act performed without the consent of a person is not necessarily against her will. The woman must have voluntarily participated in the sexual act, after the exercise of intelligence and also her choice between resistance and assent, for the consent to be valid. "Submission is not necessary consent, though consent necessary involves submission".[11] The material facts to be considered are the conduct and behaviour of the victim. It is not a rape when a woman initially objects, but subsequently gives her consent to sexual act.

Consent or its absence can be presumed from the accompanying circumstances of each case. The chief evidence of lack of consent is a sign of resistance, that is naturally expected from a woman unwilling to a sexual intercourse forced upon her. Such a

resistance may cause the tearing of clothes, injuries on the body, and even on her private parts. It is necessary to prove that the woman offered maximum resistance, and that all means had been tried to prevent sexual intercourse, including: shouting, crying, biting, beating etc. The woman may surrender from fear or exhaustion, in which case it is regarded as rape. The resistance offered depends upon the type of woman, her age, development and social status. [12] The slightest penetration of the penis within the vulva, such as the minimal passage of glans between the labia with or without emission of semen or rupture of hymen constitutes rape. Rape can be committed even when there is an inability to produce penile erection. [12]

Drugs may play a role, date rape drugs are sometimes used to assist a sexual assault, most experts prefer the term "drug facilitated assault". Sexual assault is any type of sexual activity that a person does not agree to. It can include inappropriate touching, vaginal penetration, sexual intercourse, rape and attempted rape. It is because of the effects of these drugs that victims may be physically helpless; unable to refuse sex, can't remember what happened. These drugs have no colour, smell or taste and are easily added to flavoured drinks without the victim's knowledge. Commonly used date rape drugs are:

i. GHB (gamma hydoxybutyric acid)

ii. Rophynol (flunitrazepam)

iii. Ketamine (Ketamine Hydrochloride) [13]

Rape can occur without causing any injury, and as such negative evidence does not exclude rape. The prosecution has to prove all elements of the offence. In many cases of rape, there are no signs of injury or intoxication by stupefying drugs, and the entire

allegation of lack of consent is based on fear and fraud. As per section 114 A of evidence act, if the victim states in her evidence before the court that she did not give consent for sexual intercourse, the court presumes that she did not consent in a custodial situation or when she is a victim of gang rape. The burden of proof of the consent rests on the accused. In Pakistan under the prevailing law, only a man can commit rape, and a woman cannot rape a man, she can only be tried in court for indecent assault. There is no age limit under which a boy is considered physically incapable of committing rape; the court decides the question of his potency, even old men can commit rape on very young girls. [14]

Definitions of Rape.

It is frequently defined as penetration of the anus or the vagina by penis without valid consent of one of the parties involved. In some jurisdictions the penetration need not be by penis but can be by other body parts (e.g. one or more fingers, i.e. digital penetration) or by objects (e.g. a bottle), or may involve the forcing of a vagina or anus onto a penis by a female assailant. Other jurisdictions expand the definition of rape to include other acts committed using the sexual organs of one or both of the parties, such as oral copulation and masturbation, for example, again enacted without valid consent. [15]

Types of Rape – by violation of consent.

1. **Acquaintance ("date") rape.** These are non-domestic rapes committed by someone who knows the victim. They include rapes of co-workers, schoolmates, friends, and other acquaintances, including "date rapes." [16]

    a. **Forcible date rape:** The term "acquaintance rape" or "date rape" refers to rape or non-consensual sexual activity between people who are already

acquainted, or who know each other socially as friends, acquaintances, people on a date, or even people in an existing romantic relationship, where consent for sexual activity is not given, or is given under duress. The vast majority of rapes are committed by people who already know the victim.[17]

b. Drug facilitated date rape:

In this various drugs are used by rapists to render their victims unconscious, some also cause memory loss.

2. Blitz rape:

Rape by a stranger in which the rapist assaults the victim on the street with no prior contact.

3. Spousal rape:

Also known as spouse, marital rape, wife rape, husband rape, partner rape or intimate partner sexual assault (IPSA), is rape between a married or *de facto* couple. Research reveals that victims of marital/partner rape suffer longer lasting trauma than victims of stranger rape, [18] possibly because of a lack of social validation that prevents a victim from getting access to support; a problem that domestic violence services combat.

4. College campus rape:

Some studies indicate a particular problem with rape on college campuses. According to a study carried out in 1992, one out of twelve college aged men and one in every twenty college aged women committed rape, making each responsible for an average of three rapes.[19] The Department of Justice study also found that in "about half of the incidents categorised as completed rapes, the women or man did not consider the incident to be a rape." [20] According to the *Journal of Counseling and Development*,

women aged 16–24 are at the highest risk of sexual assault. One study has concluded that as many as one in four college aged females has been a victim of either rape or attempted rape. [21]

5. Group rape:

Group rape (also known as "gang" "gang bang", "run a train", "pack" rape) occurs when a group of people participate in the rape of a single victim. Rape involving at least two or more perpetrators is widely reported to occur in many parts of the world. In Johannesburg, South Africa, surveillance studies of women attending medico-legal clinics following a rape found that one-third of the cases had been gang raped. [22] National data on rape and sexual assault in the United States reveals that about 1 out of 10 sexual assaults involve multiple perpetrators. Most of these assaults are committed by people unknown to their victims.[23] The word *tournante* is a French adjective meaning "turning" and is used as a slang term to mean a gang rape. According to the testimony of numerous victims, young muslim women who stray from traditional conduct in the immigrant neighborhoods, such as behaving and dressing like a westerner, or wanting to live as Europeans or refusing to wear the traditional clothing, have been targeted for *tournantes*.[24] According to Samira Bellil in a CNN interview, there was a trial in Lille regarding a 13-year-old girl who had been allegedly gang-raped by 80 men.[25]

6. Rape of children by parents, elder relatives, and other responsible elders:

This form of rape is incest when committed by the child's parents or close relatives such as grandparents, aunts and uncles. Edith Kriel, a social worker who helps child victims in the Eastern Cape, said: "Child abusers are often relatives of their victims even their fathers and providers."[26]

According to University of Durban-Westville anthropology lecturer and researcher Suzanne Leclerc-Madlala, the myth that sex with a virgin is a cure for AIDS is not confined to South Africa. “Fellow AIDS researchers in Zambia, Zimbabwe and Nigeria have told me that the myth also exists in these countries and that it is being blamed for the high rate of sexual abuse against young children.”[27]

7. Statutory rape.

Sex that violates age-of-consent law, but is neither violent nor physically coerced, is sometimes described as "statutory rape," a legally-recognized category in the United States.

8. Prison rape

Many rapes happen in prison. These rapes are virtually always homosexual in nature (since prisons are separated by sex). The attacker is most commonly another inmate, but prison guards may also be involved, particularly in female prisons. [28]

9. Third-party rape

These rapes occur when one person forces other people to have sex without actually sexually engaging either person. This has been used against prisoners of war in Bosnia and Herzegovania.

10. Rape as means of warfare

This type of rape is also known as 'war rape.' During war, rape is often used as means of psychological warfare in order to humiliate the enemy and undermine their morale. Rapes in war are often systematic and thorough, and military leaders may actually encourage their soldiers to rape civilians. Likewise, systematic rapes are often employed as a form of ethnic cleansing. During the Yugoslavian Civil war, it was

reported that Serbian soldiers herded enemy women into camps, who were then raped on a daily basis until pregnancy occurred.[29-30] There are numerous [31] cases of rapes conducted on Jewish women and girls by German soldiers during invasion of Poland [31]. Rapes were also committed against Polish women and girls during mass executions made primarily by Selbstschuts, which were accompanied by Wehrmacht soldiers and on territory under administration of German military, the rapes were forced before shooting female captives. Thousands of Soviet female nurses, doctors an+d field medicians fell victim to brutal German rapes when captured during the war, and often they were murdered afterwards[32]. Wehrmacht also ran brothels where some of the women were forced to work[19]. Ruth Seifert in *War and Rape: Analytical Approaches* writes, "In the Eastern territories the Wehrmacht used to brand the bodies of captured partisan women - and other women as well - with the words 'Whore for Hitler's troops' and to use them accordingly."

Norman Naimark writes in *"The Russians in Germany: A History of the Soviet Zone of Occupation, 1945-1949."* that although the exact number of women and prepubescent girls who were raped by members of the Red army in the months preceding and years following the capitulation will never be known, their numbers are likely in the hundreds of thousands, quite possibly as high as the 2,000,000 victims estimate made by Barbara Johr, in *"Befreier und Befreite"*. Many of these victims were raped repeatedly.[33] Additionally, in China during World War II, the Nanking massacre occurred, where rape was used as a tool to humiliate the civilians under Japanese oppression. As many as 80,000 women were raped by the Japanese soldiers during the six weeks of the Nanking

Massacre.[34] Comfort women is a euphemism for up to 200,000 women, who were forced into prostitution in Japanese military brothels.[35]

According to a review of *'The GI War against Japan: American Soldiers in Asia and the Pacific during World War II'*,[36] U.S. soldiers rape of Japanese women was a "general practice".[37] French Moroccan troops known as Goumiers, committed rapes and other war crimes after the Battle of Monte Cassino.[38] In 1998, the International criminal tribunal for Rwanda, estimated that 500,000 women were raped during the 1994 Rwandan genocide.[39]

11. False Accusations of Rape

There have been many examples of false accusations of rape, as vulval and vaginal injuries can be produced by fingers and instrumentation in children and virgins. Artifical bruises can be produced by marking nut juice. Vagina may be irritated by chillies. Solution of starch and egg albumin can be used to stain the clothes as it simulates semen stains. False charges can be disproved by:

i. circumstantial evidence.

ii. Statement of the victim and others.

iii. Injuries caused.

iv. Doubtful story about administration of drugs.

v. Results from laboratory specimens.

As mentioned, there are various reasons why rape victims may not be able to identify the perpetrators. On the other hand, in a small number of cases, the victims can identify the offender, but the suspect can easily refute the accusation. In such circumstances, biological evidence from the crime scene of sexual assault can lead to

vital information in the process of identifying the assailant.[40] The identification of spermatozoa as a biological evidence is most often sought in examination of alleged sexual assault victims.[41] However, in some cases, when no sperm cell is visible on cytological examination, the testimony of the victim is open to contestation.

Failure to demonstrate the presence of spermatozoa in specimens taken from victims of sexual assault can be explained by a number of factors including a prolonged post coital interval, an oligospermic or azoospermic assailant, a vasectomised or orchidectomised assailant, penetration without ejaculation, digital penetration, use of condom, douching after intercourse, use of spermicidal agents, oral contraceptives, menstruations and vaginal inflammation.[42-44] Spermatozoa are also rapidly destroyed in the mouth by salivary enzymes and in the anus by bacterial enzymes.[45] Sometimes, false negative results due to excessive inflammation or haemorrhage (menstruation) can also occur at the time of examination.[46] The longer the interval between intercourse and the sampling, the fewer the sperm cells detected. This factor often leads the forensic doctors not to take swabs beyond three days, however it has been proved to be incorrect during the present studies.

It is worth mentioning that failure to demonstrate the presence of spermatozoa does not preclude the presence of Y-chromosome from male DNA, as Y-STRs have found applications in forensic DNA analysis, such as murder and sexual assault cases,[47-48] paternity testing,[49] and male lineage studies,[50] because of their advantages over autosomal STRs:

i. Male profile can be obtained in the presence of large amounts of female DNA.

ii. Differential extraction of sperm and non-sperm (i.e., epithelial and other cells) fraction is not necessary.

iii. Analysis of azoospermic semen samples from vasectomised males is feasible.

iv. The number of male contributors can be determined in multiple rape cases because of the haploid nature of the Y-STRs.

v. Rapid exclusion of suspects can occur.

vi. Interpretation is simplified due to single allele per locus profile.

vii. Multigeneration male lineage studies can be performed.[51]

Scenarios where Y-Strs can aid forensic casework. [51]

i. Sexual assaults by vasectomised or azoospermic males (no sperm left behind for differential extraction)

ii. Sexual assault cases – when only a small amount of male DNA is present.

iii. Post-conviction cases – evidence is old and degraded.

iv. Cold cases.

v. Extending length of time after assault for recovery of perpetrator's DNA profile (greater than 48 hours)

vi. Fingernail scrapings from sexual assault victims.

vii. Assault or homicide cases – female victim fought off attacker by scratching him or suspect injured himself during attack.

viii. Male-male mixtures.

xi. Multiple males – used to clarify the number of male donors in a sample.

x. Gang rape situation to include or exclude potential contributors.

xi. Other bodily fluid mixtures (blood-blood, skin-saliva).

xii. Conventional testing yields an incomplete male DNA profile.

xiii. Screening indicates the presence of seminal fluid but a few or no sperm are present (possibly due to a vasectomy).

xiv. Identification of human remains – through comparison to a paternal relative.

xv. Kinship cases – a paternal relative can be tested in lieu of an alleged father.

Table No 1. Forensic value of Y-chromosome markers.

Use	**Advantage**
Forensic case work on sexual assault evidence.	Male specific amplification (can avoid differential extraction to separate sperm and epithelial cells).
Paternity testing.	Male children can be tied to fathers in motherless paternity cases.
Missing persons investigations.	Patrilineal male relatives may be used for reference samples.
Human migration and evolutionary studies.	Lack of recombination enables comparison of male individuals separated by large periods of time.
Historical and genealogical research.	Surnames usually retained by males; can make links where paper trail is limited.
Court acceptance.	Courts have already widely accepted STR typing, instrumentation, and software for analysis (Y-STR markers just have different PCR primers).

Butler J M.[52]

DNA, the blue print of our life.

Robert Hooke discovered the basic unit of life i.e. cell [53] and the determination of the structure of deoxyribonucleic acid (DNA) by James Watson and Francis Crick in 1953 is often said to mark the birth of modern molecular biology.[54-55]

DNA is a genetic material present in large amounts within the nucleus of our cells and is divided into chromosomes. The DNA within chromosomes is composed of both 'coding' and noncoding regions. Coding regions contain genes, which serve as the blueprint for self-replication and protein synthesis. Genes can vary in size from a few thousand to tens of thousand base pairs. It is estimated that there are 30,000-40,000 protein-coding genes contained within the human genome.[56-57] However, begun in 1990 and completed in 2003, Human Genome Project identify all the approximately 20,000 to 25,000 genes in human DNA [genomics.energy.gov], but not all the genomic scientists agree. The initial sequencing and analysis of the human genome, recently completed estimate to contain about 3.1 billion base pairs. Approximately 99.7% of human DNA is the same between individuals. The remaining 0.3%(~ one million nucleotides) varies from one individual to another individual.[58] These differences provide the opportunity for using DNA sequence information for endeavours such as human identification.

DNA Polymorphisms and Short Tandem Repeats (STRs).

DNA molecules demonstrate various types of polymorphisms (Flowchart 1), that can be categorised into the following classes:

A. Polymorphisms in the coding region
B. Polymorphisms in the noncoding regions comprising of:

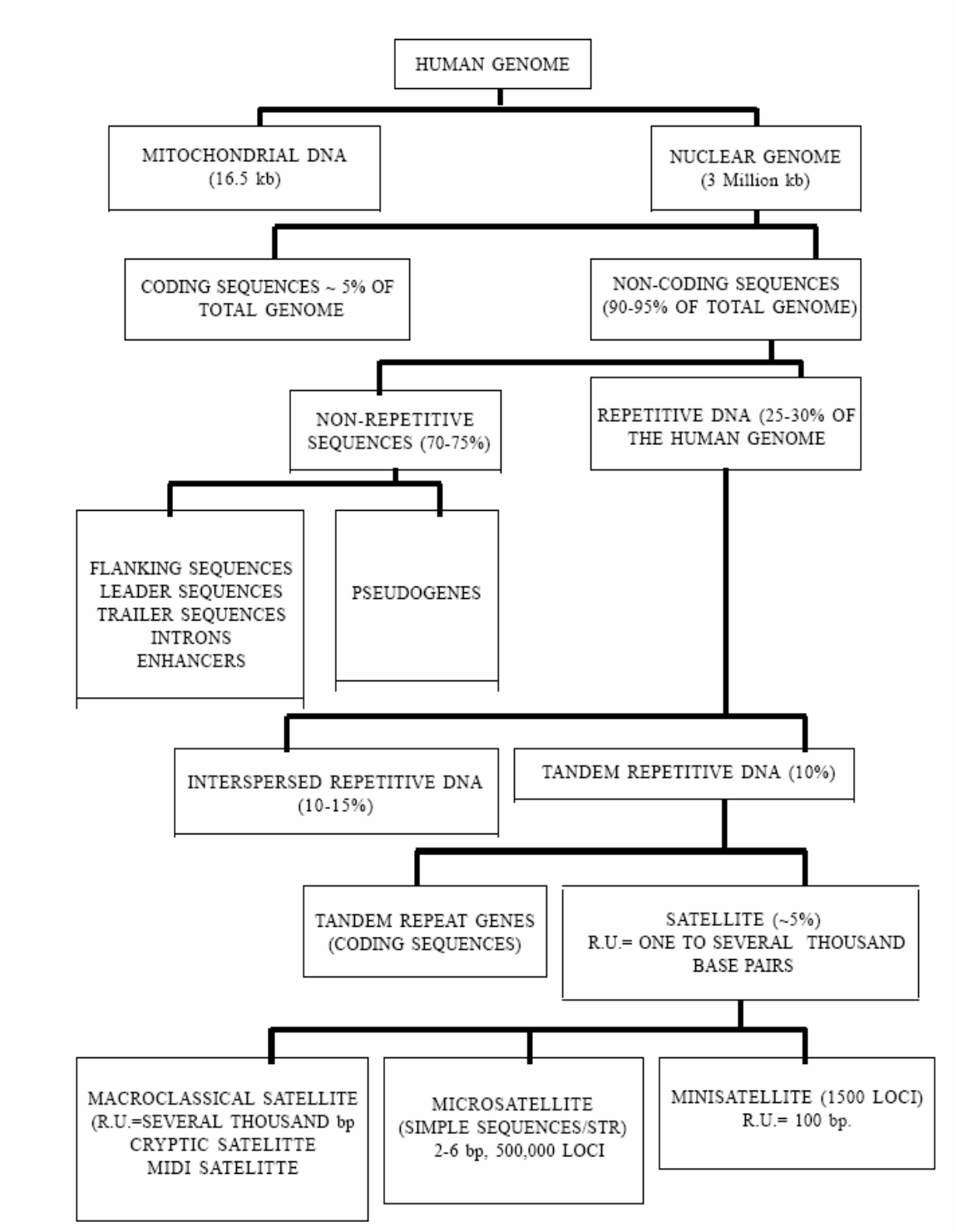
1. Variable number of Tandem Repeats
    a. Minisatellites
    b. Microsatellites
2. Single Nucleotide Polymorphisms

Minisatellites.

VNTRs are regions of DNA comprising hundreds to several thousand base pairs and are arranged as tandem repeat units. Loci with long motifs (e.g., 8-80 bp) are referred to as minisatellites [59] or variable number of tandem repeats (VNTRs).[60] The number of repeats varies greatly from person to person.[61]

The application of VNTRs for gene mapping, population genetics and personnel identification has, however, been limited by the low stability, low frequency,[62] asymmetric distribution [63] of these repeats in the genome, requirement of high molecular weight DNA template, poor amenability to PCR and inability to determine the alleles precisely with Southern hybridisation-based detection methods.

STRs first reported in the late 1980s are repeated base pair sequences that vary in length from 2 – 6 base pairs, [64] and can readily amplify with PCR. It is estimated that STR makers in the human genome occur every 10,000 nucleotides.[65] Although amplification of VNTRs with PCR enables more precise allele determinations,[66-67] the large size of the products makes them less suitable for general application than tandem repeats with amplification products of 100 to 500 bp.



Flowchart: 1 Polymorphic regions in the human genome (RU: repeat unit)

The small size of dimeric, trimeric, and tetrameric STRs facilitates their simultaneous study in a multiplex PCR, in which 9 to 16 or more loci are amplified in one reaction from a single DNA sample, [68] offering both higher throughput and greatly increased sensitivity over conventional single and multilocus DNA probe techniques. STRs found within chromosomes 1-22 are termed autosomal DNA markers. These markers are recombined with each mitotic event because half of the genetic information comes from the mother and half comes from the father. An individual is homozygous if the alleles at a specific location are identical and heterozygous if the alleles are different.[69]

Y-STRs are simply STRs that are located on the Y chromosome, most of the extraction, quantification, and amplification methods that are used for autosomal STR analysis can be applied to Y-STRs as well. However, important differences between autosomal and Y-STRs do exist, and they are mainly related to the biology of the Y chromosome. The Y chromosome is only carried by males and is passed along paternal lines. It is one of the smallest chromosome, and is more likely to remain intact in heavily degraded samples. Since all Y-STR loci are located on the single Y chromosome, these alleles do not undergo independent assortment during gametogenesis (production of sperm or ova) and are instead passed to the next generation in the same whole unit, or haplotype. Therefore, all paternally-linked males have the same Y-STR profile. Random mutations may occur along the Y chromosome, potentially resulting in a minor change in the haplotype passed to the next generation. However, these mutational events occur at about the same frequency as in autosomal STRs, that only approximately 0.1 to 0.4% (or 1-4 changes per thousand generational events). [70]

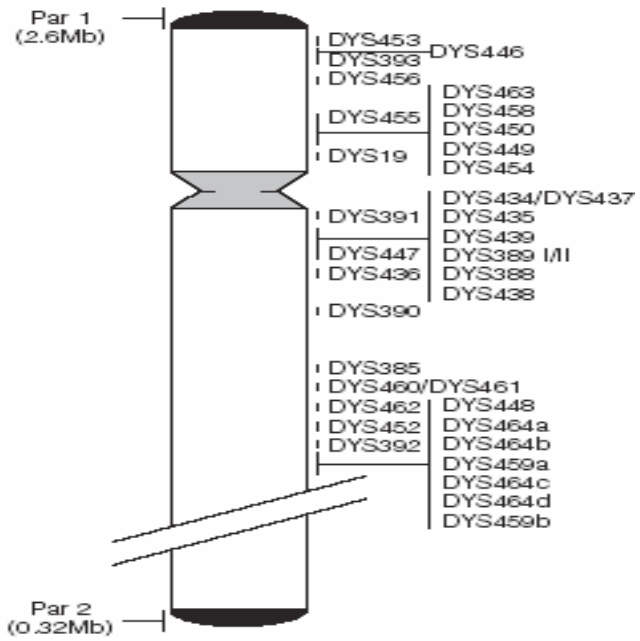


Figure: 1 The relative positions of PAR 1 and 2. Y-STRs on Y-Chromosome.

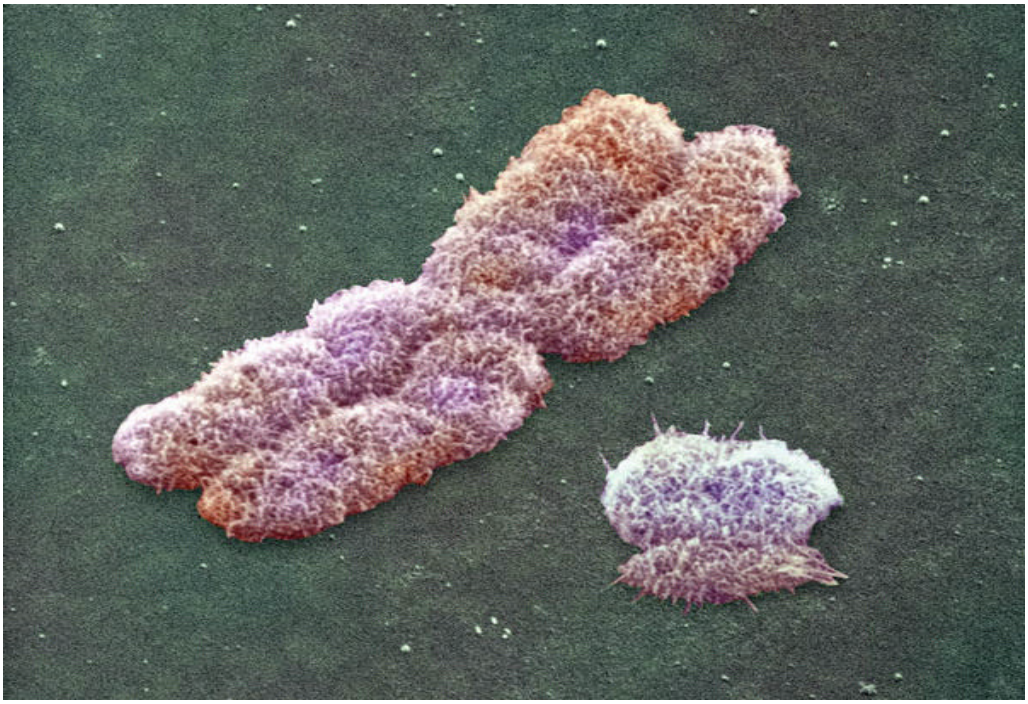


Figure: 2 A scanning electron microscope view of the X & Y chromosome pair.

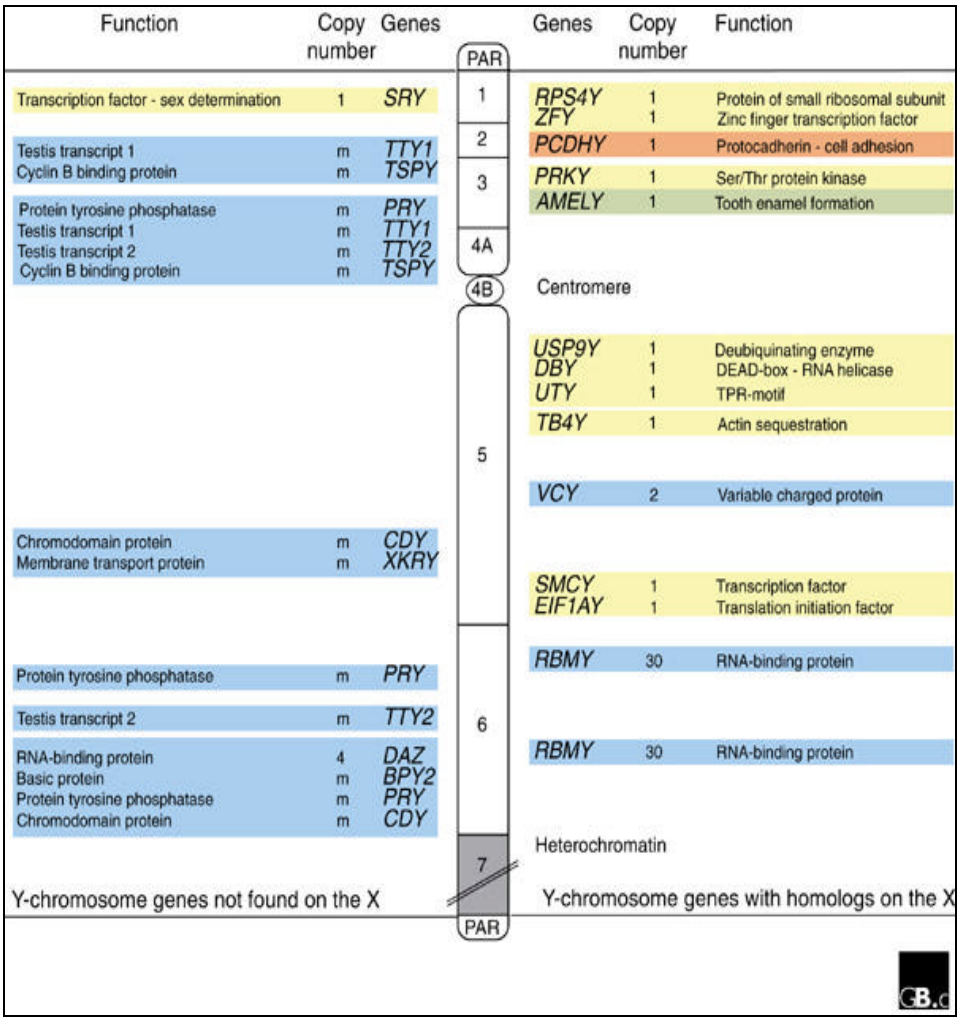


Figure 3:Genetic map of the non-recombining region of the human Y chromosome.

{** The human Y chromosome consists of a large non-recombining region (NRY, which consists roughly of 24 megabases (Mb) euchromatin and 30 Mb heterochromatin) flanked by short pseudoautosomal regions (PAR, about 2.6 Mb and 0.4 Mb, respectively). Genes specific to the Y chromosome are indicated on the left-hand side of the diagram, and genes with homologs on the X chromosome are on the right-hand side. Ubiquitously expressed genes are shown in yellow, testis-specific genes are in blue, tooth-bud-specific genes in green, and brain-specific genes in red. Some testis-specific gene families have additional locations on the NRY not shown (m: multiple copies). The extensive region of Y-chromosomal heterochromatin is indicated by the grey area. The NRY is divided into intervals (as indicated by numbers on the chromosome), defined by naturally occurring deletions.**}

Due to less variability and lack of polymorphic markers the forensic use of Y-chromosome was until recently restricted, lowering the power of discrimination and non-exclusion cases needed to be investigated further. A useful way to increase the power of

discrimination and gain acceptance within the forensic DNA community is by using robust multiplexes and use of many markers. Many Y-STR multiplexes have been described.[71-72] and more than 200 STR loci have been identified on the Y-chromosome,[73] but majority of these currently known markers have not been fully characterized with respect to their utility in forensic case work up. Therefore, only some of these loci have proven useful for the human identification applications,[74 – 78]

. The Scientific Working Group on DNA Analysis Methods (SWGDAM) has identified a set of eleven loci—DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385a/b, DYS438, and DYS439—for forensic DNA analysis in the U.S.[79] This set includes the nine core loci generating minimal haplotypes identified by the International Y-STR user group plus the DYS438 and DYS439 loci. Sinha et al.[80-81] reported on the validation and forensic application of the Y-PLEX ™ 6 and Y-PLEX ™ 5 systems, for analysis of seven and five Y-STR loci, respectively. A study by Schewale and associates [82] describes the validation and application of the Y-PLEX ™ 12 system, that provides results in a single amplification reaction for eleven Y-STR loci recommended by the SWGDAM, as well as Amelogenin, a gene that codes for proteins found in tooth enamel and is the most popular gender determination marker. Over the past ten years the utility and validity of STR typing for forensic applications such as human identity testing have been substantiated.[83 –85] The use of multiple markers from individuals are examined in order to determine a person's DNA profile. The more markers examined improves the chances of obtaining a unique STR profile. Therefore, the Y-PLEX™ 12 genotyping system can be used for forensic casework and male lineage studies.

Polymerase Chain Reaction (PCR) and Multiplex PCR.

In order to increase the number of DNA molecules to a level suitable for STR analysis, the DNA must be amplified. Although molecular cloning techniques are indispensable to modern biochemical research, the polymerase chain reaction (PCR) is often a faster and more convenient method for amplifying DNA.[86,87] Multiple cycles of this process, each doubling the amount of target DNA, exponentially amplify the DNA starting with as little as a single gene copy. The use of a heat-stable DNA polymerase, such as *Taq* polymerase isolated from *Thermus aquaticus*, eliminates the need to add fresh enzyme after each round of heating.[86] Twenty cycles of PCR increase the amount of the target sequence around a million fold with high specificity.

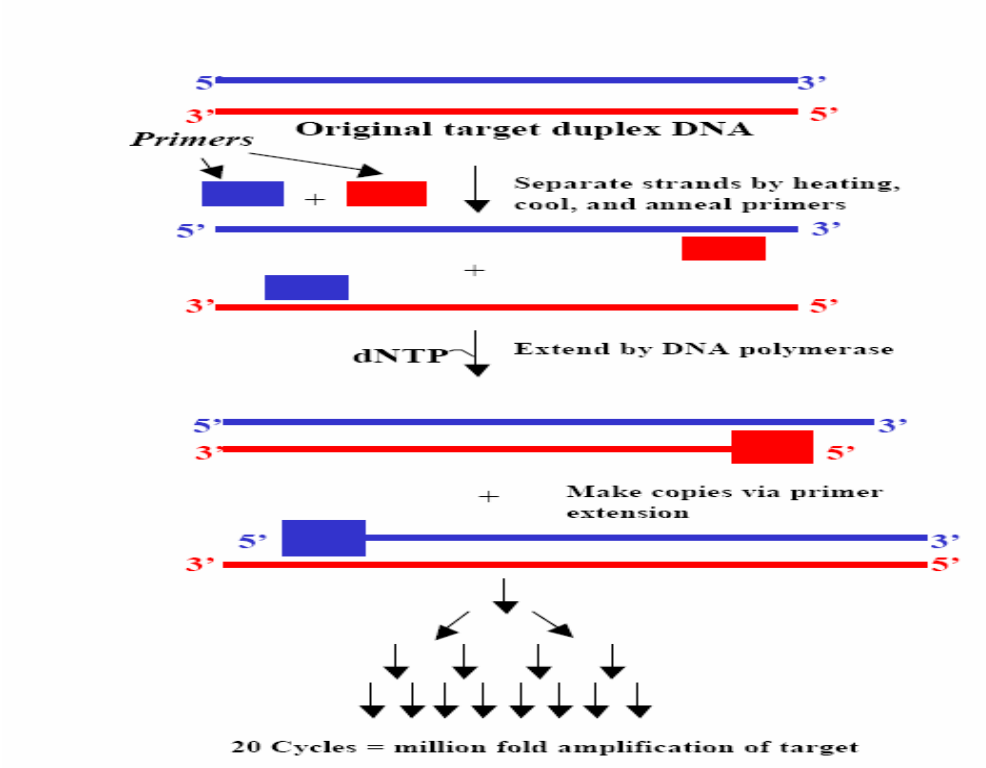


Figure 4. A diagrammatic representation of Polymerase chain reaction.

Two of the most important components of a successful PCR reaction are primer design and primer quality. The amount of PCR product, or amplicon, is directly affected by the annealing characteristics of the primers. If PCR is to work well, the forward and reverse primers must be specific for the target region, have similar annealing temperatures, and donot interact with one another. Additionally, the sequence to which the primers will bind must be fairly conserved. If the region where the primer is to bind is not conserved, primer annealing may not occur. This could result in a lack of amplification of the desired locus, termed null allele.[88]

Multiplex polymerase chain reaction (PCR).

It is defined as the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture. Fig 5 is an illustration showing the multiple PCR process. The resulting amplification products are of different sizes and can be differentiated using an appropriate analysis technique. Extensive optimisation is normally required to obtain good balance between amplicons of the various loci being amplified. [89,90]

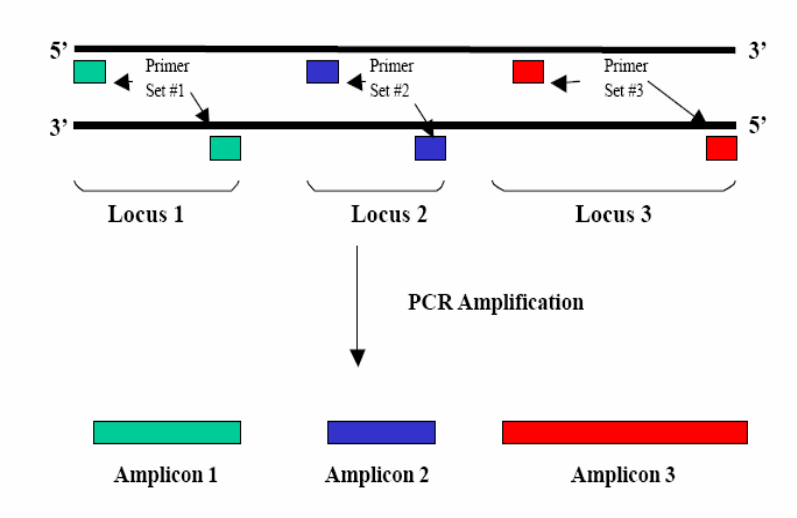


Figure 5: Diagrammatic representation of Multiplex PCR.

Simultaneous amplification of target DNA involving more than one Primer set. Each primer set is designed to amplify a different locus. Different coloured boxes represent the different primer sets. The resulting amplicons are the PCR amplification products for that particular primer set.

For a multiplex PCR reaction to work properly, primer pairs need to be compatible. Since multiplex assays are run under the same PCR conditions, the primers used need to have fairly similar characteristics such as melting temperature ™ and should not exhibit significant interactions with each other, themselves, or unwanted regions of the template. Excessive regions of complementarity between primers must be avoided to prevent the formation of primer-dimers that may cause the primers to bind to one another instead of the template DNA. Through stringent initial primer selection, the time consuming and often costly process of optimisation can be reduced. High quality primers are essential to successful multiplex amplification reactions.[91]

Since first being described in 1988,[92] PCR multiplexing has been applied in many areas of DNA testing including the analysis of deletions,[93] mutations,[94] autosomal STRs and Y chromosome STRs.[95 - 97] Furthermore, the wide availability of genetic information due to the publishing of the sequence of the human genome makes the demand for multiplex PCR even greater.

Y Chromosome STRs and Y STR Multiplex assay.

The Y chromosome has become a useful tool for tracing human evolution through male lineages [98] as well as application is to a variety of forensic situations [99] including those involving evidence from sexual assault cases containing a mixture of male and female DNA.[100,101] In 1998 the report entitled "Jefferson fathered slave's last child" used Y chromosome DNA markers to trace the Jefferson family line by linking the modern-day descendants of Thomas Jefferson and Eston Hemmings.[102] More recently, YSTR markers were used to study the Y-chromosomal lineages of the likely male- line descendants of Genghis Khan.[103]

Table 2, lists some of the Y chromosome STR markers available in the literature, including the ones studied in this work. All of the nomenclature listed for each Y chromsome STR marker follows the recommendations provided by International Society of Forensic Genetics (ISFG).[104] The ISFG is an international organization consisting of members from 49 countries that provide guidelines concerning the application of DNA polymorphisms to the area of human identification.

The information about common Y-STR markers shown in Table 2, includes the GenBank® Accession number for each locus, respective repeat motif, and number of repeats within the GenBank sequence (termed reference allele). Some sequences were made reverse and complement (R&C) in order to maintain consistency with previously used forward and reverse primer designations. Repeats motifs for each locus were defined using ISFG recommendations as a guide. [105]

Marker Name	Repeat Motif	GenBank Accession	Reference Allele
DYS19	TAGA	AC017019 (r&c)	15
DYS385 a/b	GAAA	AC022486 (r&c)	11
DYS389 I DYS389 II	(TCTG) (TCTA) (TCTG) (TCTA)	AC004617 (r&c)	12 29
DYS390	(TCTA) (TCTG)	AC011289	24
DYS391	TCTA	AC011302	11
DYS392	TAT	AC011745 (r&c)	13
DYS393	AGAT	AC006152	12
YCAII	CA	AC015978	23
DYS388	ATT	AC004810	12
DYS426	GTT	AC007034	12
DYS434	TAAT (CTAT)	AC002992	10
DYS435	TGGA	AC002992	9
DYS436	GTT	AC005820	12
DYS437	TCTA	AC002992	16
DYS438	TTTTC	AC002531	10
DYS439	AGAT	AC002992	13
DYS441	CCTT	AC004474	14
DYS442	TATC	AC004810	12
DYS446	TCTCT	AC006152	14
DYS447	TAAWA compound	AC005820	23
DYS448	AGAGAT	AC025227	23
DYS449	TTTC	AC051663	29
DYS450	TTTTA	AC051663	9
DYS456	AGAT	AC010106	15
DYS458	GAAA	AC010902	16
DYS459 a/b	TAAA	AC010682	9
DYS460 (A7.1)	ATAG	AC009235 (r&c)	10
DYS461 (A7.2)	(TAGA) CAGA	AC009235 (r&c)	12
DYS462	TATG	AC007244	11
DYS464 a/b/c/d	CCTT	AC006338	15
Y-GATA-H4	TAGA	AC011751 (r&c)	12
Y-GATA-C4	TSTA compound	G42673	21
Y-GATA-A10	TAGA	AC011751	13

Table 2. Common Y-STR markers.

Included in the Table 2, are a core set of Y STR markers that are currently being used by the European Y chromosome typing community. They have established a "minimal haplotype" and an "extended haplotype" for inclusion of common loci into a central DNA database (http://www.ystr.org). The minimal haplotype consists of results for the following Y STR markers: DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, and DYS385. The extended haplotype includes all of the markers from the minimal haplotype plus the highly polymorphic DYS 438, DYS 439.[106]

A view of the approximate location of Y –STR loci recommended by SWGDAM is shown below (Fig 6)

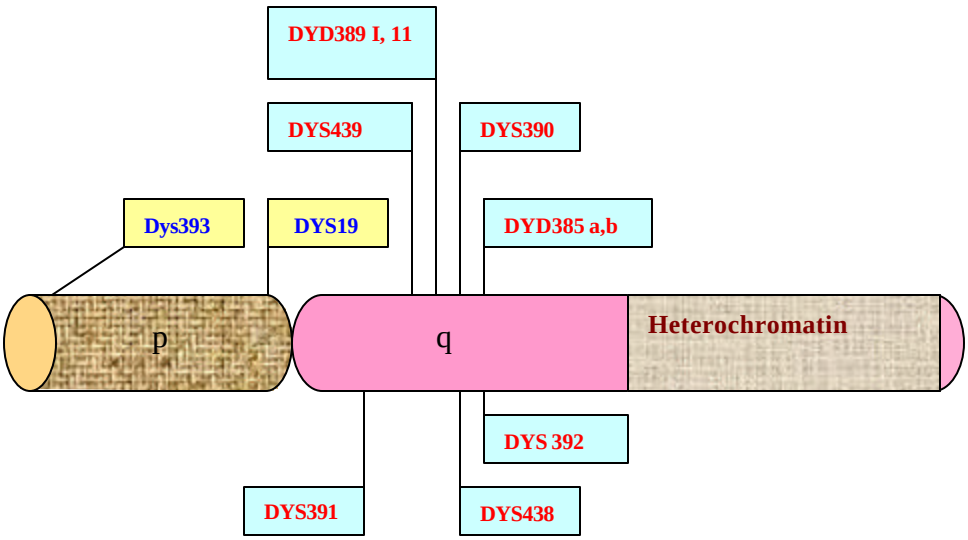


Figure 6. A detailed view of the Y Chromosome, including locations of the Y-STRs recommended by SWGDAM.

SUITABLE SAMPLE FOR FORENSIC EVIDENCE:[107]

Clothing (blouses, under garments etc), (sexual assault, rapes or murders).

Bedding (blankets, bed sheets, pillow covers) (rape and sexual assault cases).

Cars and car seats (kidnapping, car jacking, rapes etc).

Shoes (almost all types of cases).

Cigarette butts.

Hair

Teeth (identification of missing people, missing children, war casualties, reconstruction of bodies and mass disaster).

Fingernail (sexual assaults, rapes or murders).

Blood Stains (sexual assaults, rapes or murders).

Semen Stains (sexual assaults, rapes or murders).

Vaginal secretions (sexual assaults, rapes or murders).

Chewing gum (saliva)

Ski masks (saliva and hair) (bank robberies, car jacking, rapes or whenever the suspect wants to hide his identity).

Caps (hair).

Tooth brush (saliva).

Ear rings.

Nasal secretions.

Ear wax.

Watches.

Coffee cups, soft drink cans, beer bottles, etc.

Shirts (neck area of sweat).

Tooth picks.

Eyes glasses (nose piece and the back of arm).

Hearing aids.

Condoms.

Saliva stains (threatening letters, or claiming the responsibility of certain crime in a letter).

Finger rings.

Bangles.

Socks

Fingernail clippings or scrapings from a female assault victim.

Old rape kit swab tips, condoms, swab sticks, and undergarments.

New rape kit evidence including swabs, condoms, and panties.

Microscope slides which may contain male DNA.

The remarkable advances in DNA technologies have had an enormous impact on human identification, medical diagnosis, population genetics, understanding of evolution of species, wildlife management, characterization and unfolding the mysteries of antiquity of archaic specimens. The current technologies in the field of forensic genetics, their evolution and the emerging trends have opened a new frontier in forensic analysis. Research in Y-Chromosome markers, assays, and applications has seen a remarkable growth in the past several years. New and improved methods have developed over the years, with high degree of discriminatory power and an analysis period of a few hours.

VARIOUS TRENDS IN DNA PROFILING TESTS.

DNA profiling tests are of different kinds, based on the nature of variable region to be analysed, whether minisatellites, microsatellites, or the sequence of nucleotides. The methods of analysing different types of polymorphisms are:

1.0 Hybridization-based method: Restriction fragment length polymorphism (RFLP). {riflips}.

A difference in restriction maps between two individuals is called restriction fragment length polymorphism. Basically, an RLFP is an SNP that is located in the target site for restriction enzyme. It can be used as genetic marker in exactly the same way as any other marker. Instead of examining some features of the phenotype, we directly assess the genotype, as revealed by the restriction map. The basic principle of RFLP[108] is separation of the desired repetitive sequences by cleaving them out from the genome using an appropriate restriction endonuclease enzyme, electrophoresis of the digested DNA and thereafter their detection by DNA probes. This kind of analysis determines variation in the length of a defined DNA fragment. Sir Alec Jeffreys deserves credit for introducing the use of hyper-variable loci for individual identification and multi locus probes.[109] RFLP produce distinctive differences in banding patterns following electrophoresis which can be used to identify individuals by preparation of a finger print or to track the inheritance of a particular gene. However, it was difficult to interpret statistically the multi-banded pattern produced from multi-locus probes and from samples containing more than one source, as in samples from most of the sexual assault cases. Furthermore, multi-locus probes required a large amount of intact DNA. This led to the advent of SLPs (Locus specific probes).

2.0 Amplification based Technology.

Polymerase chain reaction (PCR).

The polymerase chain reaction (PCR)[110,111] technique has revolutionised the entire field of DNA study. Accuracy, precision and rapidness are the hallmarks of PCR based technology, rendering it the most informative status, also owing to its simultaneous amplification and typing of template sequence. It is also indispensable in situations where the specimen is very little or the DNA is degraded. It is an amplification procedure in which billions of copies are generated from the template in few hours.

2.10 Fluorescent nonanchored inter simple sequence repeat (FISSR) PCR.

It is a stable technology across a wide range of PCR parameters; with no prior genomic information is required for their use. Hence these markers are used in DNA fingerprinting.[112] The sensitivity, speed and informativeness of the existing ISSR-PCR method can be enhanced substantially by using fluorescent dye labeled nucleotides in the ISSR-PCR reaction, that is termed FISSR-PCR,[113] followed by separation of PCR products on an automated sequencer.

2.20 Short tandem repeat (STR) typing by monoplexing and multiplexing methods.

The most convenient approach of analysis of variations in length of the repeat sequence of microsatellites is to amplify the region of interest and determine the size of the amplified product. The use of PCR in STR analysis permits very tiny amounts of DNA, as found on a postage stamp, cigarette butt, or coffee cup, to be amplified to produce large amounts of DNA sufficient for analysis. In forensic applications, amplified and separated STR fragments are generally detected using one of the two methods. One method uses the propensity of silver or SYBR green to bind to DNA. A second prevalent

method requires that some of the primers used during the amplification contain fluorescent tags, which are incorporated into the STR fragments generated during amplification, and detected by automated platforms. Fortunately, it is possible to analyse many STR loci simultaneously in a DNA sample. Such systems (multiplexes) have been developed and they allow amplification of 3 to 16 loci in a single instance.

The advantages of STR based DNA profiling over other methods are:

i. Even highly degraded samples yield results (since shorter fragments of DNA can be analysed).

ii. Small amounts of DNA (1ng) can also be analysed because of amplification-based protocols.

iii. STR based DNA profiling involves a large number of loci, providing greater discriminatory power

iv. It is a rapid, speedy procedure and can be completed in a day or two.

v. Multiplexing and automation make it more effective.

vi. Commercially available kits and inexpensive silver staining detection method for some of the multiplexes without the requirement of expensive equipment make it a desirable option.

2.21 Manual genotyping and detection by staining.

Silver nitrate and dyes like SYBR green that show affinity to the DNA molecule, are found highly suitable for amplicon detection. In DNA profiling, silver staining procedures were highly useful for manual genotyping platforms.[114]

2.22 Automated DNA Genotyping for Fluorescence Based Multiplex STR

Presently, amongst the known DNA tests in human identification, this technique is most informative, precise, robust, rapid and hence most sought after. Nine to fifteen STR loci are simultaneously amplified and subsequently analysed by an automated DNA sequencer. The typing results are highly individual specific and have worldwide acceptance in the courts of law. DNA is extracted and subjected to multiplex amplification of the different STR loci. PCR products of STR loci can be easily resolved by size using gel electrophoresis with high resolution[115,116] or polymer-loaded capillaries.[117,118] In utilizing fluorescent dye labeling technology, STR loci with overlapping size ranges can be co-amplified in a single tube and yet be detected individually due to the different characteristic emission spectrum of each dye.[119] The laser based technology for STR analysis provides higher detection sensitivity than standard methods. Typically, between 0.5 and 2 nanograms of DNA is adequate for genotyping. Only 1 to 2% of the PCR products of a 28 PCR cycle STR amplification reaction are desired for allele typing. These fluorescence based methods are approximately 200 times more sensitive than any other standard staining technique.

Y-STR Polymorphism.

Several highly polymorphic STRs have also been identified on the human Y chromosome, like DYS389 (I and II), DYS390, DYS19, DYS385, DYS391, DYS392, etc. The Y chromosome is nuclear DNA, present in one copy per cell and only in males. It displays paternal inheritance. Like autosomal STR markers, Y-STRs are amenable to typing small or degraded samples of DNA and can be analyzed on the same instrumentation platforms. DNA polymorphism on the human Ychromosomes is a prized tool for identity testing as well as for evolution and migration studies. Y chromosome

loci are not independent and hence the product rule cannot be used to estimate population frequencies at multiple loci. The Y chromosome markers are collectively inherited as a single locus having a large number of alleles; the population frequency of a given haplotype is determined by counting in a population database. In forensics, Y chromosome markers are particularly useful in multiple rape cases, and in identification of mutilated bodies, if control samples of a few genetically related individuals from the paternal side are only available for comparison.

2.23 Improved analysis of STRs with Time-of-Flight Mass Spectrometry

Before the advent of mass spectrometry DNA analysis technology, in automated DNA genotyping, instruments could handle only a few dozen samples per day. This technology allows several thousand samples to be processed daily in seconds, rather than minutes or hours, and with improved accuracy compared with conventional electrophoresis methods. Mass spectrometry is a versatile analytical technique involving detection of ions and measurement of their mass-to-charge ratio. As these ions are separated in a vacuum environment, analysis times can be extremely rapid, often within microseconds. The new ionization technique for measuring biomolecules known as matrix-assisted laser desorptionionization (MALDI) coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) [120] overcomes molecular photo-dissociation of the sample ions induced by direct laser irradiation. This technology focuses on STR loci that have been developed by commercial manufacturers and studied extensively by forensic scientists.[121,122]

Positive features of mass spectrometry for STR analysis include:

i. Rapid results: STR typing at the rate of seconds per sample

ii. Accuracy: no allelic ladders

iii. Direct DNA measurement: no fluorescent or radioactive labels

iv. Automated sample preparation and data collection

v. High-throughput capabilities of thousands of samples every day per system

vi. Flexibility: single nucleotide polymorphism (SNP) assays can be run on the same platform

2.30 RAPD analysis in species identification

RAPD[123,124] (Rapid amplification of polymorphic DNA) requires fewer steps to identify and map markers when compared to RFLP analysis. The steps for RAPD consist of DNA extraction, PCR, separation of amplified fragments on an agarose gel, and detection using ethidium bromide-stained gel. RAPD analysis has been used for many studies on animals and plants, and is a very useful technique for species identification. The significance of the method is that no prior information of template sequence is required and universal primers can be used.

3.0 Sequencing Based Technology

Single nucleotide polymorphisms (SNPs) are single base-pair changes in the DNA sequence, which can be detected by sequencing, RFLPPCR or single-strand conformational polymorphism (SSCP) techniques. A set of SNPs decoding identification of an individual demands only a short stretch of DNA (<100 bp) for analysis. This is of great advantage over the conventional methods in genotyping highly degraded forensic and archaic samples. The presence of ~1.8 million SNPs in the human genome makes it imperative to include SNPs in forensic investigations. The forensic DNA community

already has experience in applying SNP markers for a screening process. Due to amenability to automation, SNPs can prove very helpful for excluding the innocent from prosecution.

Single nucleotide polymorphisms represents alterations in DNA sequence at a single nucleotide position, either due to base changes, insertion or deletion of one or a few bases. One occurs every ~ 1330 bases in the human genome. Defined by this SNPs, every human being is unique. while DNA sequence analysis is still relatively cumbersome today, SNP analysis can be performed by a range of simpler and more rapid methods.

Methods for SNP Detection

SNPs can be detected by various means ranging from direct comparisons of sequence to mass spectroscopy or bio-chemical methods that produce differences based on sequence variations in a defined region. The observed frequency of SNPs per genome predicts that, over the human population as a whole there should be >10 millions SNPs that occur at a frequency of >1%. Already > 1 million have been identified. There are at least 11 methods for detecting and analysing single nucleotide polymorphism (SNPs) they include:

Genetic bit analysis,[125] direct sequencing,[126] denaturing high performance liquid chromatography (HPLC),[127] real-time PCR employing molecular beacons [128] and Taq Man 5 / –nuclease assay,[129] fluorescence polarization[130], mass spectrometry,[131] high density arrays (Affymetrix chip),[132,133] electronic dot-blot (nanogen chip),[134] oligonucleotide ligation assay (OLA) [135] and Tm – shift genotyping.[136]

The advantages of SNPs are:

i. These markers are numerous in mammalian genomes.

ii. Multiple methods of SNP detection are available.

iii. The amplification of alleles is not prone to preferential amplification and robust multiplex amplification is relatively easy to achieve.

iv. Amenable to digitalization on the basis of presence or absence of SNP thus simplifying the identification technique.

3.10 Reverse Dot Blot Assay in detection of HLA DQA1 and PM (LDLR, GYPA, HBGG, D7S8 and GC Loci)

Other than determining the extent of allelic diversity by sequence analysis, one can detect the presence of specific alleles in a PCR-amplified sample by dot blot hybridization with labeled oligo-nucleotide probes, exploiting variations in the DNA sequence, e.g. HLADQA1 [137] and PM systems. One can use the procedure either with p32, biotin or horseradish peroxidase labeled oligonucleotide probes for signal detection.

3.20 mtDNA sequence analysis.

A novel approach in DNA analysis is the application of mitochondrial DNA (mtDNA) sequencing and haplotype frequency calculations in human identification,[138,139] population studies, evolutionary biology[140] and anthropology[141].

3.30 Single Stranded Conformational Polymorphism (SSCP).

SSCP is a method of detecting single nucleotide polymorphisms in any region of the genome without the need for sequencing the homologous DNA fragment isolated from a large number of individuals.[142]

4.0 Microchip Technology

Akin to the microchip technology, which has been the backbone in the area of personal computers, micro-fluidic systems are the very basis of microelectromechanical system (MEMS) devices in the domain of 'laboratories on a chip'. The basic molecular biology techniques of electrophoresis, thermal cycling and hybridization can be accomplished by microchip formats. Forensic analysis is bound to be immensely benefited by the success of these efforts. DNA typing of biological material in the field will then be a matter of a few minutes and could be performed without any academic expertise in molecular biology.

NATIONAL AND INTERNATIONAL STATUS OF DNA PROFILING IN CRIME INVESTIGATIONS.

In 1985, Sir Alec Jeffreys first applied DNA profiling in UK in a much publicised immigration case [35] and a sexual assault and murder case of two teenage girls in Leicester, which have since then tremendously helped the British courts to form an opinion on accepting DNA evidence. In USA, the first DNA case was reported in 1992. Till date, over two million cases of murder, rape and parentage disputes have been presented before courts globally. The maximum number of such cases has been successfully tried in USA followed by UK. CODIS is an electronic 4 tier database of DNA profiles that is maintained by the Federal Bureau of Investigation (FBI), USA, It is one of the leaders in implementing DNA typing technology in the identification of perpetrators of violent crime, developed under provisions of legislation. This DNA database comprises the following categories of DNA records:

i. Convicted offenders - DNA identification records of convicted persons.

ii. Forensic - analyses of DNA samples recovered from crime scenes.

iii. Unidentified human remains.

iv. Relatives of missing persons.

All CODIS STRs are tetrameric repeat sequences. The Combined DNA Index System (CODIS) blends forensic science and computer technology into effective tools for solving violent crimes. CODIS enables central, state, and local crime laboratories to exchange and compare DNA profiles electronically, thereby linking crimes to each other to convict offenders.

At present DNA profiling is carried out in about 60 laboratories of USA and 15 laboratories of UK. DNA profiling is the method of choice in about 45 advanced countries for crime investigation. We should also equip ourselves with these necessary tools. We are a developing nation, with scarcity of sources. Punjab, the most thickly populated province shows an alarming state of rise in the crime rate, particularly rape cases. The presence of spermatozoa as a biological evidence in the semen analysis is not sufficient enough to meet the modern era requirements, therefore Y-STR analysis of rape cases with negative results is the clarion call of the time. So, we should equip our crime investigation laboratories with modern, computerised, time saving and cost effective tools and techniques for the provision of authentic results and quality-controlled services to our masses.

MATERIALS AND METHODS:

The different types of biological evidence can be used to associate or to exclude an individual from involvement with the crime, but the most common materials tested in forensic laboratories are blood and semen, or blood stains and semen stains. The

importance of proper DNA evidence collection cannot be overemphasized, if the DNA sample is contaminated at the beginning, an important investigation is likely to be compromised.

Samples for our study were carefully chosen to prevent needless redundancy in the evidence for a case. These samples were cervico-vaginal, anal and oral, secured in triplicate.

CHART FOR THE METHODOLOGY:

1020 rape victims from 35 districts of Punjab.

↓

Biological test for spermatozoa performed.

↓

300 cases of negative cytology observed. (29.41%)

↓

Swabs from 300 cases with negative cytology processed for Y-STR analysis.

↓

Y-STR analysis found positive in 108 of 300 cases (36%)

↓

Allele frequencies and haplotypes for Punjabi population studied on Y-STR loci.

SAMPLE COLLECTION AND PRESERVATION.

DNA testing techniques are very sensitive hence the sample collection was performed carefully to produce DNA profiles that are meaningful. The samples for this research work were received from all the 35 districts of Punjab.

Following precautions were observed during sample collection for it's proper and detailed analysis:

i. Proper caps, masks, latex gloves were worn to avoid contamination.

ii. Gloves were changed between handling of different items of evidence.

iii. Each item of evidence was packed separately.

iv. Swabs containing semen stains were air dried prior to sealing the package.

v. Samples were packed in paper envelopes or paper bags after drying.

vi. Plastic bags were avoided as water condenses in them, especially in areas of high humidity, hence water can speed up the degradation of DNA molecule.

Packs were clearly marked with:

i. Case number

ii. Item number

iii. Collection date

iv. Initials across the packs to maintain a proper chain of custody.

STORAGE AND TRANSPORT OF SAMPLES.

Carelessness or ignorance of proper handling procedures during storage and transport of DNA from the site of collection to the laboratory can make the specimen unfit for analysis.

Following precautions were observed during storage and transport:

i. Stains were properly dried prior to the transport.

ii. Best preservation was achieved by storing at cool, dark and dry place, thus reducing the rate of bacterial growth and degradation of DNA.

iii. Samples were stored as non-extracted tissues, until they reach the laboratory.

After proper packing, the samples were transported by overnight delivery to our laboratory and stored at –20 C until used.

DNA EXTRACTION.

A biological sample obtained from a crime scene contains a number of substances besides DNA. DNA molecule must be separated from other cellular material before they can be examined. Cellular proteins that harbour and protect DNA in the environment of the cell can inhibit the ability to analyse the DNA.

EZNA (easy nucleic acid isolation kit) by Omega Bio Tek was used to provide a rapid and easy method for the isolation of genomic DNA from forensic samples.

DNA EXTRACTION PROCESS:

i. Post coital vaginal swabs were incubated overnight at 37°C in 400µl of DNA extraction buffer (100mM NaCl + 10 mM Tris-Hcl + 25 mM EDTA + 0.5% SDS) + 0.1 mg/ml Proteinase K.

ii. Swab remnants were removed to a spin-ease basket, the basket inserted back into the original tube and centrifuged at 14,000 x g for 5 minutes. The resultant supernatant, containing the non-sperm DNA fraction was removed.

iii. Cell pellet was transferred to a new microfuge tube, 200µl of STL extraction buffer was added and incubated at 55°C for 15 minutes and samples were vortexed every 2 minutes..

iv. 25µl of OB Protease solution was added and mixed by vortexing.

v. Incubated for 45 minutes at 60°C with occasional mixing.

vi. 225µl buffer BL was added and mixed by vortexing.

vii. Incubated at 60°C for 10 minutes.

viii. Briefly centrifuged to remove any droplets inside the lid.

ix. 225 µl absolute ethanol was added and mixed thoroughly by vortexing.

x. Briefly centrifuged to remove any droplets from the lid.

xi. Each Hi Bind DNA minicolumn was inserted into a 2 ml collection tube, (provided with the kit).

xii. Entire sample from step xiii was transferred into the column, including any precipitate that may have formed.

xiii. Centrifuged at 8000 x g for 1 minute.

xiv. Collection tube and flow through liquid was discarded.

xv. Each column was placed into a second 2 ml tube and washed by pipetting 500µl of HB buffer into column.

xvi. Centrifuged at 8000 x g for 1 minute. Flow through liquid was disposed and collection tube was re used.

xvii. Each column was placed into the same 2 ml tube from step xviii and washed by pipetting 750µl of wash buffer diluted with ethanol into column.

xviii. Centrifuged at 8000 x g for 1 minute. Collection tube and flow through liquid disposed.

xix. Column was washed again, using a new collection tube with 750µl of wash buffer and centrifuged as above.

xx. Flow through was discarded and collection tube re-used.

xxi. The same collection tube was centrifuged at a speed > 13,000 x g for 2 minutes to dry the column.

xxii. Column was placed into a nuclease-free 1.5 ml microfuge tube and 100µl of elution buffer preheated to 70°C was added.

xxiii. Tube was held to sit for 3 minutes at room temperature.

xxiv. DNA was eluted from the column by centrifugation at 8000 x g for 1 minute.

xxv. Elution was repeated with a second volume of 100µl elution buffer to increase the DNA concentration.

The quality and quantity of DNA was determined with the help of spectrophotometer. The quantity was assessed by measuring absorbance at both 260nm and 280nm. A ratio of (A_{260} / A_{280}) of 1.7 - 1.9 corresponded to 85% - 95% purity.

PCR AMPLIFICATION:

Development of Multiplex System.

Due to complexity involved in the design of multiplex PCR, a successfully designed, optimised and validated multiplex Y-Plex ^{TM} 12 system, was used as it provides results in a single amplification reaction for eleven Y-STR loci recommended by the SWGDAM, as well as Amelogenin.

The selection criteria for STR loci.

i. High discriminating power, usually > 0.9 with observed heterozygosity > 70%.

ii. Separate chromosomal locations to ensure that closely linked loci were not chosen.

iii. Robustness and reproducibility of results when multiplexed with markers.

iv. Lower stutter characteristics.

v. Low mutation rate.

vi. Predicted length of alleles with smaller sizes preferred.

Markers	**Gen bank accession**	**Position (MB)***	**Mutation rate***	**Repeat motiff***	**Allele range***	**Ref allele**	**PCR product size bp**	**Reference**
DYS 19	AC017019 (r & c)	10.12	0.20%	TAGA	10-19	15	174 – 210	Rewer & Epplen 1992
DYS 385 a / b	AC022486	19.23	0.23%	GAAA	7 – 28	11	220 – 288	Schneider et al. 1998
DYS 389 I	AC004617 (r & c)	13.05	0.20%	TCTG	9 – 17	12	179 – 207	Kayser et al 1997
DYS 389 II	AC004617 (r & c)	13.05	0.31%	TCTA	24-34	29	292 – 332	Kayser et al 1997
DYS 390	AC011289	15.71	0.32%	TCTA TCTG	17-28	24	163 – 207	Kayser et al 1997
DYS 391	AC011302	12.54	0.40%	TCTA	6 – 14	11	230 – 262	Kayser et al 1997
DYS 392	AC011745 (r & c)	20.97	0.05%	TAT	6 –20	13	103 – 139	Kayser et al 1997
DYS 393	AC006152	3.17	0.05%	AGAT	9 –17	12	100 – 136	Kayser et al 1997
DYS 438	AC002531	13.38	0.09%	TTTTC	6 –14	10	292 – 327	Ayub et al 2000
DYS 439	AC002992	12.95	0.38%	AGAT	8 – 15	13	230 – 258	Ayub et al 2000

Table 3: shows details of loci selected for the present study.

All loci characteristics, including repeat unit structure, size and general chromosome location were obtained from published sources. [143-145] Sequences for each locus were obtained from GenBank® using the standard nucleotide BLAST (Basic Local Alignment Search Tool) [146,147]

Allele and size range determination: The allele and size ranges of the Y-STR were determined by the review of YSTR literature. The list of Y STR literature examined for the allele and size range determinations is available at website. [148]

Primer design.

Parameter	**Optimal value**
Primer length	18 – 30 bases
Primer melting temperature (T_m)	55 – 72c
Percentage GC content	40 – 60%
No self –complementarity (hair pin structure)	< 3 contiguous bases
No complementarity to other primer (Primer Dimer)	< 3 contiguous bases.
Distance between 2 primers on target sequence	< 2000 bases apart.
Unique oligonucleotide sequence	Best match in BLAST search
T_m difference between forward and reverse primers in pairs	< 5c
No long runs with the same base	< 4 contiguous bases.

Table 4 . Parameters for primer selection.

The primers were selected and designed based on the published sequences. [149-151]

Table 5.
Primer Sequences for PCR amplification of loci. **[152]** Predicted primer melting temperatures Tm were calculated using a total primer concentration = 0.05 mM amd [Na+] = 50 mM

Locus	**Primer sequences (5'-to-3')**	**Primer concentration (µM)**	**Melting temperature Tm(c)**
DYS	**F**ACTACTGAGTTTCTGTTATAGTGTTTTT	1.8	55.0

19	R GTCAATCTCTGCACCTGGAAAT	1.8	60.5
DYS 385 a,b	F AGCATGGGTGACAGAGCTA R GCCAATTACATAGTCCTCCTTTC	0.6 0.6	56.9 54.7
DYS 388	F GAATTCATGTGAGTTAGCCGTTTAGC R GAGGCGGAGCTTTTAGTGAG	1.8 1.8	63.4 59.2
DYS 389 I, II	F CCAACTCTCATCTGTATTATCTATG R GTTATCCCTGAGTAGTAGAAGAATG	1.3 1.3	54.2 59.0
DYS 390	F TATATTTTACACATTTTTGGGCC R GTGACAGTAAAATGAAAACATTGC	0.2 0.2	57.2 57.0
DYS 391	F TTCAATCATACACCCATATCTGTC R GATAGAGGGATAGGTAGGCAGGC	0.2 0.2	57.9 60.0
DYS 392	F TAGAGGCAGTCATCGCAGTG R GACCTACCAATCCCATTCCT	1.8 1.8	60.2 57.3
DYS 393	F GTGGTCTTCTACTTGTGTCAATAC R GAACTCAAGTCCAAAAAATGAGG	0.4 0.4	54.7 57.7
DYS 438	F CCAAAATTAGTGGGGAATAGTTG R GATCACCCAGGGTCTGGAGTT	0.2 0.2	58.9 62.6
DYS 439	F TCGAGTTGTTATGGTTTTAGGTCT R GTGGCTTGGAATTCTTTTACCC	0.2 0.2	58.3 60.3

The Primers were purchased from BIO BASIC Inc. East Markham Ontario,Canada. In order to perform several parallel reactions, we used 2x Superhot PCR.[Bioron, Germany] strategy using recombinant monoclonal antibody against Taq

DNA polymerase. It is an optimised ready to use PCR mixture of all components for PCR, except DNA template and Primers.

Amplification.

2X Superhot Master Mix composition contained:

1. 25 µL of 2X PCR Master Mix.
    i. Taq DNA Polymerase (recombinant) in reaction buffer. 0.1 units / µl.
    ii. 32 mM $(NH_4)_2 SO_4$
    iii. 130 mM Tris Hcl, pH 8.8 at 25C.
    iv. 0.02% Tween-20
    v. 3mM $MgCl_2$
    vi. dNTPs: 0.4 mM each.
    vii. Anti bodies to Taq DNA Polymerase {concentration adjusted}
2. 10_{pg} - 1µg/ 50µl DNA template.
3. Sterile deionised water to raise the volume to 50 µL.
4. Finally, the 2X Y-PLEX^{TM} 12 Primer mix was prepared by combining the forward and reverse primers for all twelve loci, with contents mentioned above. Final concentration of the buffer in an amplification reaction was 1.0X.

Reaction mixture set up.

i. All contents were gently vortexed and briefly centrifuged after thawing.

ii. Added, in a thin-walled PCR tube, on ice.

iii. The sample was gently vortexed briefly centrifuged to collect all drops from walls of tube.

iv. Samples were placed in a thermocycler and PCR started.

Amplification reactions were performed in a GeneAmp R_ PCR systems 9700 (Applied Biosystems, Foster City, CA) with conditions as follows: 95?C, 10 min; 30 cycles of 94?C, 1min; 58?C,1min and 70?C, 1min; 60?C, 60 min and 4?C until the samples were removed from the thermal cycler.

Performance and purity tests.

Tested for the absence of endodeoxyribonucleases and exodeoxyribonucleases.

No detectable conversion of covalently closed circular DNA to nicked DNA was observed after incubation of 25µl of 2x Superhot PCR Master mix with 1µg of pUC 19 DNA in 50 µl for 4 hours neither at 37c nor at 70c.

CONTROLS USED FOR MONITORING.

Controls were used to monitor the effectiveness of the chosen experimental conditions and the technique of the experimenter.

Negative control, was the entire PCR reaction mixture without DNA template, that was replaced by the PCR grade water of the same volume as of DNA template.

A positive control was also valuable indicator of whether or not any of the PCR component have failed or were not added during the reaction set up phase of the experiment. A standard DNA template with good quality DNA was used as the positive control.

Detection and analysis of PCR products in Y-STR analysis was accomplished with the **Agilent 2100 bioanalyser (Agilent Tecnologies).**

It facilitates the analysis of high-resolution multiples PCR reactions, measuring precise size and concentration of each fragment. High sensitivity due to the use of laser detection and broad linear dynamic range able to detect weak bands also with a minimum exposure to hazardous material such as ethidium bromide.

Purification of PCR products for Genetic data collection.

i. 20 µl PCR product was mixed with 60 µl 0f 70% chilled ethanol.

ii. Contents were centrifuged at full speed for 5 minutes.

iii. Supernatant was taken off.

iv. 20 µl of de-ionised PCR grade water was added to the DNA pellet.

v. Tube was vortexed for 5 minutes.

vi. Purified PCR products were ready for sequential analysis.

SEQUENCER ANALYSIS.

The amplified and purified PCR products were analysed using 310 ABI Prism ™ Genetic Analyser (Applied Bio systems). The results were analysed using GeneScan® analysis v 3.1 software(Applied Biosystems) and genotypes were determined by Genotyper® v 2.5 software(Applied Biosystems) with the PowerTyper ™ Y Macro (Promega). The numerical allele designations were obtained by comparison of the sample fragments with those of allelic ladders provided with the kit. The Arlequin ver 3.0 was used for the calculation of results- the software has become the standard software for haplotype analysis.[153]

Protocol Used Arlequin ver 3.0

RUN NUMBER 1 (29/01/08 at 16:19:11)

Project information:

NbSamples = 1
DataType = MICROSAT
GenotypicData = 0

Settings used for calculations
General settings:

Deletion weight = 1
Transition weight = 1
Tranversion weight = 1
Epsilon value = 1e-07
Significant digits for output = 5
Use original haplotype definition
Allowed level of missing data = 0.05

Active Tasks:

i. Standard indices:

Standard diversity indices : (Punjab)
Molecular diversity indices : (Punjab)
No. of gene copies : 108
No. haplotypes : 68
No. of loci : 10
No. of usable loci : 10 loci with less than 5.00 % missing data
No. of polymorphic loci : 10

ii. Haplotypic frequency estimation made at:

Haplotypic level
Locus level

iii. Summary of computations performed within populations

Basic properties

Statistics	Punjab	Mean	s.d.
No. of gene copies	108	108.000	0.000
No. of loci	10	10.000	0.000
No. of usable loci	10	10.000	0.000
No. of polym. loci	10	10.000	0.000

END OF RUN NUMBER 1 (29/01/08 at 17:53:11))
Total computing time for this run : 0h 0m 0s 94 ms

RESULT.

A total of 930 cases of sexual assaults were analysed during the study period, from all the 35 districts of Punjab, Pakistan. Among them a total of 300 cases (32.25%) were found to be negative for spermatozoa. These were processed for Y-STR analysis, - and the core loci recommended by SWGDAM were analysed through Y- Plex^{TM} 12 system. The application of Y- Plex^{TM} 12 system provided results in a single amplification reaction for eleven Y-STR loci and Amelogenin. It also demonstrated that the Y- Plex^{TM} 12 system was successfully used for the forensic case work up and in finding out the allele frequencies and haplotypes for Punjabi population. Therefore, we performed a study for the Y-STR DNA amplification as an evidence in sexually assaulted female victims with no cytological evidence of spermatozoa and calculated the Y- chromosome STR haplotypes and allele frequencies in Punjabi population of Pakistan.

The characteristics of the alleged victims are summarised in the tables shown below, however the most important issue was that all the medico-legal examinations of the alleged rape victims were carried out on the directions of the courts and were brought by the police. Among the 930 cases medico-legally evaluated, the number of victims reported was 1020 during the study period. The relevant history of each victim was obtained, along with the details from the accompanying relatives and the police personal.

Table 6: The number of rape cases in Punjab.

Period	**Cases**	**Victims**	**Age Group**		**Marital status**			
			Minor	Adults	Married	Un married	Widows	Divorced
July 06 Dec 07	930	1020	293 28.73%	727 71.27%	598 58.63%	360 35.29%	33 3.24%	29 2.84%

Table 7. The number of rape / gang rape cases.

Period	Cases	Victims	Rapes	Gang Rapes	Assailants
July 2006 Dec 2007	930	1020	692	238	1172

Briefly, the age range was from 5 – 60 years with a median age of 20. The number of young victims was about 68 % of the total, whereas the number of minor vicitms (under the age of 12) was 293 (28.73 %). The marital status indicated that 598 victims (58.63 %) were married and 360 (35.29 %) were unmarried, 33 (3.24 %) were widows and 29 (2.84%) were divorced. The number of assailants was more than one in (238 / 930) 25.59 % of the cases. Though gang rape is not exclusive to any of the geographical location or any one culture, it is more prevalent in the developing countries and is related more to revenge seeking for honour. The word 'tournante' is the French word used to narrate the gang rape. Most of the gang rapes were the end result of kidnapping carried out for the sake of revenge, later leading to the molestation and rape due to aggressive behaviour of the victims.

Table 8. The age ranges of 108 cases and their percentage.

Sr. No	Age Group	Cases	Percentage
1.	0 – 9	9	8.33 %
2.	10 – 19	45	41.67 %
3.	20 – 29	29	26.86 %
4.	30 – 39	16	14.81 %
5.	40 – 49	8	7.41 %
6.	50 – 59	1	00.92 %
7.	60 – above	0	00.oo %

As far as the occupation is concerned, about 60 % of the victims had a poor, rural background. Poverty made their daily life more dangerous (e.g) walking alone at nights,

less parental supervision. It also made them more dependent on men for survival and therefore less able to control their sexuality, consent to sex, recognise their own victimisation or to seek help when victimised. These issues increase their vulnerability to sexual victimisation. In addition, poor women may be at risk for sexual violence because their economic (and, often, educational) status necessitates that they engage in high-risk survival activities, for example trading sex for food, money, or other items. Poverty also puts women at increased risk of intimate partner violence, of which sexual violence is often one aspect.

Table 9. Occupation of the victims is shown both in number and percentage.

Sr. No	**Occupation**	**Cases**	**Percentage**
1.	House Wife	42	38.89 %
2.	Labourer	27	25.00 %
3.	Domestic work	17	15.74 %
4.	Service	12	11.11 %
5.	Student	10	9.26 %

Drugs may also play a role, alcohol and/or other drug users are frequently involved in rape. In 12% of rapes, both the victims and the perpetrators have been drinking. In 7 % only the perpetrators and 5% of the cases, only the victims have been drinking. Rapes where neither the victim nor the perpetrators have been drinking were 76% of all rapes. The situation is different in this part of the world, because of religious obligations. Alcohol is not available easily so Dhatura and other indigenous materials are in use. These are the drugs that are sometimes used to assist a sexual assault, and because of the effects of these drugs, victims may be physically helpless, unable to refuse sex, and can't remember what happened. The drugs often have no colour, smell, or taste and are easily added to flavoured drinks without the victim's knowledge making a person

experience a black out and forget things that happen. Both girls and guys who have been given these drugs report feeling of paralysis, having blurred vision, and lack of memory.

Table 10. Number of individuals, under the influence of drugs at the time of rape.

Sr. No	**Character Under Influence**	**Cases**	**Percentage**
1.	Victim	05	4.63 %
2.	Assailant	08	7.40 %
3.	Both	13	12.04 %
4.	None	82	75.93 %

When people talk of rape, they might think of a stranger jumping out of shadowy place and sexually attacking someone. However, it's not only strangers who rape, in fact, about half of all people who are raped know the person who attacked them. Most friendships, acquaintances, and dates of course never lead to violence, but, sadly enough sometimes it happens. In our cases the assailant was a stranger / unknown in 28% cases. whereas it was a familiar face in 72 % of the cases. When forced sex occurs between two people who know each other, it is known as "date rape" or "acquaintance rape". Rape is always the fault of the rapist, that is also the case when two people are dating – or even during an intimate relationship. One person never owes the other person sex. If it is forced against someone's will, that is a rape.

Healthy relationships involve respect – including respect to the feelings of others. Someone who really cares about you will respect your wishes and will not force or pressurises you to have sex.

Table 11. Number of victims known / unknown to the assailants and their percentage.

Sr. No	**Relation**	**Cases**	**Percentage**
1.	Friends	41	37.96 %
2.	Acquaintance	30	27.78 %
3.	Other Close	07	6.48 %
4.	Strangers	28	25.93 %
5.	Unknown	02	1.85 %

Most experts assert that primary cause in majority of the rape cases is the aggressive desire to dominate the victim rather than an attempt to achieve sexual fulfillment. In the present series over 2/3rd of all rapes occurred in someone's home, 31.48 % occurred at the assailants' place, 26.85 % at victim's and 10.18 % at the residence of common friends.

Table 12. Places of incidence of sexual assault.

Sr. No	**Place**	**Cases**	**Percentage**
1.	Of Assailant	34	31.48 %
2.	Of Victim	29	26.85 %
3.	Of common friend	11	10.18 %
4.	At parties	05	4.62 %
5.	In vehicles	05	4.62 %
6.	At hotels	09	8.33 %
7.	Outdoors	15	13.92 %

Vaginal, oral and anal penetration were alleged in 63%, 7%, 15% of the cases respectively, however there was no history of penetration in 15% of cases, but body licking and kissing took place in 74% cases. Perineal trauma occurred in 30.55%, anal trauma in 19.44 % and other physical injuries were seen in 50.93 % of the cases, majority of them seen over the breast and the cheeks.

Table 13. Penile penetration at various sites of victims.

Sr. No	**Penetration**	**Cases**	**Percentage**
1.	Vaginal	68	62.97 %
2.	Oral	08	7.41 %
3.	Anal	06	5.56 %
4.	No Penetration	26	24.06 %

Table 14. Sites of injuries commonly observed during sexual assault.

Sr. No	**Site**	**Cases**	**Percentage**
1.	Perineum	33	30.55 %
2.	Anal	04	3.70 %
3.	Others	55	50.94 %
4.	None	16	14.81 %

The lapse of time between alleged sexual assaults and the medical examination was from 6 – 192 hrs. The cause of the delay was of procedural nature, whereas 17 cases got delayed because of compromising efforts, only in two cases the delay could not be explained. None of the victims had any sexual intercourse during the interval between the sexual assault and the medical examination.

Table 15. Lapse of time occurring between the incidence and medical examination.

Hours	**Cases**	**Percentage**
0 – 12	12	11.11 %
12 – 24	32	29.63 %
24 – 36	26	24.08 %
36 – 48	21	19.45 %
48 – 60	10	9.25 %
60 – 72	06	5.56 %
72 – above	01	.92 %
Total =	108	

The legal system of Pakistan is such that only the reported cases are dealt with, so despite our best efforts we were unable to find the exact number of unreported case. However, we believe that the number of unreported incidents could be 5 – 6 times the number reported.

Table 16. Summary of the cases.

Number reported	**Number of cases**	**Percentage**
Cases reported	930	
Victims	1020	
Cases found cytological negative	300/930	32.25 %
Cases found Y-STR positive	108/300	36.00 %
Cases withdrawn under political pressure	36	3.92 %
Cases left	894	96.08 %
Out of 894 cases F I R lodged	855	95.61 %

Undertaking of the project of Y-STR multiplex PCR assay development and testing was defined through extensive literature searches, and to achieve the amplification of all eleven Y-STR loci recommended by SWGDAM in a single PCR, the Y-PLEX^{TM} 12 system was preferred. Validation studies of the Y-PLEX ^{TM} 12 system were confirmed according to the FBI Director's Quality Assurance Standards. The repeat motifs, known alleles, and observed sizes of PCR products for these twelve Y-STR loci are summarized in Table 17. The repeat units at the Y-STR loci amplified with Y-PLEX^{TM} 12 system are four bases in size, except for DYS438 and DYS392. The repeat unit size at the DYS438 locus is five bases and at the DYS392 locus is three bases.

Table 17. Loci and the observed details in 108 cases of Punjabi population.

Marker's name	**Repeat motiff**	**Allele range**	**Observed allele range**	**Ref Allele**	**PCR product size bp**	**Observed product size**
DYS 19	TAGA	10-19	13-17	15	174-210	188-205
DYS 385 a / b	GAAA	7 –28	9-22	11	220-288	226-282
DYS 389 I	TCTG	9-17	12-14	13	179-207	187-196
DYS 389 II	TCTA	24-34	26-32	30	292-332	296-325
DYS 390	TCTA TCTG	17-28	22-26	23	163-207	179-197
DYS 391	TCTA	6-14	10-14	10	230-262	242-259
DYS 392	TAT	6-20	10-17	11	103-139	111-128
DYS 393	AGAT	9-17	11-15	13	100-142	103-120
DYS 438	TTTTC	6-14	7-13	11	292-327	290-325
DYS 439	AGAT	8-15	9-14	12	230-258	234-255
Amelogenin	……..	X,Y	X,Y	--	104-110	103-110

The length of amplified fragments range between 100 – 350 bases. Amplification using the Y-PLEX^{TM} 12 kit was performed in the Px2 thermal cycler. All twelve loci amplified well and the cycler provided best results.

Allelic Ladder

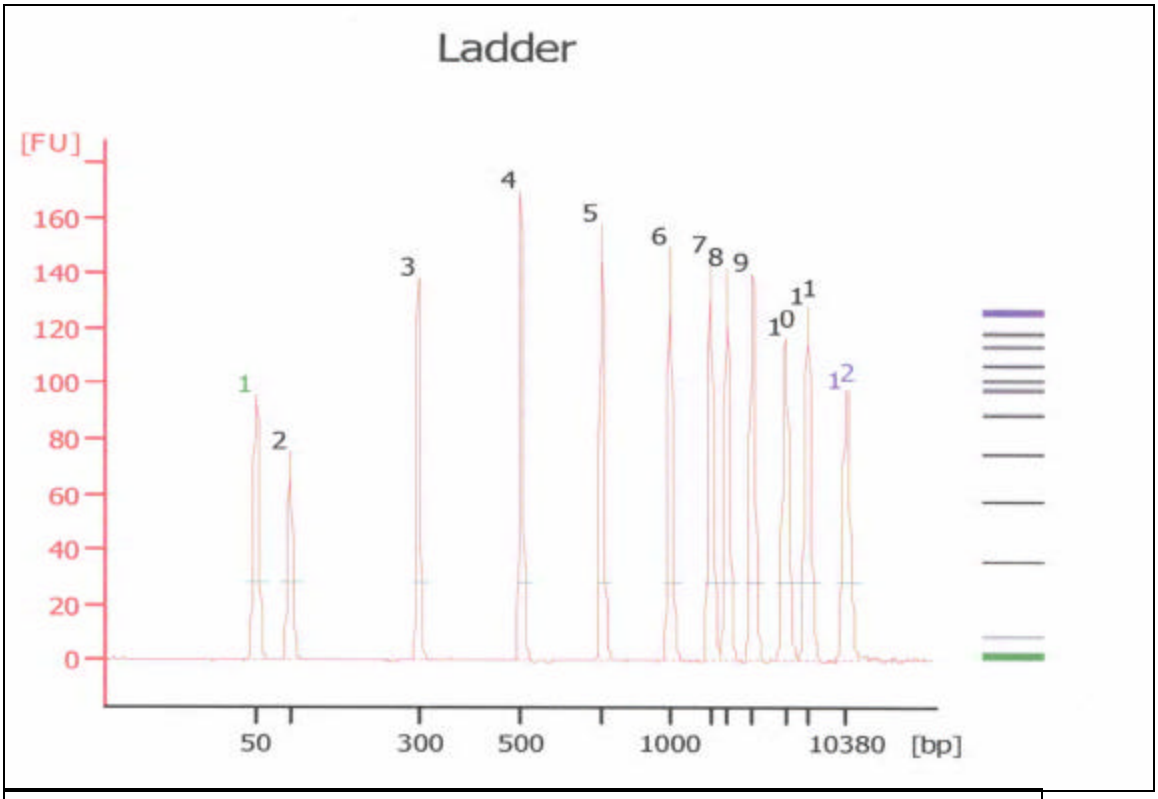
The allelic ladder was provided in the kit by the Agilent Technologies in yellow capped DNA ladder vial, with the well marked ladder symbol. (Electropherogram 1). A confirmation was made that the ladder included was in concordance to the guidelines provided by "The Commission of the International society for Forensic genetics" (ISGF).

Elecropherogram 1.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: 11/29/2007 2:55:32 PM
Modified: 11/29/2007 3:29:44 PM

Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary.



Peak table for Ladder

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2	100	4.00	60.6	Ladder Peak
3	300	4.00	20.2	Ladder Peak
4	500	4.00	12.1	Ladder Peak
5	700	4.00	8.7	Ladder Peak
6	1,000	4.00	6.1	Ladder Peak
7	1,500	4.00	4.0	Ladder Peak
8	2,000	4.00	3.0	Ladder Peak
9	3,000	4.00	2.0	Ladder Peak
10	5,000	4.00	1.2	Ladder Peak
11	7,000	4.00	0.9	Ladder Peak
12	10,380	4.20	0.6	Upper Marker

Precision of allele sizing and reproducibility.

The precision of computing the size of each allele was calculated by measurement from a series of electrophoresis runs on 2100 Genetic Analyzer. The observed size range, mean size, and standard deviation for each allele are summarised in the following tables (18 – 28).

Table 18. Observed size range, mean size, and standard deviation values computed from the precision study of locus 393.

Locus	**Allele**	**Observed range**	**Mean**	**s.d**
393	11	102.85 – 103.53	103.19	0.13
	12	106.84 – 107.53	107.19	0.13
	13	110.86 – 111.58	111.21	0.13
	14	114.94 – 117.61	115.27	0.12
	15	119.05 – 119.63	119.34	0.11

Table 19. Observed size range, mean size, and standard deviation values computed from the precision study of locus 392.

Locus	**Allele**	**Observed range**	**Mean**	**s.d**
392	10	111.42 – 112.61	112.01	0.19
	11	114.55 – 115.61	115.08	0.17
	12	117.65 – 118.62	118.14	0.16
	14	120.71 – 121.67	121.19	0.16
	15	123.83 – 124.73	124.28	0.15
	17	127.00 - 127.76	127.39	0.13

Table 20.
Observed size range, mean size, and standard deviation values computed from the precision study of locus 390.

Locus	Allele	Observed range	Mean	S.D
390	22	179.84 – 180.56	180.2	0.15
	23	183.81 – 184.58	184.2	0.16
	24	187.80 – 188.54	188.2	0.15
	25	191.79 – 192.34	192.12	0.15
	26	195.78 – 196.48	196.13	0.15

Table 21. Observed size range, mean size, and standard deviation values computed from the precision study of locus 389 I.

Locus	Allele	Observed range	Mean	s.d.
389 I	12	187.17 – 188.09	187.63	0.21
	13	191.03 – 192.01	191.52	0.20
	14	195.13 – 195.09	195.11	0.20

Table 22. Observed size range, mean size, and standard deviation values computed from the precision study of locus 19.

Locus	Allele	Observed range	Mean	s.d.
19	13	188.51 – 189.36	188.93	0.17
	14	192.45 – 193.26	192.85	0.16
	15	196.39 – 197.14	196.76	0.15
	16	200.33 – 201.00	200.66	0.15
	17	204.24 – 204.90	204.57	0.14

Table 23. Observed size range, mean size, and standard deviation values computed from the precision study of locus 385 a,b.

Locus	Allele	Observed range	Mean	s.d.
385	9	226.80 – 227.20	227.00	.09
a,b	10	234.45 – 234.83	234.71	.08
	11	238.30 – 238.72	238.51	.08
	12	242.12 – 242.57	242.35	.09
	13	245.98 – 246.39	246.18	.08
	14	249.72 – 250.39	250.05	.11
	15	253.76 – 254.17	253.97	.09
	16	257.65 – 258.09	257.87	.09
	17	261.54 – 261.94	261.74	.08
	18	265.47 – 265.85	265.66	.09
	19	269.35 – 269.77	269.56	.09
	20	272.91 – 273.66	273.46	.10
	21	276.83 – 277.65	277.24	.10
	22	280.81 – 281.63	281.22	.09

Table 24. Observed size range, mean size, and standard deviation values computed from the precision study of locus 439.

Locus	Allele	Observed range	Mean	s.d.
439	9	234.45 – 235.15	234.75	.14
	10	238.47 – 239.28	238.87	.14
	11	242.48 – 243.08	242.63	.13
	12	246.13 – 247.10	246.61	.17
	13	250.47 – 251.12	250.80	.12
	14	254.50 – 255.12	254.81	.13

Table 25. Observed size range, mean size, and standard deviation values computed from the precision study of locus 393.

Locus	Allele	Observed range	Mean	s.d.
391	10	242.46 – 242.85	242.65	.09
	11	246.47 – 246.91	246.69	.10
	12	250.56 – 251.06	250.90	.11
	13	254.59 – 255.08	254.83	.12
	14	258.64 – 259.17	258.90	.13

Table 26. Observed size range, mean size, and standard deviation values computed from the precision study of locus 438.

Locus	Allele	Observed range	Mean	s.d.
438	7	290.88 – 291.41	291.14	.10
	8	296.28 – 296.86	296.57	.12
	9	301.63 – 302.36	301.99	.15
	10	307.05 – 308.09	307.57	.21
	11	312.49 – 313.70	313.09	.25
	12	317.79 – 319.19	318.49	.28
	13	323.08 – 324.50	323.79	.29

Table 27. Observed size range, mean size, and standard deviation values computed from the precision study of locus 389 II.

Locus	Allele	Observed range	Mean	s.d.
389 II	26	296.40 – 297.78	297.08	.28
	27	300.63 – 302.11	301.37	.32
	28	305.02 – 306.73	305.87	.37
	29	309.33 – 311.22	310.27	.41
	30	313.63 – 315.68	314.66	.43
	31	317.92 – 320.05	319.44	.44
	32	322.11 – 324.37	323.24	.46

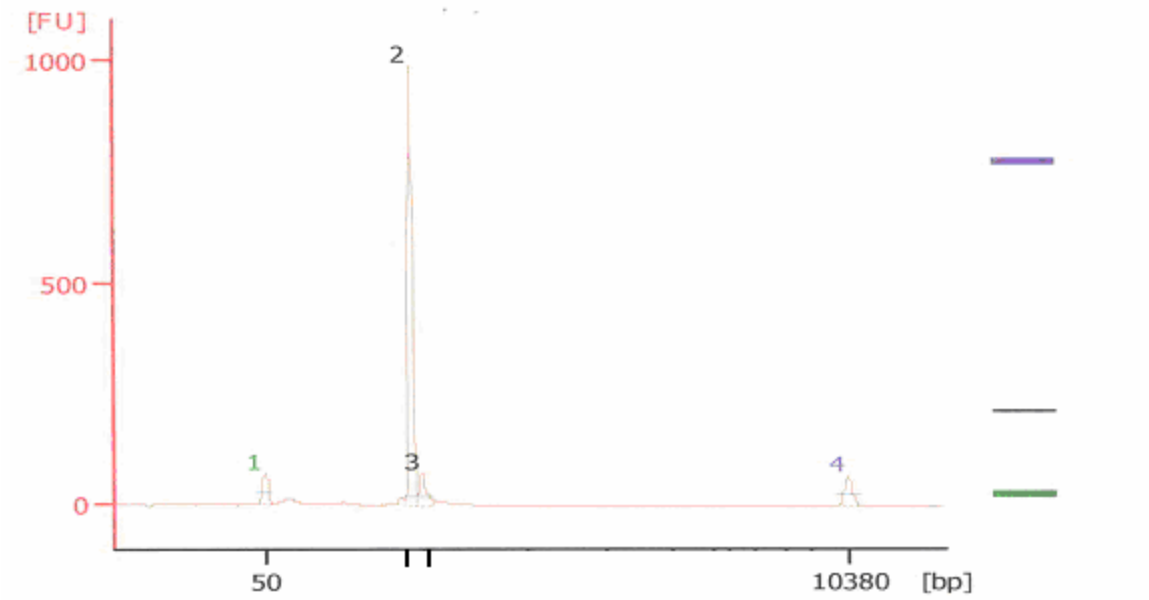
Table 28. Observed size range, mean size, and standard deviation values computed from the precision study of Amelogenin.

Locus	Allele	Observed range	Mean	s.d
Amelogenin	X	103.62 – 104.26	103.94	0.14
	Y	109.22 – 109.91	109.57	0.13

Electropherogram 2.
Sample 1.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 1.
Number of peaks found : 2

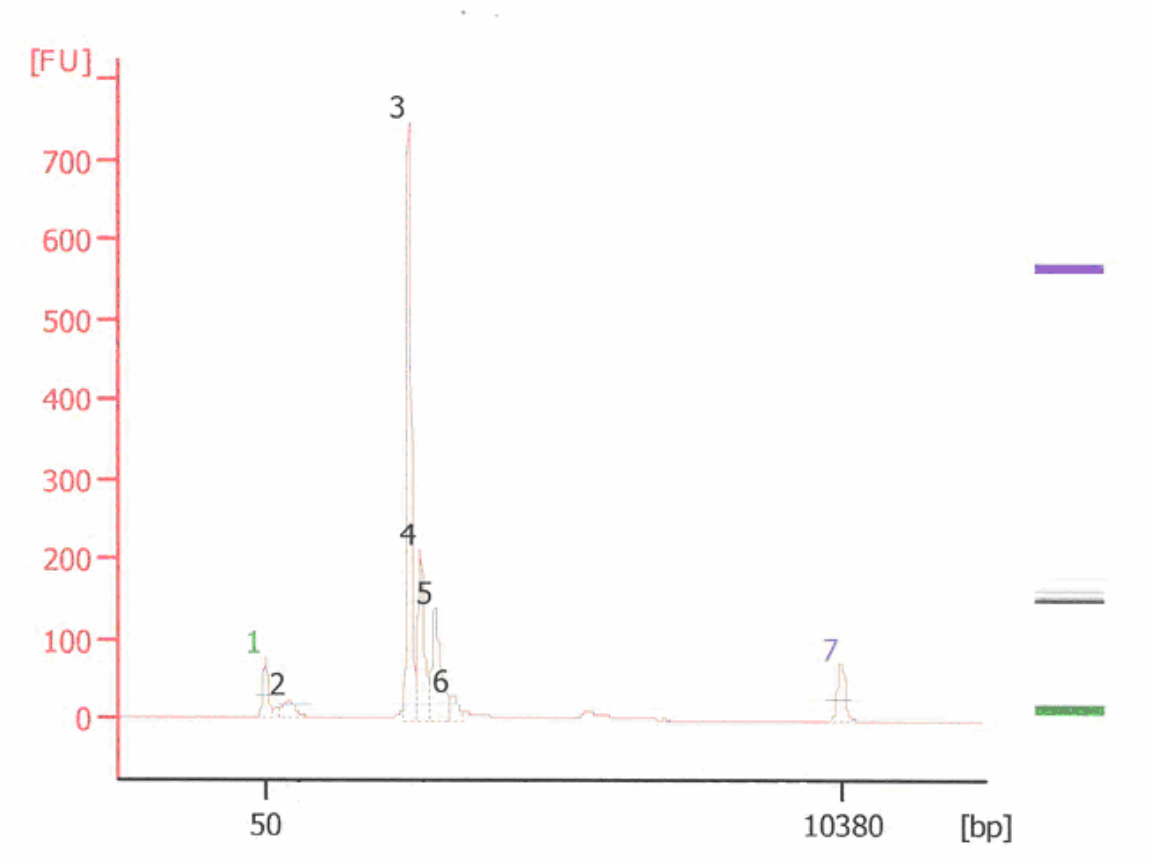
Peak table for sample 1.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		226	54.94	300.1	(DYS 385 a,b)
3.		315	5.82	29.8	(DYS 438)
4.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 3.
Sample 2.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 2.
Number of peaks found : 5

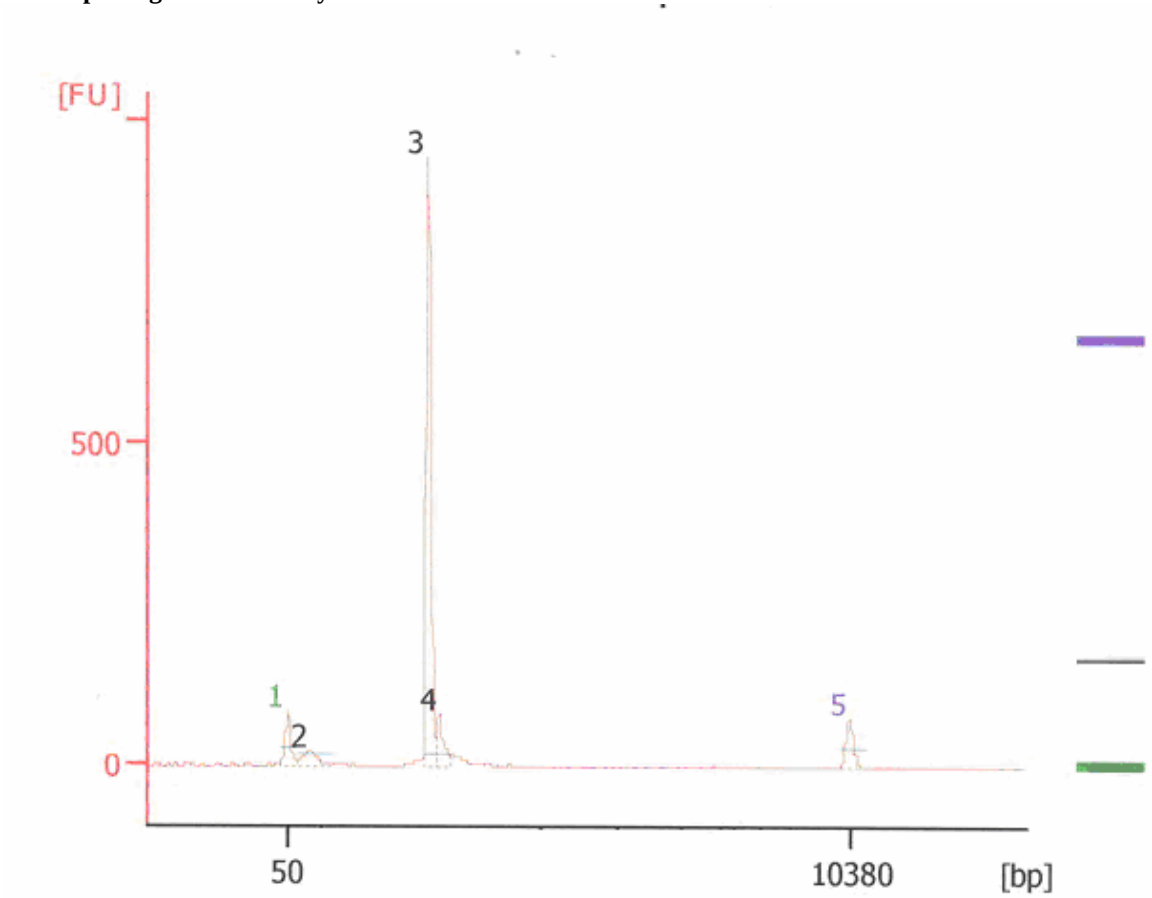
Peak table for sample 2.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		105	4.95	88.4	(DYS 393)
3.		180	2.52	10.6	(DYS 390)
4.		200	40.69	222.4	(DYS 19)
5.		296	14.70	75.3	(DYS 389II)
6.		324	14.80	69.1	(DYS 438)
7.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 4.
Sample 3.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summar y



Overall Results for sample 3.
Number of peaks found : 3

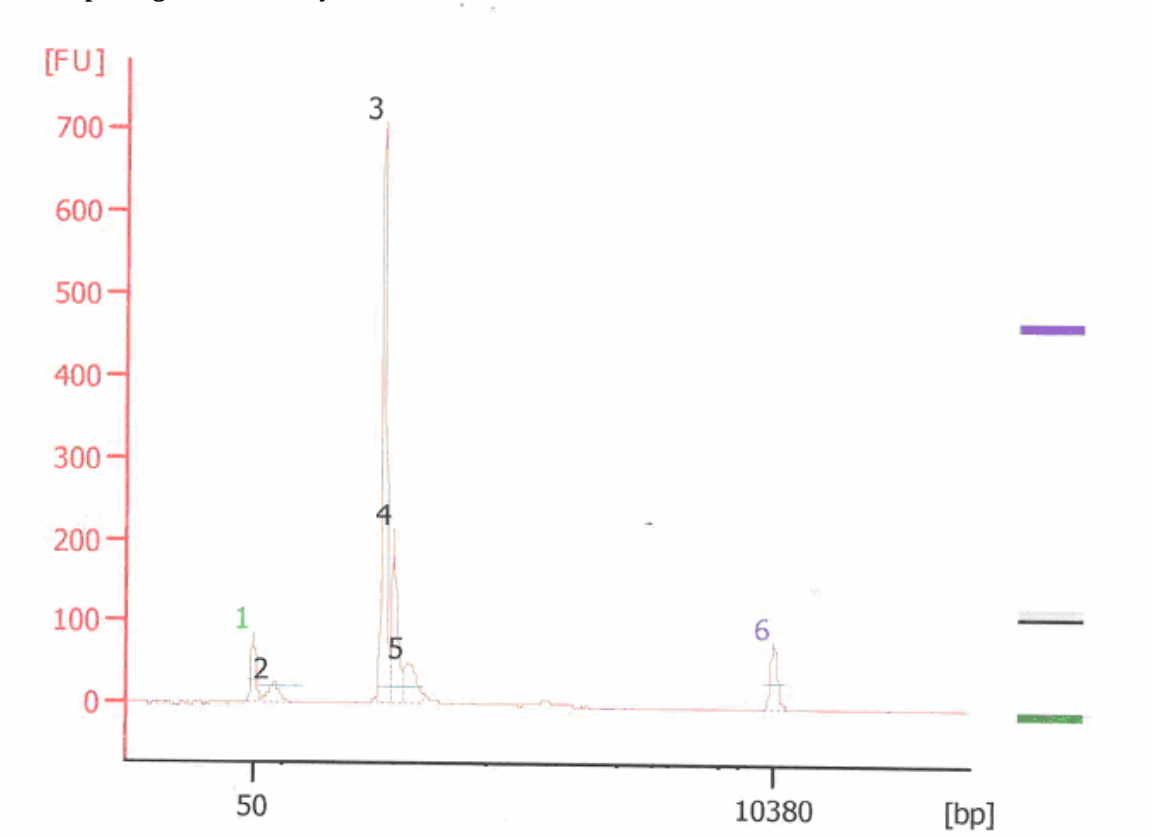
Peak table for sample 3.

Peak	Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2.	115	4.84	86.9	(DYS 392)
3.	246	6.21	31.8	(DYS 439)
4.	258	52.42	286.1	(DYS 391)
5.	10,380	4.20	0.6	Upper Marker

Electropherogram 5.
Sample 4.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 4.
Number of peaks found.: 4

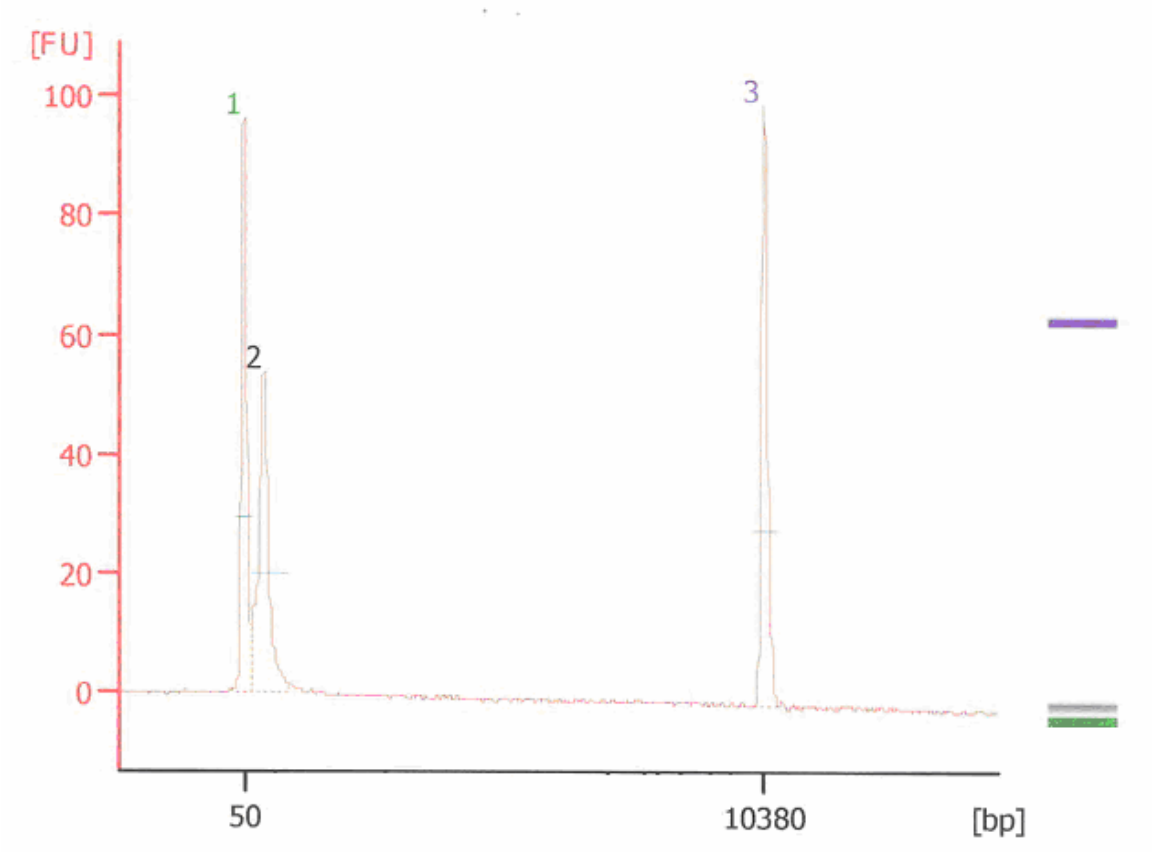
Peak table for sample 4.

Peak	Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2.	105	4.98	89.2	(DYS 393)
3.	196	40.47	220.7	(DYS 389 I)
4.	197	15.90	81.1	(DYS 390)
5.	255	7.39	34.4	(DYS 439)
6.	10,380	4.20	0.6	Upper Marker

Electropherogram 6.
Sample 5.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 5:
Number of peaks found: 1

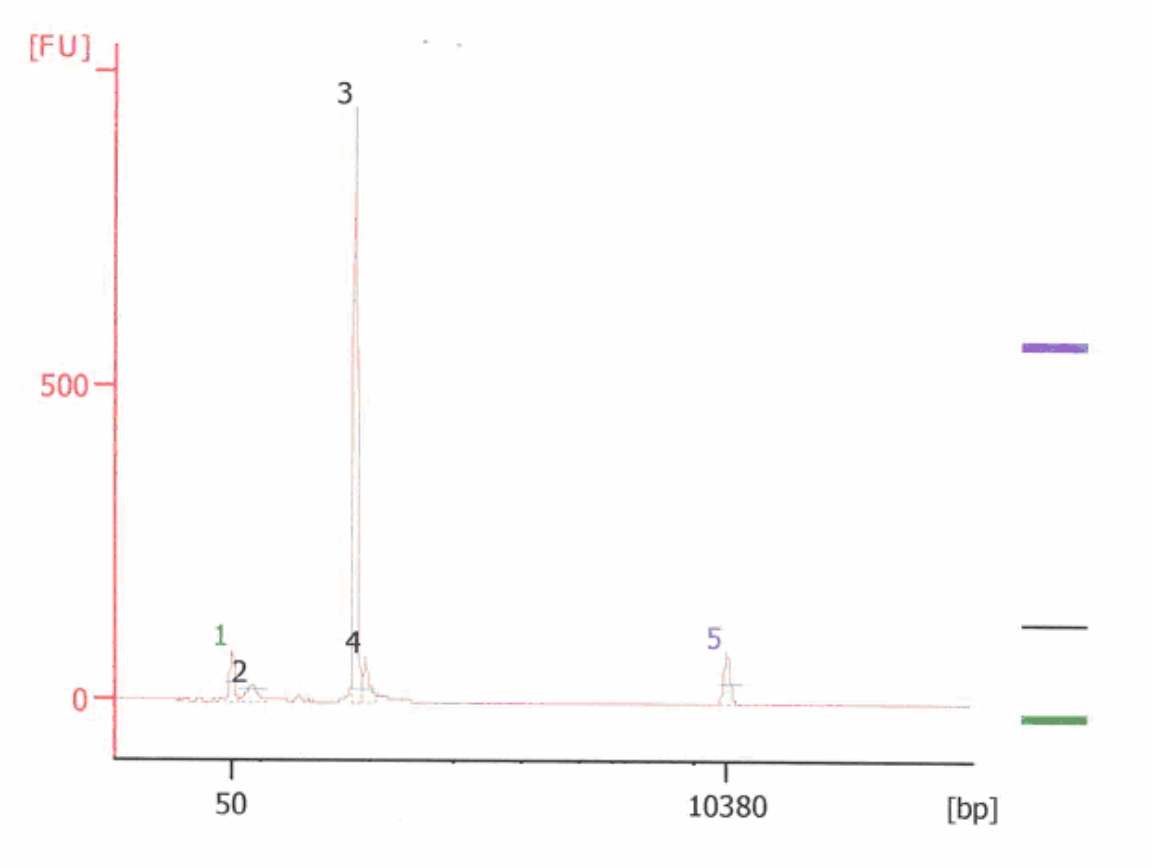
Peak table for sample 5.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		105	7.74	141.1	(Amelogenin)
3.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 7.
Sample 6.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 6:
Number of peaks found : 3

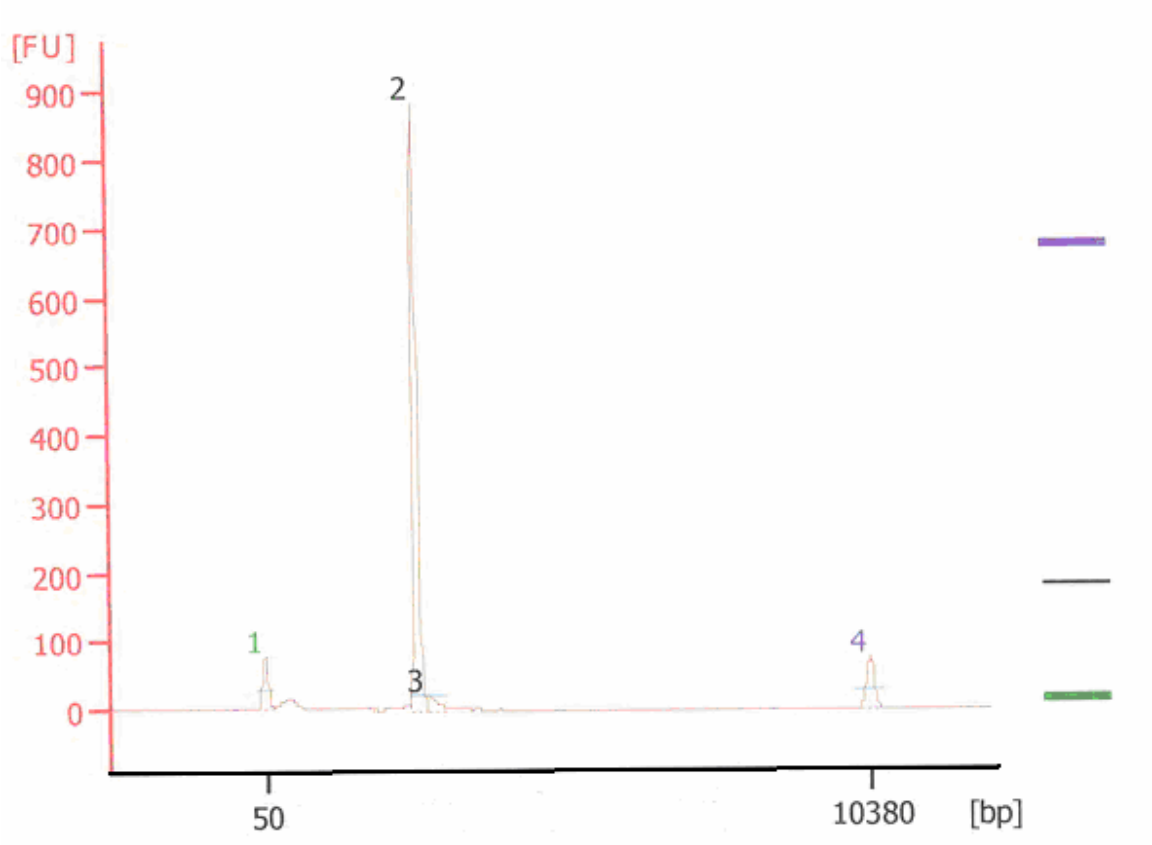
Peak table for sample 6.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		110	4.42	78.9	(DYS 393)
3.		205	48.46	264.9	(DYS 19)
4.		297	5.51	28.1	(DYS 389II)
5.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 8.
Sample 7.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 7:
Number of peaks found : 2

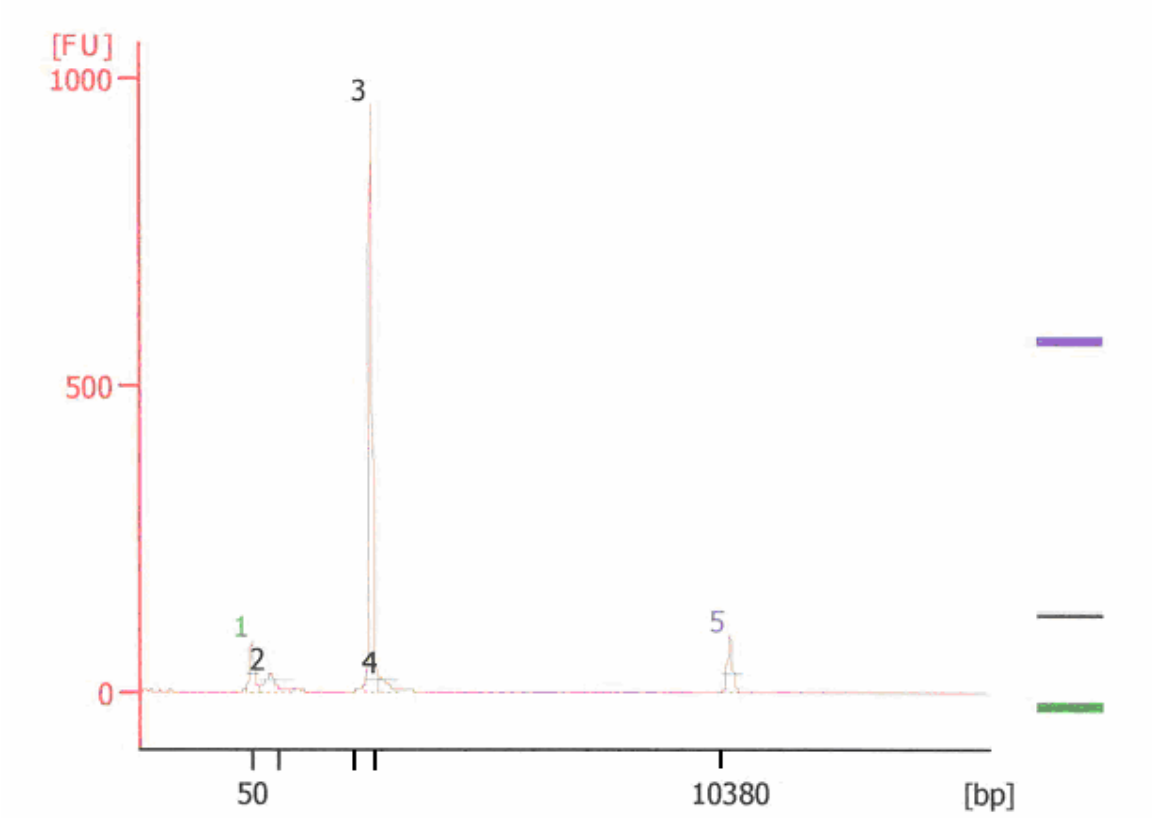
Peak table for sample 7.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		278	48.98	266.6	(DYS 385 a,b)
3.		296	2.24	11.5	(DYS 438)
4.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 9.
Sample 8.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 8:
Number of peaks found : 3

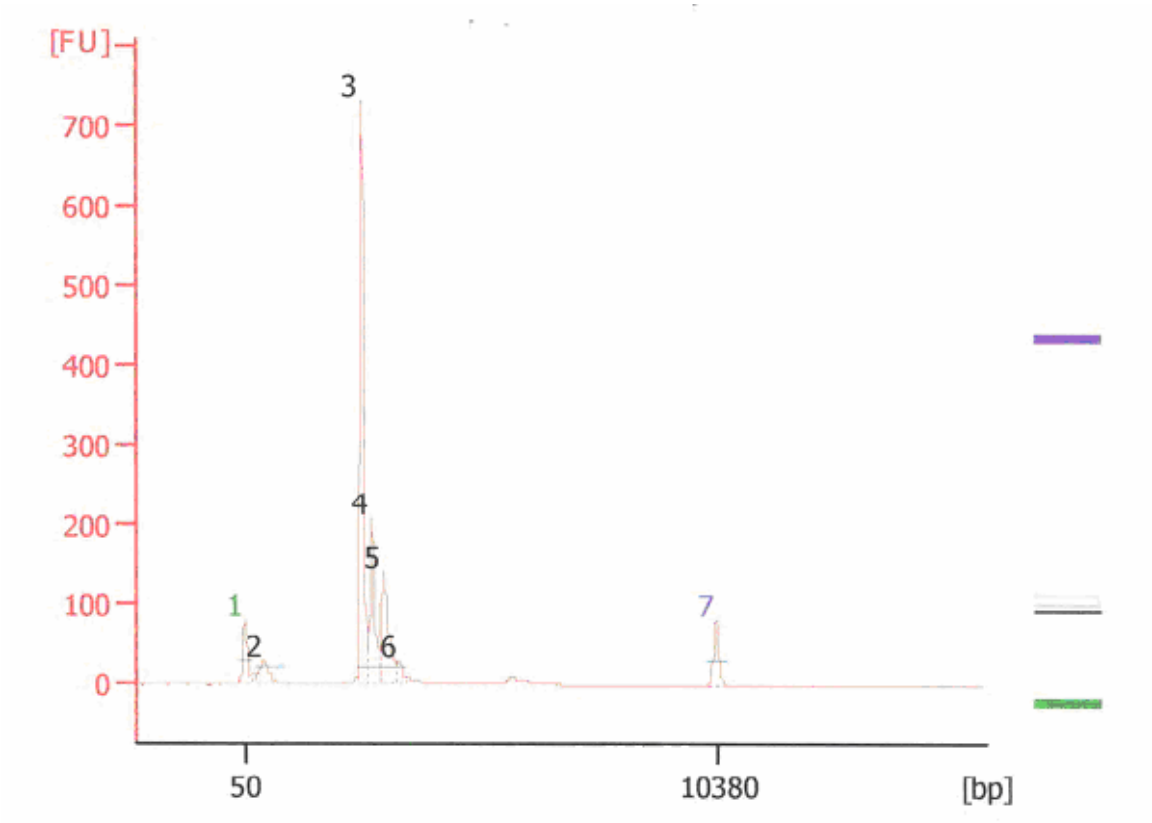
Peak table for sample 8.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		125	4.93	89.2	(DYS 392)
3.		277	50.77	277.3	(DYS 385 a,b)
4.		296	2.34	12.0	(DYS 438)
5.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 10.
Sample 9.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 9:
Number of peaks found : 5

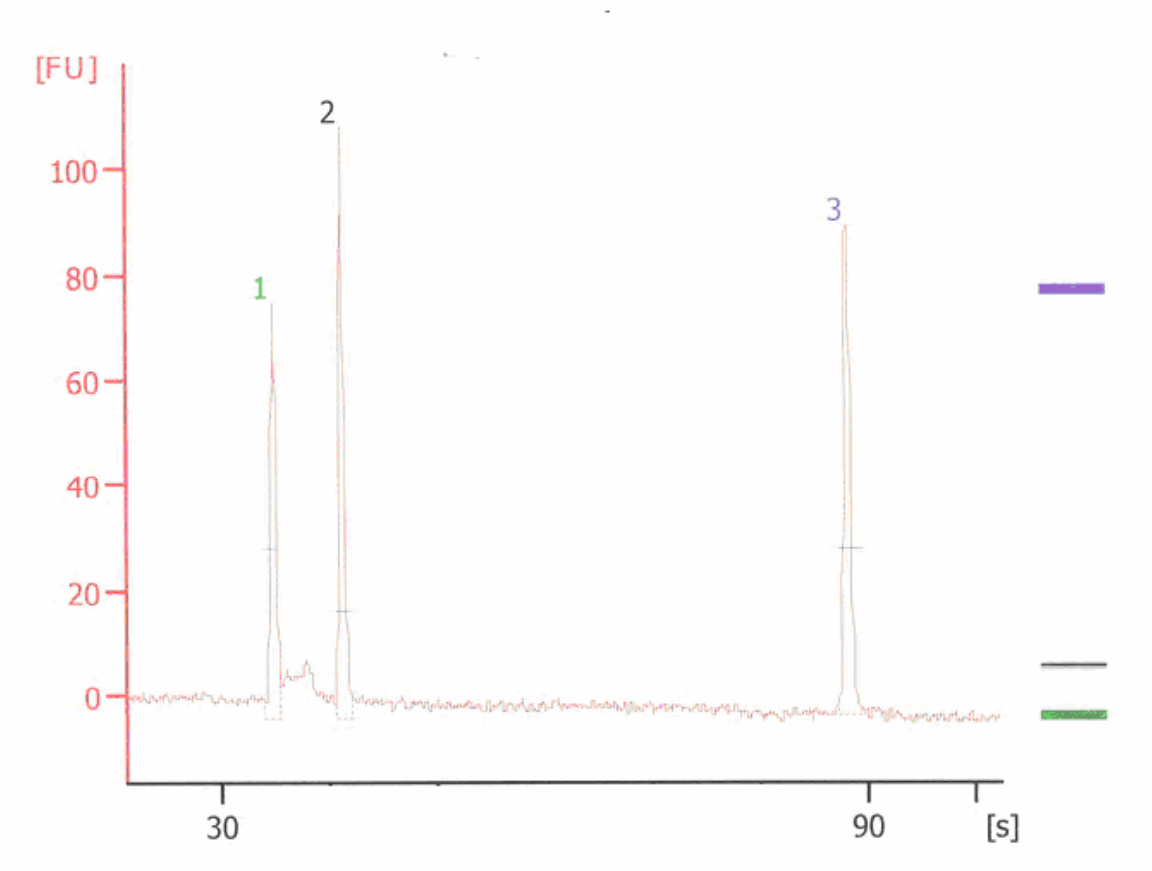
Peak table for sample 9.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		125	4.95	88.4	(DYS 392)
3.		187	40.69	222.4	(DYS390
4.		192	14.70	75.3	(DYS 389 I)
5.		200	14.80	69.1	(DYS 19)
6.		245	2.52	10.6	(DYS 439)
7.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 11.
Sample 10.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 10:
Number of peaks found : 1

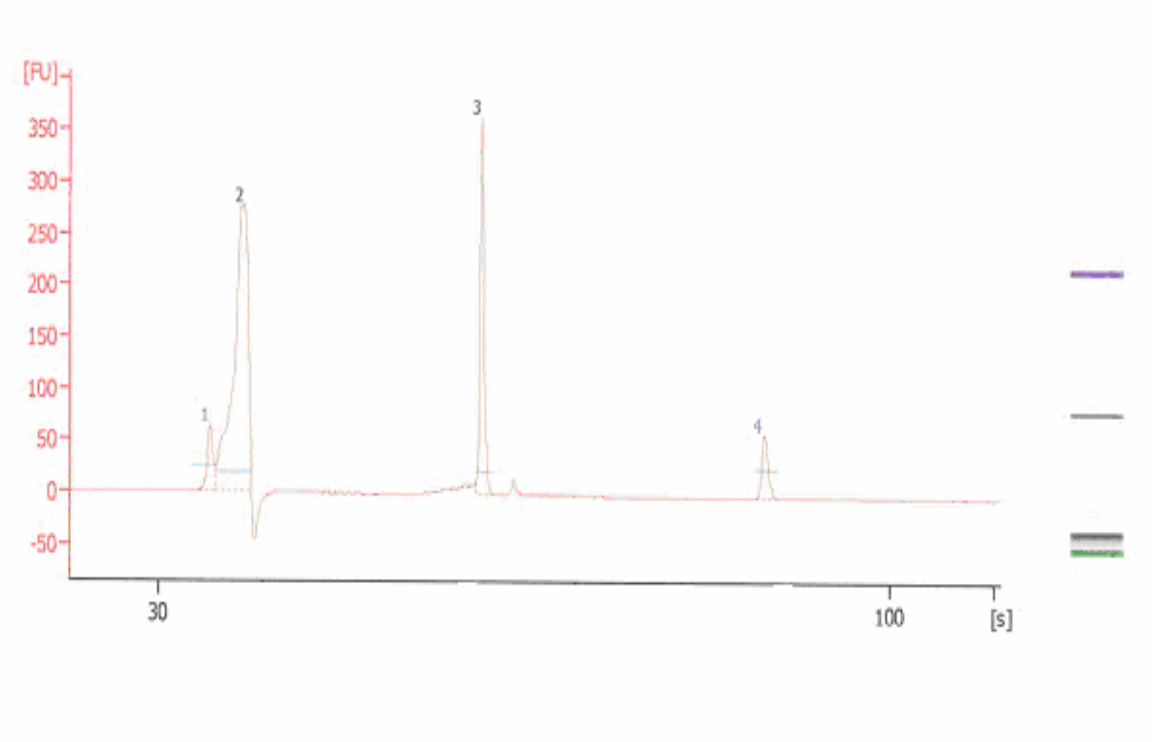
Peak table for sample 10.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		106	7.41	72.5	(DYS 393)
3.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 12.
Sample 11.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 11.
Number of peaks found : 2

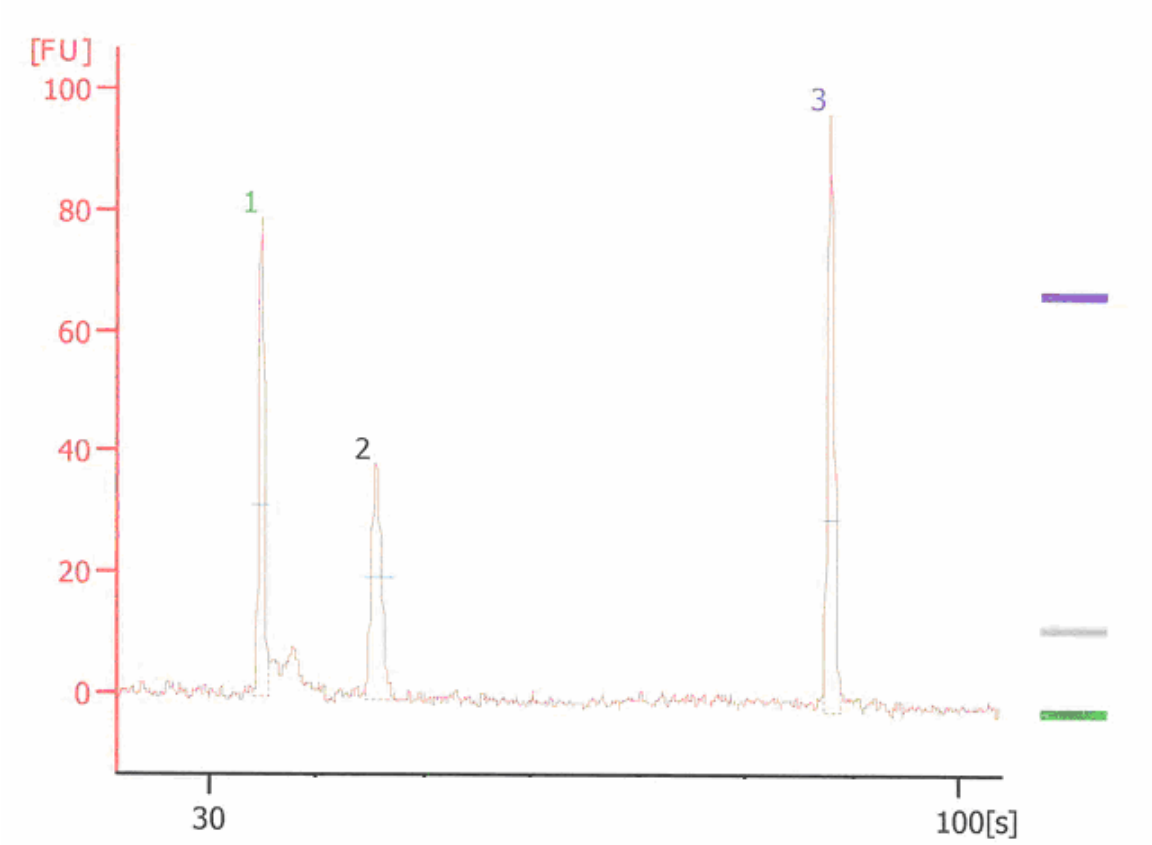
Peak table for sample 11.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		103	94.70	1386.8	(Amelo)
3.		110	20.60	56.4	(DYS 393)
4.	♦	10,380	4.20	0.6	Upper Marker

Electropherogram 13.
Sample 12.

2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad
Created: **11/29/2007** **2:55:32 PM**
Modified: **11/29/2007** **3:29:44 PM**
Assay Class: DNA 7500
Data Path: D:\...-29\2100 expert_DNA 7500_DE72904781_2007-11-29_14-55-32.xad

Electropherogram Summary



Overall Results for sample 12.

Number of peaks found : 1

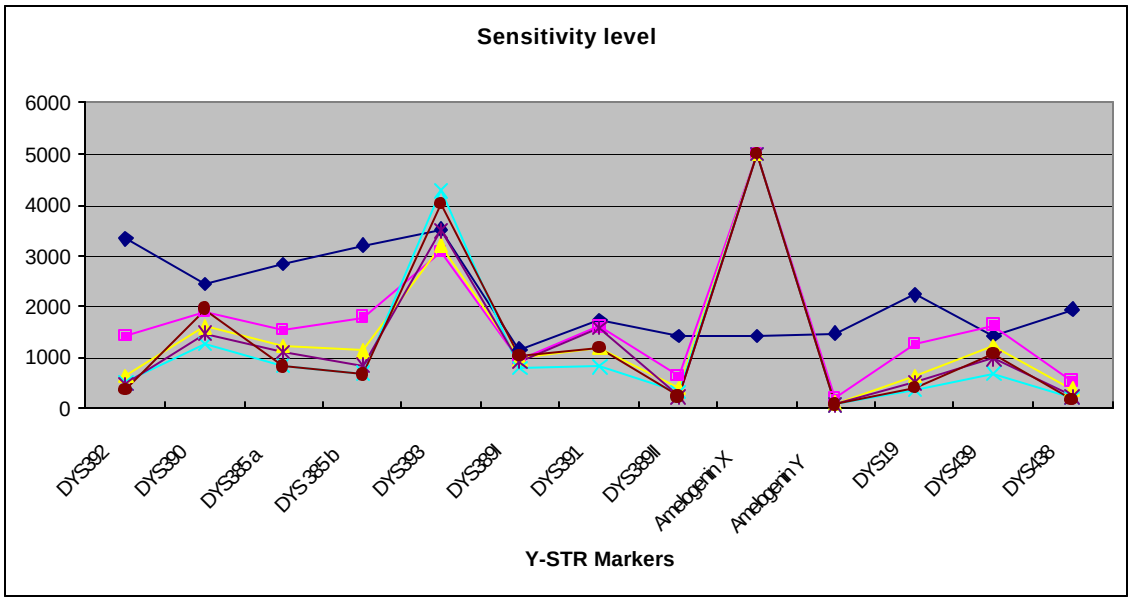
Peak table for sample 12.

Peak		Size [bp]	Conc. [ng /ul]	Molarity [nmol/l]	Observations
1	♦	50	8.30	251.5	Lower Marker
2.		232	4.31	28.2	(DYS 385 a,b)
3.	♦	10,380	4.20	0.6	Upper Marker

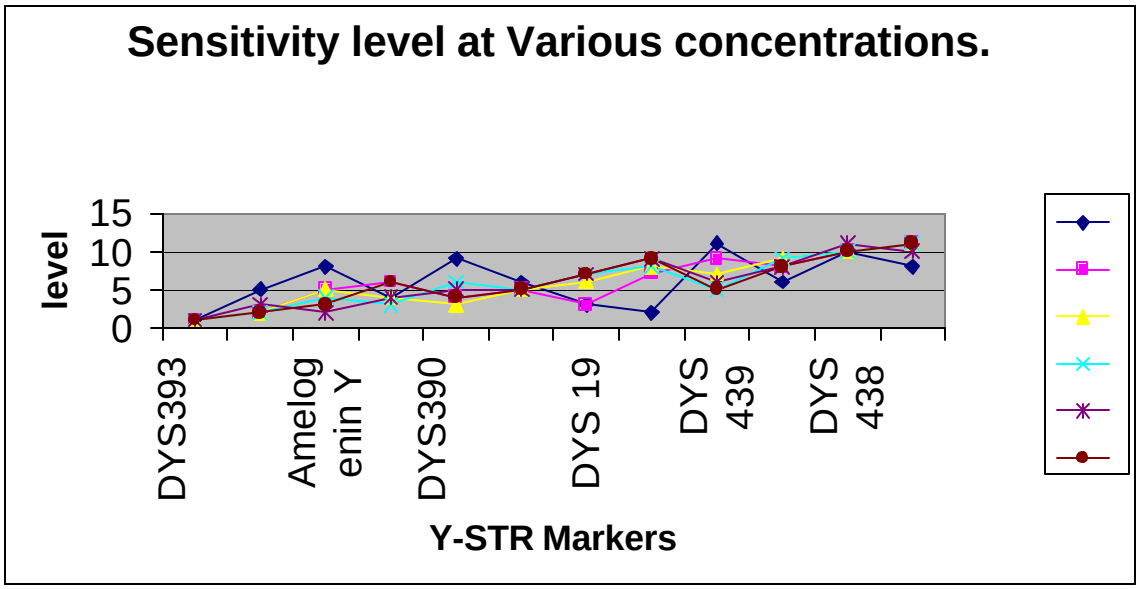
The degree of sensitivity of various markers was also assessed on amplification. It was observed that the markers with smaller allele range proved to be more sensitive than with wider range. (Table 29) The sensitivity level was further assessed at various concentrations (Graph 1,2) and it was found that the most sensitive marker was DYS 393 followed by others. Amelogenin was sensitive in a number of cases however variations were observed in some of the cases. Discrepancy was noted in 16.7% of the cases as some of the loci were found positive, whereas others failed to give any value, the reason for that has been explained as that it was due to reduction in binding of primers with male template DNA in the presence of a far excess amount of female DNA.

Table 29. Degree of the sensitivity of various markers as observed in 108 sexual assault cases.

S.No	**Marker**	**Allele Range**	**Sensitivity Level**
1.	DYS 393	102 – 120	1
2.	Amelogenin X,Y	103 – 110	2
3.	DYS 392	111 – 128	3
4.	DYS 390	179 – 197	4
5.	DYS 389 I	187 – 196	5
6.	DYS 19	188 – 205	6
7.	DYS 385	226 – 282	7
8.	DYS 439	234 – 255	8
9.	DYS 391	242 – 259	9
10.	DYS 438	290 – 325	10
11.	DYS 389 II	296 - 325	11



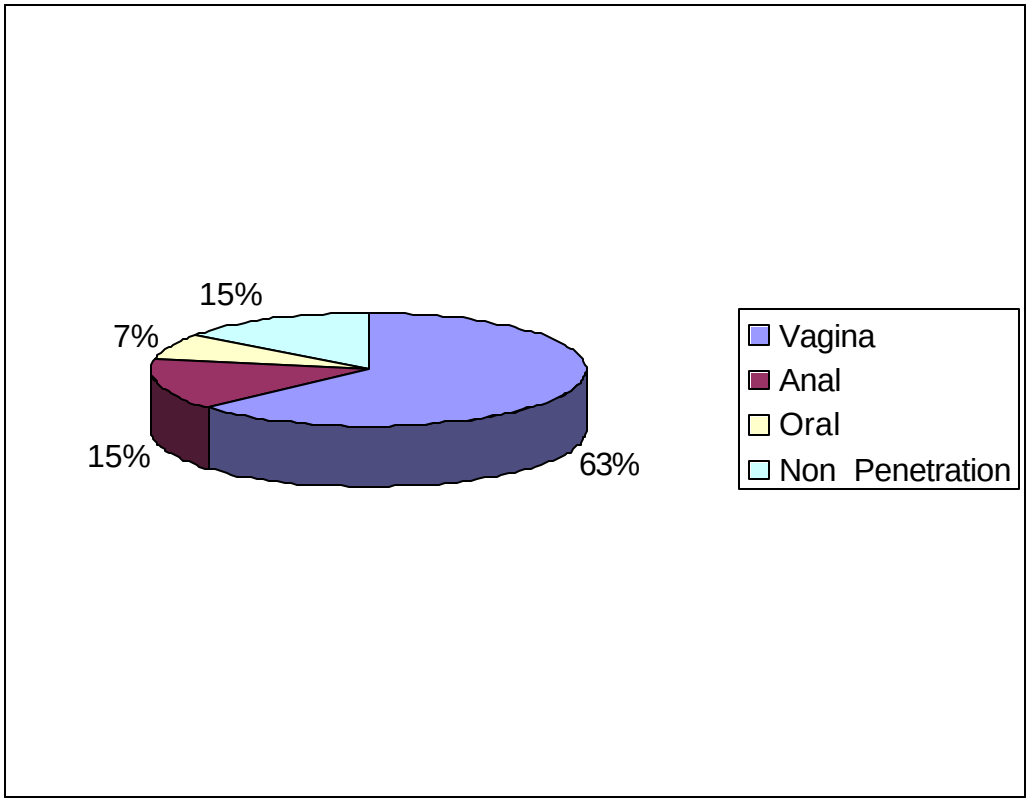
Graph 1. Computerised Graphic representation of the sensitivity of Y-STR markers.



Graph 2. showing the sensitivity pattern of markers.

In 192/300 cases (64%) no amplification of Y Chromosome material was observed. It as observed in 108/300 cases (36%) and the various sites of detection are shown in Fig 4. The most common place was the vagina (63%), followed by 15% anal and 7% oral penetration. The non-penetration cases were 15% and these were the cases where penetration was not reported but DNA was detected from the swabs taken from the surrounding regions of vagina, mouth and anus.

Figure 7: Y DNA was detected out of 108 cases as :



After making an assessment of the Y-STR DNA amplification as biological evidence in 300 cytologically negative cases, the genetic data on Y Chromosome STR haplotypes and allele frequency was found out for 108 people of Punjabi population and a comparison made with the studies performed by Qamar and associates [192] on 12 ethnic groups form Pakistani population, Frank E W et al[191] on Caucasion, Africo-American, Hispanics, native Americans, Xing et al[193] on population from Shaanxi province China, Kido et al on genetic data from South Africa[194], Mendes Junior CT on population from Sao Paulo state, South eastern Brazil[195}, Morikawa T on Japanese population.[196]

Among 300 cases found cytologically negative, processed for Y-STR analysis 108 cases were found positive, with a percentage of about 36%. The amplified products were detected using the ABI PRISM^{TM} 310 Genetic Analyzer (Applied BioSystems). The results were analysed using GeneScan® analysis v 3.1 software(Applied Biosystems) and genotypes were determined by Genotyper® v 2.5 software(Applied Biosystems) with the PowerTyper ^{TM} Y Macro (Promega). Gene and haplotype diversities were calculated according to Nei and Arlequin ver 3.0 - the software has become the standard software for haplotype analysis. [153]

//

RUN NUMBER 1 (29/01/08 at 16:19:11)

//

Project information:

Nb samples = 1
Data type = MICROSAT
Genotypic data = 0

Settings used for calculations
General settings:

Deletion weight = 1
Transition weight = 1
Tranversion weight = 1
Epsilon value = 1e-07
Significant digits for output = 5
Use original haplotype definition
Allowed level of missing data = 0.05

Active tasks:

---

1. Standard indices:
   i. Standard diversity indices (Punjab)
   ii. Molecular diversity indices (Punjab)

2. Haplotypic frequency estimation:
   Make estimations at the:
   i. Haplotypic level
   ii. Locus level

Analysis at the Intra population level.
Sample : Punjab, Pakistan

1.(i) **Standard diversity indices : (Punjab)**[154,155]

No. of gene copies : 108
No. haplotypes : 68
No. of loci : 10
No. of usable loci : 10 loci with less than 5.00 % missing data
No. of polymorphic loci : 10 [Ref. Nei M, Garza J C]

Table 30. Results are shown for polymorphic loci.

Locus#	Numb Gene copies	Num. alleles	Gene diversity	Allelic range
DYS19	108	5	0.67030	4
DYS385I/II	108	(30)	0.89806	(13)
DYS389I	108	3	0.49844	2
DYS389II	108	7	0.73399	6
DYS390	108	5	0.74645	4
DYS391	108	5	0.50190	4
DYS392	108	6	0.58584	7
DYS393	108	5	0.65196	4
DYS438	108	7	0.70786	6
DYS439	108	6	0.73278	5
Mean	108.000	6.273	0.67619	5.636
s.d.	0.000	2.093	0.10293	2.460

Haplotype-level computations

Sum of square freqs. : 0.0324
Haplotype diversity : 0.9766 +/- 0.0074
(Standard deviation is for the sampling process)

1(ii)
Table 31: Molecular diversity indices : (Punjab)[156-158]

Sample size	: 108.0000
No. of haplotypes	: 68
Allowed level of missing data	: 5.0000 %
Number of usable loci	: 10
Distance method	: No. of different alleles
Mean number of pairwise differences :	6.727587 +/- 3.196717
Average gene diversity over loci :	0.672759 +/- 0.354019

(Standard deviations are for both the sampling and the stochastic processes)
Unable to compute some theta values for microsatellite data type

Infinite allele model:
Theta(Hom) : 39.983900
S.D. Theta(Hom) : 13.588903

2(i)

Table 32: Haplotypes frequency estimation : (Punjab)[159-162]

Haplotype database content:

No.	Freq.	s.d.	Haplotypes												
			19	385 a	385 b	389 I	389 II	390	391	392	393	438	439	n
1	0.120370	0.031457	15	9	16	13	29	22	10	14	12	11	11	13
2	0.074074	0.025318	16	11	14	13	30	25	11	11	13	10	12	8
3	0.046296	0.020314	14	15	19	14	30	23	10	11	12	11	11	5
4	0.027778	0.015887	14	13	18	13	29	24	10	10	15	10	12	3
5	0.027778	0.015887	16	11	14	13	30	25	10	11	13	9	10	3
6	0.018519	0.013033	13	15	21	14	31	23	10	12	13	11	13	2
7	0.018519	0.013033	14	13	16	12	28	22	10	14	11	9	12	2
8	0.018519	0.013033	14	13	17	13	29	23	14	17	13	10	11	2
9	0.018519	0.013033	14	13	18	13	29	24	10	10	14	11	10	2
10	0.018519	0.013033	15	10	14	13	29	25	12	11	13	9	12	2
11	0.018519	0.013033	15	11	15	13	30	25	11	11	13	11	11	2
12	0.018519	0.013033	15	12	14	13	31	25	10	11	13	10	10	2
13	0.018519	0.013033	15	13	19	14	30	23	10	10	14	11	12	2
14	0.018519	0.013033	15	9	16	13	29	22	10	14	14	10	11	2
15	0.018519	0.013033	16	11	13	14	31	25	10	11	13	9	11	2
16	0.018519	0.013033	16	11	14	13	30	23	11	11	13	11	12	2
17	0.018519	0.013033	16	11	14	13	30	24	11	11	13	10	12	2
18	0.018519	0.013033	16	11	14	13	31	25	11	11	13	12	10	2
19	0.009259	0.009259	13	16	17	12	28	23	11	12	12	10	10	1
20	0.009259	0.009259	14	11	14	13	28	24	11	14	12	9	12	1
21	0.009259	0.009259	14	13	13	12	28	22	11	11	14	11	12	1
22	0.009259	0.009259	14	13	18	13	28	24	10	10	15	11	10	1
23	0.009259	0.009259	14	13	18	14	30	23	10	10	14	11	12	1
24	0.009259	0.009259	14	13	19	13	29	24	10	11	13	9	13	1
25	0.009259	0.009259	14	13	20	14	31	22	11	12	13	10	10	1
26	0.009259	0.009259	14	13	22	13	30	23	10	12	13	11	9	1
27	0.009259	0.009259	14	14	18	12	28	22	10	14	11	8	10	1
28	0.009259	0.009259	14	14	18	14	30	23	10	10	14	11	11	1
29	0.009259	0.009259	14	14	19	13	28	23	10	10	14	9	10	1
30	0.009259	0.009259	14	14	21	14	31	23	10	10	14	10	12	1
31	0.009259	0.009259	14	16	18	14	30	23	10	11	12	9	10	1
32	0.009259	0.009259	15	10	14	13	29	24	11	11	13	10	12	1
33	0.009259	0.009259	15	10	14	13	29	25	11	11	13	10	13	1
34	0.009259	0.009259	15	10	19	13	30	23	10	11	12	11	14	1

35	0.009259	0.009259	15	11	11	13	30	25	10	11	13	12	10	1
36	0.009259	0.009259	15	11	11	14	31	23	10	11	13	11	12	1
37	0.009259	0.009259	15	11	14	13	29	25	11	11	13	13	10	1
38	0.009259	0.009259	15	11	14	13	30	24	10	11	13	9	11	1
39	0.009259	0.009259	15	11	14	13	31	25	10	11	13	9	10	1
40	0.009259	0.009259	15	11	14	13	32	25	10	11	13	11	13	1
41	0.009259	0.009259	15	11	14	14	31	24	10	11	13	9	12	1
42	0.009259	0.009259	15	11	14	14	31	25	11	11	14	10	12	1
43	0.009259	0.009259	15	11	14	14	32	25	11	11	13	9	12	1
44	0.009259	0.009259	15	11	15	13	30	25	12	11	14	10	12	1
45	0.009259	0.009259	15	11	15	13	30	26	11	11	13	9	13	1
46	0.009259	0.009259	15	11	16	14	32	25	10	11	13	9	10	1
47	0.009259	0.009259	15	12	17	12	28	24	10	11	12	11	12	1
48	0.009259	0.009259	15	13	17	12	28	24	11	11	13	9	10	1
49	0.009259	0.009259	15	14	14	12	27	22	10	11	14	10	10	1
50	0.009259	0.009259	15	14	18	12	28	23	10	11	12	11	12	1
51	0.009259	0.009259	15	14	19	14	31	23	10	11	13	7	11	1
52	0.009259	0.009259	15	15	16	13	29	22	10	11	12	10	13	1
53	0.009259	0.009259	15	15	16	14	31	23	10	11	12	9	10	1
54	0.009259	0.009259	15	16	19	14	30	22	10	11	12	9	12	1
55	0.009259	0.009259	15	18	19	12	26	23	10	11	14	11	12	1
56	0.009259	0.009259	15	9	15	13	30	22	10	15	12	10	12	1
57	0.009259	0.009259	15	9	16	13	29	22	11	14	12	10	10	1
58	0.009259	0.009259	15	9	16	13	29	23	10	14	13	9	12	1
59	0.009259	0.009259	16	11	11	13	30	23	11	11	13	8	13	1
60	0.009259	0.009259	16	11	14	13	30	25	11	11	14	9	11	1
61	0.009259	0.009259	16	11	14	13	31	26	11	11	13	11	13	1
62	0.009259	0.009259	16	11	15	13	30	25	10	11	13	10	10	1
63	0.009259	0.009259	16	11	15	13	30	25	11	11	13	10	10	1
64	0.009259	0.009259	16	11	15	13	31	25	10	11	13	11	13	1
65	0.009259	0.009259	16	12	14	12	29	25	10	11	13	10	10	1
66	0.009259	0.009259	16	9	16	13	31	22	11	14	12	12	10	1
67	0.009259	0.009259	17	11	15	13	30	25	13	11	13	9	12	1
68	0.009259	0.009259	17	16	21	12	29	22	10	12	14	10	13	1

													= 108	

Freq= Frequency, S.D= Standard deviation, n= number of individuals.

2(ii)
Allele Data base content:

Table 33. Alleles for the locus DYS19:

No.	Freq.	s.d.	Allele:
1	0.462963	0.048204	15
2	0.250000	0.041861	16
3	0.240741	0.041331	14
4	0.027778	0.015887	13
5	0.018519	0.013033	17

Table 34. Alleles for the locus DYS389I:

No.	Freq.	s.d.	Allele:
1	0.666667	0.045572	13
2	0.222222	0.040191	14
3	0.111111	0.030382	12

Table 35. Alleles for the locus DYS389II:

No.	Freq.	s.d.	Allele:
1	0.370370	0.046684	30
2	0.305556	0.044532	29
3	0.175926	0.036809	31
4	0.101852	0.029239	28
5	0.027778	0.015887	32
6	0.009259	0.009259	27
7	0.009259	0.009259	26

Table 36. Alleles for the locus DYS390:

No.	Freq.	s.d.	Allele:
1	0.250000	0.041861	22
2	0.333333	0.045572	25
3	0.259259	0.042365	23
4	0.138889	0.033433	24
5	0.018519	0.013033	26

Table 37. Alleles for the locus DYS385I/II:

No.	Freq.	s.d.	Allele:
1	0.166667	0.036028	9,16
2	0.250000	0.041861	11,14
3	0.046296	0.020314	15,19
4	0.064815	0.023801	13,18
5	0.018519	0.013033	15,21
6	0.018519	0.013033	13,16
7	0.027778	0.015887	13,17
8	0.037037	0.018257	10,14
9	0.074074	0.025318	11,15
10	0.027778	0.015887	12,14
11	0.027778	0.015887	13,19
12	0.018519	0.013033	11,13
13	0.009259	0.009259	16,17
14	0.009259	0.009259	13,13
15	0.009259	0.009259	13,20
16	0.009259	0.009259	13,22
17	0.027778	0.015887	14,18
18	0.018519	0.013033	14,19
19	0.009259	0.009259	14,21
20	0.009259	0.009259	16,18
21	0.009259	0.009259	10,19
22	0.027778	0.015887	11,11
23	0.009259	0.009259	11,16
24	0.009259	0.009259	12,17
25	0.009259	0.009259	14,14
26	0.018519	0.013033	15,16
27	0.009259	0.009259	16,19
28	0.009259	0.009259	18,19
29	0.009259	0.009259	9,15
30	0.009259	0.009259	16,21

Table 38. Alleles for the locus DYS391:

No.	Freq.	s.d.	Allele:
1	0.638889	0.046435	10
2	0.305556	0.044532	11
3	0.018519	0.013033	14
4	0.027778	0.015887	12
5	0.009259	0.009259	13

Table 39. Alleles for the locus DYS392:

No.	Freq.	s.d.	Allele:
1	0.203704	0.038935	14
2	0.601852	0.047323	11
3	0.111111	0.030382	10
4	0.055556	0.022144	12
5	0.018519	0.013033	17
6	0.009259	0.009259	15

Table 40. Alleles for the locus DYS393:

No.	Freq.	s.d.	Allele:
1	0.277778	0.043300	12
2	0.500000	0.048337	13
3	0.037037	0.018257	15
4	0.027778	0.015887	11
5	0.157407	0.035207	14

Table 41. Alleles for the locus DYS438:

No.	Freq.	s.d.	Allele:
1	0.379630	0.046915	11
2	0.314815	0.044899	10
3	0.231481	0.040775	9
4	0.037037	0.018257	12
5	0.018519	0.013033	8
6	0.009259	0.009259	13
7	0.009259	0.009259	7

Table 42. Alleles for the locus DYS439:

No.	Freq.	s.d.	Allele:
1	0.277778	0.043300	11
2	0.351852	0.046166	12
3	0.250000	0.041861	10
4	0.101852	0.029239	13
5	0.009259	0.009259	9
6	0.009259	0.009259	14

Table 43. Summary of computations performed within populations.
Basic properties.

Statistics	Punjab	Mean	s.d.
No. of gene copies	108	108.000	0.000
No. of loci	10	10.000	0.000
No. of usable loci	10	10.000	0.000
No. of polym. loci	10	10.000	0.000

Table 44. Number of alleles

Locus#	Punjab	Mean	s.d.	Total number
1	5	5.000	0.000	5
2	30	30.000	0.000	30
3	3	3.000	0.000	3
4	7	7.000	0.000	7
5	5	5.000	0.000	5
6	5	5.000	0.000	5
7	6	6.000	0.000	6
8	5	5.000	0.000	5
9	7	7.000	0.000	7
10	6	6.000	0.000	6
Mean	**7.900**	**7.900**	**0.000**	**7.900**
s.d.	**7.449**	**7.449**	**0.000**	**7.449**

END OF RUN NUMBER 1 (29/01/08 at 17:53:11))
Total computing time for this run : 1h 34m 0s= 94 ms

The most frequent haplotype and their frequency distribution are presented in the Table 46 showing values for Haplotype frequency estimation. A total of 108 individuals produced 68 haplotypes , of which 50 were unique, 13 were found in 2 individuals and 2 were found in 3 individuals, 13, 8 and 5 individuals had single haplotype.

The most common haplotype, found in 13 individuals is shown below.

Table 45. Common Haplotype found.

Allele	**15**	**9**	**16**	**13**	**29**	**22**	**10**	**14**	**12**	**11**	**11**
Locus	DYS 19	DYS 385a	DYS 385b	DYS 389I	DYS 389II	DYS 390	DYS 391	DYS 392	DYS 393	DYS 438	DYS 439

Haplotype diversity is 0.9766 +/- 0.0074

Gene diversity 0.67619, SD 0.10293,

Sum of square frequencies is 0.0324.

The comparison between our studies in Punjab, and 12 ethnic groups of Pakistan reported in a study by Qamar et al is presented in Tables 47 - 56.

Table 46. Locus: DYS19. Comparison of allele frequency in Punjabi population and other Pakistani groups.

	Other Pakistani n = 503		**Punjabi n = 108**	
Allele	Count	%	Count	**%**
12	-	-	-	-
13	24	4.77	3	2.78
14	172	34.19	26	24.07
15	188	37.37	50	46.30
16	94	18.67	27	25.00
17	25	4.97	2	1.85
18	-	-	-	-

Table 47. Locus: DYS 389 I.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
9	91	18.09	-	-
10	252	50.09	-	-
11	152	30.20	-	-
12	8	1.62	12	11.11
13	-	-	72	66.67
14	-	-	24	22.22
15	-	-	-	-

Table 48. Locus: DYS 389 II.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
20	-	-	-	-
25	-	-	-	-
26	-	-	1	.93
27	6	1.10	1	.93
28	64	12.55	11	10.18
29	170	33.85	40	37.04
30	155	30.88	33	30.55
31	83	16.5	19	17.60
32	22	4.45	3	2.77
33	3	.67	-	-

Table 49. Locus:DYS 390.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	19	3.78	-	-
22	103	20.48	27	25.00
23	153	30.42	33	30.56
24	121	24.05	15	13.89
25	91	18.09	31	28.70
26	13	2.58	2	1.85
27	1	.20	-	-
28	1	.20	-	-
29	1	.20	-	-

Table 50. Locus: DYS 391.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	15	2.98	69	63.88
11	20	3.97	33	30.55
12	167	33.20	3	2.77
13	210	41.74	1	.93
14	83	16.50	1	.93
15	8	1.61	-	-

Table 51. Locus: DYS 392.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
8	1	.19	-	-
9	-	-	-	-
10	44	8.76	12	11.11
11	307	61.04	65	60.18
12	44	8.76	6	5.56
13	23	4.57	-	-
14	64	12.72	22	20.37
15	17	3.37	1	.93
16	3	.59	-	-
17	-	-	2	1.85
18	-	-	-	-

Table 52. Locus: DYS 393.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
10	15	2.98	-	-
11	20	3.97	3	2.77
12	167	33.20	30	27.78
13	210	41.76	54	50.01
14	83	16.50	17	15.74
15	8	1.59	4	3.70
16	-	-	-	-
17	-	-	-	-

Table 53. Locus: DYS 439.Comparison of allele frequency in other Pakistani groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	**%**
9	4	.79	1	.93
10	126	25.05	27	25.00
11	178	35.83	38	35.18
12	138	27.45	30	27.78
13	55	10.94	11	10.18
14	2	.39	1	.93

Table 54. Locus: DYS 438.Comparison of allele frequency in other Pakistani ethnic groups and Punjabi population.

	Other Pakistani	**n = 503**	**Punjabi**	**n = 108**
Allele	Count	%	Count	%
7	1	.20	1	.93
8	2	.40	2	1.85
9	121	24.05	25	23.14
10	160	31.80	34	31.48
11	202	40.17	41	37.97
12	16	3.18	4	3.70
13	1	.20	1	.93
14	-	-	-	-

Table 55. Locus: DYS 385 a,b.Comparison of allele frequency in other Pakistani ethnic groups and Punjabi population.

	Other Pakistani	n = 503	Punjabi	n = 108
Allele	Count	%	Count	**%**
8	-	-	-	-
9	18	1.70	19	8.80
10	8	.80	5	2.31
11	74	7.36	42	19.44
12	108	10.74	4	1.86
13	195	19.38	21	9.72
14	200	19.88	44	20.37
15	156	15.50	18	8.33
16	135	13.42	27	12.50
17	53	5.27	5	2.32
18	31	3.08	12	5.56
19	28	2.78	13	6.01
20	-	-	1	.46
21	-	-	4	1.86
22	-	-	1	.46
24	-	-	-	-
25	-	-	-	-

Comparison between 12 ethnic groups from Pakistan, Punjabi population, Caucasians, African Americans, Hispanics, Native Americans, Shaanxi Province of China, South Africa, Sao Paulo State, Southeastern Brazil and Japan is shown below. (Table 56)

Comparison between 12 ethnic groups from Pakistan, Punjabi population, Caucasians, African Americans, Hispanics, Native Americans, Shaanxi Province of China, South Africa, Sao Paulo State, Southeastern Brazil and Japan is shown below. (Table 57)

Table 56.

DYS 19

Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistani, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.

Locus	Allele	Caucasian n= 1243 count	%	Africa-Ameri n= 1605 count	%	Hispanics n= 454 count	%	Native Ameri n= 104 count	%	Pakistani n= 503 count	%	Chinese n= 137 count	%	African n= 73 count	%	Brazilian n= 617 count	%	Japanese n= 153 count	%	Punjabi n= 108 count	%
DYS 19	12	01	.08	02	.13	01	.22	-	-	-	-	02	1.46	-	-	-	-	-	-	-	-
	13	77	6.19	33	2.05	88	19.38	22	21.15	24	4.77	07	5.11	04	5.48	84	13.62	09	5.88	02	1.85
	14	**816**	**65.65**	400	24.92	**245**	**53.96**	**58**	**55.76**	172	34.19	36	26.27	**23**	**31.44**	**304**	**49.28**	12	7.84	17	15.74
	15	240	19.30	**614**	**38.25**	84	18.50	17	16.34	**188**	**37.37**	**62**	**45.26**	22	30.17	144	23.34	**74**	**48.38**	**60**	**55.56**
	16	80	6.45	305	19.00	25	5.50	06	5.77	94	18.67	26	18.98	22	30.17	67	10.85	30	19.60	27	25.00
	17	28	2.25	249	15.52	11	2.42	01	.96	25	4.97	04	2.92	01	1.37	17	2.75	28	18.30	02	1.85
	18	01	.08	02	.13	-	-	-	-	-	-	-	-	01	1.37	01	.16	-	-	-	-

DYS 389 I

Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistan, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.

Locus	Allele	Caucasian n= 1243 count	%	Africa-Ameri n= 1605 count	%	Hispanics n= 454 count	%	Native Ameri n= 104 count	%	Pakistani n= 503 count	%	Chinese n= 137 count	%	African n= 73 count	%	Brazilian n= 617 count	%	Japanese n= 153 count	%	Punjabi n= 108 count	%
DYS 389 I	9	-	-	-	-	-	-	-	-	91	18.09	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	**252**	**50.09**	-	-	04`	5.48	-	-	-	-	-	-
	11	03	.24	18	1.12	07	1.54	01	.96	152	30.20	-	-	-	-	-	-	07	4.58	-	-
	12	238	19.15	259	16.18	57	12.56	17	16.35	08	1.62	-	-	**25**	**34.25**	-	-	21	13.73	12	11.11
	13	**806**	**64.84**	**1062**	**66.17**	**281**	**61.89**	**70**	**67.31**	-	-	-	-	19	26.02	-	-	49	32.03	**72**	**66.67**
	14	186	14.96	261	16.26	107	23.57	16	15.38	-	-	-	-	25	34.25	-	-	**72**	**47.05**	24	22.22
	15	10	.81	05	.31	02	.44	-	-	-	-	-	-	-	-	-	-	04	2.61	-	-

DYS 389 II

Locus	Allele	Caucasian n= 1243 count	%	Africa-Ameri n= 1605 count	%	Hispanics n= 454 count	%	Native Ameri n= 104 count	%	Pakistani n= 503 count	%	Chinese n= 137 count	%	African n= 73 count	%	Brazilian n= 617 count	%	Japanese n= 153 count	%	Punjabi n= 108 count	%
DYS 389II	20	-	-	-	-	-	-	-	-	-	-	02	1.46	-	-	-	-	-	-	-	-
	25	01	.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	01	0.06	-	-	-	-	-	-	02	1.46	-	-	-	-	-	-	01	.93
	27	17	1.36	14	.87	06	1.32	01	.96	06	1.10	09	6.57	07	4.11	-	-	10	6.54	01	.93
	28	210	16.89	147	9.15	47	10.35	17	16.36	64	12.55	38	27.74	**23**	**31.50**	-	-	17	11.11	11	10.18
	29	**568**	**45.69**	361	22.49	**180**	**39.64**	**44**	**42.30**	**170**	**33.85**	**44**	**32.12**	04	5.48	-	-	41	26.80	**40**	**37.04**
	30	315	25.36	**562**	**35.02**	144	31.71	36	34.62	155	30.88	30	21.90	10	13.70	-	-	**54**	**35.29**	33	30.55
	31	104	8.36	396	24.68	53	11.67	6	5.76	83	16.5	09	6.57	21	28.77	-	-	23	15.03	19	17.60
	32	24	1.94	105	6.55	24	5.31	-	-	22	4.45	03	2.18	12	16.44	-	-	05	3.27	03	2.77
	33	04	.32	19	1.18	-	-	-	-	03	.67	-	-	-	-	-		03	1.96	-	-

DYS 390

Locus	Allele	Caucasian n= 1243 count	%	Africa-Ameri n= 1605 count	%	Hispanics n= 454 count	%	Native Ameri n= 104 count	%	Pakistani n= 503 count	%	Chinese n= 137 count	%	African n= 73 count	%	Brazilian n= 617 count	%	Japanese n= 153 count	%	Punjabi n=108 count	%
DYS 390	18	-	-	-	-	-	-	-	-	-	-	-	-	02	2.74	-	-	-	-	-	-
	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20	-	-	28	1.75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	21	19	1.53	**887**	**55.27**	26	5.73	03	2.89	19	3.78	-	-	**39**	**53.43**	58	9.40	-	-	-	-
	22	146	11.75	146	9.10	34	7.49	01	.96	103	20.48	09	6.57	03	4.11	76	12.32	**41**	**26.80**	27	25.00
	23	320	25.5	169	10.53	114	25.11	**48**	**46.15**	**153**	**30.42**	**60**	**43.80**	-	-	175	28.36	36	23.53	**33**	**30.56**
	24	**570**	**45.86**	265	16.51	**231**	**50.88**	37	35.58	121	24.05	41	29.93	06	8.22	**239**	**38.66**	24	15.69	15	13.89
	25	177	14.24	98	6.11	46	10.13	15	14.42	91	18.09	25	18.24	04	5.48	15	2.43	38	24.84	31	28.70
	26	09	.72	11	.69	03	.66	-	-	13	2.58	01	.73	18	24.65	48	7.78	11	7.18	02	1.85
	27	02	.16	01	.06	-	-	-	-	01	.20	01	.73	01	1.37	02	.35	03	1.96	-	-
	28	-	-	-	-	-	-	-	-	01	.20	-	-	-	-	04	.70	-	-	-	-
	29	-	-	-	-	-	-	-	-	01	.20	-	-	-	-	-	-	-	-	-	-

DYS 391

		Caucasian n= 1243		Africa-Ameri n= 1605		Hispanics n= 454		Native Ameri n= 104		Pakistani n= 503		Chinese n= 137		African n= 73		Brazilian n= 617		Japanese n= 153		Punjabi n= 108	
Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistan, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.																					
Locus	Allele	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%
DYS 391	7	-	-	01	.22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	02		-	-	-	-	-	-	01	.73	-	-	-	-	01	.65	-	-
	9	28	2.25	24	1.49	24	5.28	16	15.38	-	-	10	7.30	-	-	66	10.70	02	1.30	-	-
	10	579	46.58	**1131**	**70.46**	**242**	**53.30**	**50**	**48.08**	15	2.98	**96**	**70.07**	**64**	**87.67**	**318**	**51.54**	**133**	**86.94**	**70**	**64.82**
	11	**616**	**49.55**	424	26.44	182	39.88	36	34.62	20	3.97	30	21.90	09	12.33	220	35.66	17	11.11	33	30.55
	12	19	1.54	23	1.43	06	1.32	01	.96	167	33.20	-	-	-	-	12	1.94	-	-	03	2.77
	13	01	.08	01	.06	-	-	01	.96	**210**	**41.74**	-	-	-	-	01	.16	-	-	01	.93
	14	-	-	-	-	-	-	-	-	83	16.50	-	-	-	-	-	-	-	-	01	.93
	15	-	-	-	-	-	-	-	-	08	1.61	-	-	-	-	-	-	-	-	-	-

DYS 392

		Caucasian n= 1243		Africa-Ameri n= 1605		Hispanics n= 454		Native Ameri n= 104		Pakistani n= 503		Chinese n= 137		African n= 73		Brazilian n= 617		Japanese n= 153		Punjabi n= 108	
Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistan, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.																					
Locus	Allele	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%
DYS 392	8	-	-	01	.06	-	-	-	-	01	.19	-	-	-	-	-	-	-	-	-	-
	9	-	-	02	.12	01	.22	01	.97	-	-	-	-	-	-	-	-	-	-	-	-
	10	04	.32	06	.37	02	.44	00	00	44	8.76	-	-	03	4.11	35	5.67	-	-	12	11.11
	11	394	31.69	**1166**	**72.65**	143	31.49	24	23.07	**307**	**61.04**	-	-	**70**	**95.89**	248	40.19	59	38.57	**65**	**60.18**
	12	51	4.02	76	4.73	20	4.40	04	3.85	44	8.76	-	-	-	-	40	6.48	17	11.11	06	5.56
	13	**684**	**55.03**	299	18.64	**231**	**50.88**	**42**	**40.38**	23	4.57	-	-	-	-	**257**	**41.64**	**66**	**43.14**	-	-
	14	101	8.13	51	3.19	33	7.28	29	27.88	64	12.72	-	-	-	-	20	3.24	09	5.88	22	20.37
	15	09	.73	03	.18	15	3.30	03	2.88	17	3.37	-	-	-	-	15	2.43	02	1.30	01	.93
	16	-	-	01	.06	07	1.55	01	.97	03	.59	-	-	-	-	02	.35	-	-	-	-
	17	-	-	-	-	01	.22	-	-	-	-	-	-	-	-	-	-	-	-	02	1.85
	18	-	-	-	-	01	.22	-	-	-	-	-	-	-	-	-	-	-	-	-	-

DYS 393

		Caucasian n= 1243		Africa-Ameri n= 1605		Hispanics n= 454		Native Ameri n= 104		Pakistani n= 503		Chinese n= 137		African n= 73		Brazilian n= 617		Japanese n= 153		Punjabi n= 108	
Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistan, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.																					
Locus	Allele	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%
DYS 393	10	-	-	-	-	-	-	-	-	15	2.98	-	-	-	-	02	.35	-	-	-	-
	11	02	.16	04	.24	04	.88	-	-	20	3.97	04	2.92	-	-	94	15.24	5	3.27	03	2.77
	12	116	9.35	61	3.80	66	14.53	22	21.15	167	33.20	**64**	**46.73**	01	1.37	**402**	**65.12**	38	25.50	30	27.78
	13	**974**	**78.35**	**898**	**55.95**	**334**	**73.57**	**65**	**62.51**	**210**	**41.76**	36	26.27	**46**	**63.02**	99	16.05	**83**	**59.46**	**54**	**50.01**
	14	125	10.05	441	27.49	41	9.04	15	14.42	83	16.50	28	2.43	10	13.70	19	3.08	13	8.50	17	15.74
	15	25	2.01	191	11.90	08	1.76	02	1.92	08	1.59	05	3.65	15	20.54	01	.16	05	3.27	04	3.70
	16	01	.08	08	.50	01	.22	-	-	-	-	-	-	01	1.37	-	-	-	-	-	-
	17	-	-	02	.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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DYS 438

		Caucasian n= 1243		Africa-Ameri n= 1605		Hispanics n= 454		Native Ameri n= 104		Pakistani n= 503		Chinese n= 137		African n= 73		Brazilian n= 617		Japanese n= 153		Punjabi n= 108	
Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistan, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.																					
Locus	Allele	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%
DYS 438	7	-	-	-	-	-	-	-	-	01	.20	-	-	-	-	-	-	-	-	01	.93
	8	02	.16	21	1.30	01	.22	-	-	02	.40	-	-	-	-	-	-	-	-	02	1.85
	9	49	3.94	25	1.55	47	10.36	04	3.85	121	24.05	-	-	-	-	-	-	01	.65	25	23.14
	10	316	25.42	181	11.28	98	21.59	22	21.15	160	31.80	-	-	06	8.22	-	-	**88**	**57.52**	34	31.48
	11	112	9.01	**998**	**62.18**	78	17.18	33	31.73	**202**	**40.17**	-	-	**60**	**82.19**	-	-	17	11.11	**41**	**37.97**
	12	**732**	**58.89**	371	23.13	**225**	**49.55**	**42**	**40.38**	16	3.18	-	-	07	9.59	-	-	02	1.30	04	3.70
	13	31	2.50	09	.56	05	1.10	03	2.89	01	.20	-	-	-	-	-	-	45	29.42	01	.93
	14	01	.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistan, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.

DYS 439

Locus	Allele	Caucasian n= 1243 count	%	Africa-Ameri n= 1605 count	%	Hispanics n= 454 count	%	Native Ameri n= 104 count	%	Pakistani n= 503 count	%	Chinese n= 137 count	%	African n= 73 count	%	Brazilian n= 617 count	%	Japanese n= 153 count	%	Punjabi n= 108 count	%
DYS 439	9	01	.08	-	-	-	-	-	-	04	.79	-	-	-	-	-	-	-	-	01	.93
	10	77	6.19	36	2.24	30	6.60	02	1.92	126	25.05	-	-	-	-	-	-	-	-	27	25.00
	11	443	35.63	492	30.65	135	29.73	41	39.42	**178**	**35.83**	-	-	34	46.58	-	-	32	20.92	**38**	**35.18**
	12	**565**	**45.46**	**794**	**49.48**	**217**	**47.79**	**50**	**48.09**	138	27.45	-	-	**39**	**53.42**	-	-	**86**	**56.21**	30	27.78
	13	140	11.26	257	16.01	69	15.19	11	10.58	55	10.94	-	-	-	-	-	-	30	19.60	11	10.18
	14	17	1.38	26	1.62	03	.69	00	00	02	.39	-	-	-	-	-	-	05	3.27	01	.93

DYS 385 a,b

		Caucasian	Africa-Ameri	Hispanics	Native Ameri	Pakistani	Chinese	African	Brazilian	Japanese	Punjabi
	385a	n= 1243	n= 1605	n= 454	n= 104	n= 503	n= 137	n= 73	n= 617	n= 153	n= 108
	385b	n= 1243	n= 1605	n= 454	n= 104	n= 503	n= 137	n= 73	n= 617	n= 153	n= 108

Comparison of Allele Frequency in Caucasian, African American, Native American, Hispanics, Pakistani, Chinese, South African, Brazilian, Japanese and Punjabi Population groups.

Locus	Allele	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%	count	%
DYS 385 a,b	8	-	-	01	.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	02	.08	06	.18	-	-	-	-	18	1.79	-	-	-	-	-	-	01	.33	19	8.80
	10	31	1.25	10	.31	08	.88	01	.48	08	.80	01	.36	-	-	-	-	43	14.05	05	2.31
	11	762	30.65	355	11.06	223	24.56	45	21.64	74	7.36	44	16.06	06	4.11	-	-	16	5.23	42	19.44
	12	139	5.59	68	2.12	38	4.19	06	2.89	108	10.74	**73**	**26.64**	01	.68	-	-	26	8.50	04	1.86
	13	264	10.62	114	3.55	85	9.36	13	6.25	195	19.38	48	17.52	04	2.74	-	-	**50**	**16.34**	21	9.72
	14	**830**	**33.39**	447	13.93	**242**	**26.65**	**48**	**23.08**	**200**	**19.88**	24	8.76	**30**	**20.55**	-	-	23	7.52	**44**	**20.37**
	15	252	10.14	409	12.74	112	12.34	42	20.19	156	15.50	15	5.48	21	14.38	-	-	09	2.94	18	8.33
	16	101	4.06	**613**	**19.10**	76	8.37	18	8.65	135	13.42	15	5.48	26	17.81	-	-	13	4.25	27	12.50
	17	47	1.89	588	18.32	49	5.40	22	10.58	53	5.27	20	7.30	19	13.01	-	-	40	13.07	05	2.32
	18	40	1.61	391	12.81	45	4.96	06	2.89	31	3.08	16	5.84	07	4.80	-	-	23	7.52	12	5.56
	19	13	.52	172	5.36	24	2.64	04	1.92	28	2.78	13	4.74	04	2.74	-	-	31	10.13	13	6.01
	20	04	.16	27	.84	06	.66	03	1.44	-	-	05	1.82	25	17.13	-	-	25	8.15	01	.46
	21	-	-	09	.28	-	-	-	-	-	-	-	-	03	2.05	-	-	04	1.31	04	1.86
	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	01	.46
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	01	.33	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	01	.33	-	-

DISCUSSION:

Investigation of violent crime has always been the decisive issue for law enforcing agencies in any country and more so in societies of developing countries like Pakistan. Pakistan is a developing country with a scarcity of sources and modern technology. Along with the developments made in various fields of technology, the crime rate has also risen to an alarming extent. Pakistan has great extremes in the distribution of wealth, for much of its 61 years of independence, it had a tormented existence and most of that period was ruled by a series of more or less military regimes. The country is racked by inter-communal and intersect strife. The language and style of competitive politics goes beyond adversarial debate in a framework of respect, taking the form of hostile, confrontational and self-interested manoeuvering. There is a small, but very rich class (frequently described as Feudal Lords) from which most of the political elite come, a large majority of very poor people, and a relatively small middle class in between. Law enforcing agencies have traditionally been used more to serve the narrow interests of those in office than to defend the rule of law. So, there ought to be significant human right abuses including torture, honour killings and sexual assault.

Sexual assault is an emotionally charged crime in which passions for prevention and prosecution run at a high level. Many people involved in sexual assault work, take on the role of advocates and support persons for victims of sexual assault.

Sexual violence starts very early in life. Leslie et al stated that mean age of rape victim in their study was 23.3 years.[163] According to our study 41.67 % were, in the age group between 10 and 19 years, whereas the mean age of 10 – 60 years was 20 years. Among them 38.89 % of victims were house wives followed by labourers (25%),

domestic workers 15.74 %, students 9.26 % and 11.11 % working women. The majority (60.5%) of rape victims was from lower socio-economic group having monthly income of less than Rs 3000 (US$: 47). Among them 9.5% were from middle class having monthly income of Rs 10,000 (US $:156) or more. We found that 20.5% of victims were literate and 6% completed their graduation.

Majority of victims (58.63%) were married, 3.24% were divorced and 2.84% were widows. Most (75%) of the women were from rural background as majority of the cases brought were from surrounding villages. In 72% of cases the rapist knew the victim (38% friends, 28% acquaintances, and 6% close relations). In one case due to property dispute, the victim was kidnapped by his relative and kept in a room for a week, where he and his two sons repeatedly raped her. Groth and Burgess stated that rape is not so much about sex as it is about other issues such as power show, aggression and violation.[164] Lim et al stated in his study of sex assault cases in Singapore, that, slightly more than half (53%) of rape victims were strangers to the offender, and majority of known offenders were boyfriends, followed by ex lover, ex spouses and relatives.[165] This change in pattern is due to different life style and social setup. As per prevalent Pakistani traditions and culture a girl/woman usually does not go out alone with unknown person or entertain them when they are alone at home.

Austin, stated that victims are chosen not for their attire but rather more for their vulnerability. In his study eight victims were related to the rapist, two were daughters aged 12 and 13 years, two were step daughters aged 9 and 14 years, two were cousins aged 16 and 18 years, one was the perpetrators wife's niece aged 9 years and another a relative's daughter aged 14 years. All these victims were sexually assaulted in the home

of the perpetrator except for one, who was assaulted on a beach.[166] In our study 68.51% of victims were sexually assaulted at their home/ perpetrator's residence or a common friend's place. Apart from them 13.92% were raped at farm / jungle / railway yard or at place where she was working alone. In addition, 8.33% had sexual intercourse at hotel, where they had gone for consensual sex, these were professionals, and ultimately were caught by police. Some (4.62%) victims were assaulted at parties, in five cases victims were offered a drink after which they became drowsy and then taken to a farm house / house and raped. In one of the two cases, victims who were staying alone with her small children, stated that she was sexually assaulted while sleeping in night and the accused ran away when her children woke up due to noise. In our study 22.23 % of victims were threatened by the accused of dire consequence if they reported the matter to police, or informed their relatives.

In 36.11 % of cases, patients had taken bath and washed her clothes before the medical examination was conducted, as a result of it valuable trace evidences were lost. This can be attributed to delay in reporting the matter to the police. Only 11.11 % of victims were brought before us for medical examination within 12 hours of rape, 29.63% were brought in 13 to 24 hours after rape. This delay may be due to lack /absence of an examining Gynaecologist at Primary level Hospitals (75% of cases reported were from rural areas) as a result patients were referred to higher centers, for medical examination. Some of the victims took days to get them medically examined, this delay was due to indecisiveness on the part of victim's parents, relatives or the victim to report the case to police, due to the fear of Pakistani male dominated society and considering the future life of victim and her sisters, and in some cases the victim had voluntarily ran away to some

other city, with the accused due to love affair and were captured by the police after some days, as the parents of the victim had reported the case of kidnapping of their daughter.

In 80 cases (74.40 %) the perpetrator of assault was alone, in 15 cases (13.87%) they were two in number, but in 13 cases (12.03%) three or more than three persons were involved in the act. Seto and Kuban stated in their study of gang rape, that the two accused were drunk and other reportedly being on drugs, and their motive was only robbery, and during robbery they molested the victim, but when the victim became aggressive then they raped her so as to cause the victim to be ashamed enough to report robbery.[167]

In 52.78 % only one sexual act was done, as the victim was present only for a few minutes with the assailant, but in 25 % of the cases multiple sexual acts were done, as the victims were in custody of the assailant for days, until recovered by the court through bailiff or were discovered by the police. In 5.56% of cases pregnancy had resulted due to rape. In 14.81% of cases no injuries and signs of struggle were present on the body of victim. Majority of the injuries (48.15 %) were seen on breast, 25% were on cheeks, 77% of them were finger nail abrasions and bite marks. Ramin et al, found an injury rate of 18% in comparison group of 129 females aged 14 to 49 years, injuries seen were abrasions, oedema, haematoma and lacerations. [168] In our study 30.55% of genital injuries were seen of which 82% were contusions, and in 2% cases it was laceration of genitalia. Slaughter et al. included in her comprehensive study of genital injuries in sexual assault patients, the colposcopic examination of 75 women (aged 13 to 48 years) within 24 hours of consensual penile penetration of the vagina. Trauma (defined as tears,

ecchymosis, abrasion, redness, swelling) was limited to one site and had resolved by the follow up visit in those who returned.[169]

In 25% of cases bite marks were found on breast (67%) and cheeks (33%) of victims. The presence of injury marks on breast and genitals of victims lends further support to the concept of rape being a method of violent offence rather being for sexual enjoyment. Groth et al, attempted to categorize rape into three categories – **power rape** (sexuality used primarily to express power), **anger rape** (sexuality used to express anger), **sexual rape** (sexuality used to enjoy rape). In our study 27 patients (25%) were from urban area (city), and amongst them 8 victims did not show any signs of struggle or injuries on their bodies, except for bite marks on breast, as most of these cases were of consensual sexual intercourse.[170]

In an incidence at Ghotkee, Sindh, Pakistan. A TV net work narrated the story on 26th April 2008, as " The young son of a 65 years old lady (victim) came back from USA. He developed illicit relations with the sister of 4 brothers (assailants). During the course of affair the young man developed photographs of some of the abnoxious and objectionable scenes of the girl and started blackmailing her. On getting the information from others, the 4 brothers got flared and decided to take revenge, and so they gang raped the 65 years old mother of the young man and also made a movie of the incidence.

In another incidence reported by the same TV channel on 3rd May, 2008 a 20 years old woman was kidnapped as a revenge to molest her father, a school teacher, for not obeying the landlord. She was gang raped and then murdered. All the assailants are still at large.

The act of rape was committed by the perpetrators at any time depending upon the availability, opportunity and vulnerability. However, as per our study half of rape victims (50%) were sexually assaulted between 6 pm and midnight, and 9.26 % were assaulted between 6 am and 12 noon as the victim was in the custody of accused for several days, and they used to have sexual intercourse 3 to 4 times a day. Leslie and Lim et al hypothesised that timing of assault was significantly different; rape was more likely to be committed under the cover of darkness, after midnight and in secluded places, whereas molestation occurred in crowded places, inside public transport, or along staircase, other places such as hotels were significantly more likely to be chosen for rape than molestation.[154] In the present series 22.22 % victims were in a state of mild mental instability, whereas two individuals 1.85% were ready to marry the accused as they were in love with him, but were caught by police after a few days, on complain of kidnapping by their parents. About 75.93 % rape victims were not able to give any opinion on, proposed punishment to the rapists.

Although rape victims have often been portrayed as young, attractive, sexually provocative females, this notion was dismissed by Groth and Birnbaum and Taylor who observed that there is no typical victim profile, young females, elderly females, and even pregnant women have been raped. The victims were not extraordinarily striking in appearance and almost never dressed in seductive clothing.[171,172] In the present study, victims' attractiveness did not seem to be an important reason for rape, but it was a contributory factor in certain cases. However there was a strong element of opportunism present in most of the cases. According to Rodabaugh and Austin victims are chosen not

for their attire but rather more for their vulnerability, [173] and that the psychopaths were more likely to be opportunistic rapists.

Sexual violence is associated with many short- and long-term problems, including physical injury and illness, psychological symptoms, economic costs, and even death. Survivors often isolate themselves from their support network either physically or emotionally. The survivor may feel disconnected from peers as a result of the perceived personal experience. The shattering of trust can adversely affect intimate relationships, as survivors may have a heightened suspicion of others' motives and feelings.Another area of research referred to as "second victimisation," has to do with the caustic and interrogatory way the police and medical staff sometimes treat people who allege sexual assault. Sexual assault can affect an individual forever, changing them into someone living in a constant state of turmoil. In extreme cases the outcome may be suicide.[174]

Despite the underestimation of the true magnitude of the problem, research has increased understanding of factors that make some populations more vulnerable to sexual violence, victimisation and more at risk for sexual violence perpetration. Vulnerability factors increase the likelihood that a person will suffer harm. Risk factors increase the likelihood that a person will cause harm. However, neither vulnerability nor risk factors are the direct causes of sexual violence — they are *contributing* factors to sexual violence. Vulnerability factors for victimisation and risk factors for perpetration comprise a combination of individual, relational, community and sociable factors.

Vulnerability factors for victimisation. [175]

i. Prior history of sexual violence.

ii. Gender.

iii. Young age. Young women are at higher risk of being raped than older women.

iv. Drug or alcohol use. Binge drinking and drugs use are related to increased rates of victimisation.

v. High-risk sexual behaviour. As with drug/alcohol use, researchers are trying to understand the complex relationships between sexuality and sexual violence — their causality, directionality, and other aetiological factors that increase vulnerability for victimization are not well understood.

vi. Poverty.

Factors for Perpetration [175]

Individual Factors

i. Alcohol and drug use.

ii. Coercive sexual fantasies.

iii. Impulsive and antisocial tendencies.

iv. Preference for impersonal sex.

v. Hostility towards women.

vi. Hypermasculinity.

vii. Childhood history of sexual and physical abuse.

viii. Witnessed family violence as a child.

Relationship Factors [175]

i. Association with sexually aggressive and delinquent peers.

ii. Family environment characterized by physical violence and few resources.

iii. Strong patriarchal relationship or familial environment.

iv. Emotionally unsupportive familial environment.

Community Factors [175]

i. Lack of employment opportunities

ii. Lack of institutional support from police and judicial system

iii. General tolerance of sexual assault within the community

iv. Settings that support sexual violence

v. Weak community sanctions against sexual violence perpetrators

Societal Factors [175]

i. Poverty

ii. Societal norms that support sexual violence

iii. Societal norms that support male superiority and sexual entitlement

iv. Societal norms that maintain women's inferiority and sexual submissiveness

v. Weak laws and policies related to gender equity

vi. High tolerance levels of crime and other forms of violence

Protective Factors

Protective factors may lessen the likelihood of sexual violence victimization or perpetration, and exist at individual, relational, community, and societal levels. Although less is known about protective factors, the literature suggests measures to prevent

potential perpetrators. Some examples for youth are connectedness with school, friends and adults in the community, and emotional health.[175]

The present study is supposed to provide some context for examining practice and prevention of sexual assault by offering a critical analysis of the situation and its' solution. The past decade has seen great advances in a powerful criminal justice tool, Deoxyribonucleic acid DNA: DNA evidence is now widely accepted as a standard forensic technique for the investigation and detection of a wide spectrum of crime types from volume crime (burglary and autocrime) to serious and major crime such as rape and murder. Evaluation was made for the feasibility of Y-STR analysis in negative cytology specimens of rape cases and collection of Y Chromosome STR data for the various Punjabi population groups. Punjab is the largest province of Pakistan with more than 60 million of population. Pakistan lies on the postulated southern coastal route followed by anatomically modern *H. sapiens* out of Africa, and so may have been inhabited by modern humans as early as 60,000–70,000 years ago. However, earliest evidence found scattered around the Soan river Valley in Northern Pakistan may dates back to 200,000 – 400,000 years.[176,177] Wolpert (2000) mentioned that there is evidence of cave dwellers in Pakistan's northwest frontier, but fossil evidence from the Paleolithic has been fragmentary. [178] Evidence has been uncovered at Mehrghar, in southwestern Pakistan, indicating Neolithic settlements from as long ago as 7,000 B.C.[179], which were followed by the Indus Valley civilizations (including the cities of Harappa and Mohenjodaro) that flourished in the 3rd and 2nd millennia B.C. [180]. Around 1500 B.C. the Indo-European–speaking nomadic pastoral tribes from further north—often called the Aryans—crossed the Hindu Kush Mountains into the subcontinent. Subsequent historical events include

the invasion of Alexander the Great (327–325 B.C.) and the Arab and Muslim conquests from 711 a.d. onwards [181].

The present population of Pakistan consists of >160 million individuals (according to current WHO figures) belonging to at least 18 ethnic groups and speak >60 languages.[181] Most of these languages are Indo-European, but they also include an isolate, Burushaski; a Dravidian language, Brahui; and a Sino-Tibetan language, Balti. Punjabi-speaking individuals form the majority population of Pakistan, but they represent a complex admixture of ethnic castes and groups and are analysed here. The evaluation process was carried out through a systematic approach of testing, analysing and simultaneous amplification of chosen Y-STR markers, as the potent tool in the detection of serious crimes such as sexual assault and lineage studies in Punjabi population. In our country the primary investigation sought for sexual assault victims is the biological evidence i.e. identification of spermatozoa from cervico-vaginal smears, negative results are particularly frequent. Absence of spermatozoa in specimens from victims of sexual assault has been explained by a number of factors.[44] However, the failure to demonstrate the presence of spermatozoa in the cytological examination of a rape case does not exclude the presence of male DNA (Y-chromosome).

Cases having negative cytology, were detected by using Y-STR amplification, and Y-chromosome analysis provided evidence of the presence of male cells in up to 36 % of alleged female victims of sexual assault victims. This was also useful for cases with non-penile penetration. Presence of Y-DNA in cases with negative cytology, provided proof of sexual contact and so could be used to corroborate the testimony of the sexual assault female victims. Y-STRs can also provide evidence in instances involving old and

degraded samples leading to difficult DNA extraction.

The rationale for this protocol was that the primers designed to hybridize to Y-STR sequences would not hybridize the female DNA. Thus, Y-STR analysis was still possible when the male to female DNA ratio is well below the 1/50 threshold for detection of male autosomal STRs.

The most sensitive marker (present in more cases) was DYS 393 followed in descending order of sensitivity by DYS393> Amelogenin Y> DYS 392> DYS 390> DYS 389I> DYS 19> DYS 385> DYS 439> DYS 391> DYS 438> DYS 389II>. The pattern observed regarding the sensitivity of Y STR markers during the study was that sensitivity was indirectly proportional to the number of base pairs. The markers with small number of base pairs were found more sensitive than otherwise.

Another important point of our study was that Y-chromosome was detected in 36% victims after 72 hours of sexual assault, biologically spermatozoa are rarely detected so late, so we stress that swabs ought to be taken from the victims of sexual assault for Y Chromosome DNA typing even after long delays between sexual assault and medical examination.

Thus our study has shown the feasibility of Y-STR analysis in providing evidence of sexual assault with:

i. Cases of negative cytology
   - ♦ With or without penetration.
   - ♦ Independent of ejaculation.

ii. Old and degraded samples.

iii. The method simplified by multiplex amplification showed the feasibility of haplotype determination.

As we know that most of the human Y-chromosome does not recombine during meiosis and remains unchanged from generation to generation. Therefore, the Y-STR markers chosen were highly polymorphic and used commonly by the forensic workers. We benefited ourselves from the Y-STRs identified, in January 2003, by the Scientific Working Group on DNA Analysis Methods (SWGDAM) recommending the use of the European Minimal Haplotype in conjunction with two additional Y-STR loci. However, unlike the minimal and extended haplotypes, that require two or three or four multiplexes to obtain the results, the Y-STR 12 plex system was used and we achieved a high power of discrimination in a single amplification. In the case of Y STRs, the discriminatory power of a set of markers is defined as the probability of obtaining a non-unique haplotype based on the marker used. A unique haplotype is defined as one that only occurs once in a given population.

After the successful assessment of the reliability of Y-STR multiplex analytical platform, we hope to demonstrate that the analytical platform described herein can provide accurate and reliable analysis of Y-STR markers for forensic case works and lineage studies.

In some of the studies stress has been laid on a robust multiplex, but it should be well understood that by increasing the number of loci, simultaneously amplification is restricted by several factors.

i. Majority of these markers have not been fully characterized with respect to their utility in forensic casework.

ii. Studies, while providing an extensive overview, showed less detail on specific loci, with a small number of loci being identified as the "most variable".[182 – 185]

iii. Gene diversity values are based on a smaller size of groups.[186 – 190].

iv. While some of these markers may exhibit individually high gene diversity values, a few studies have demonstrated the ability of these markers to aid in resolving coincidental matches.[190-193]

v. Only a few novel non-'core' markers have undergone the extensive developmental validation studies required. Without such developmental validation studies it is not possible to evaluate whether the loci are sufficiently robust for use with degraded and limiting quantities of sample in a multiplex analysis format, or can provide sufficient additional discrimination potential when used in combination with other core Y-STR markers.[194-195]

vi. The size of the Amplicons should be designed for at least 75 base pairs in size. The most common algorithm used for determining the DNA fragment size is known as the local southern method that works very well for accurate sizing of DNA fragments over the 100–450 bp size range. [196 -199]

vii. The more polymorphic a particular locus is the larger the size range it will encompass. One may have to decide between two less polymorphic markers or one with a larger potential size range of alleles. [196]

viii. The lack of adequate instrumentation may curtail the amount of amplicons that can be simultaneously detected. Multicolour fluorescence detection is necessary to resolve similar sized PCR products that are labeled with

spectrally distinguishable dyes. Genetic Analyser, with 16-capillary array system, with five dye capability can expand the analysis of multiple PCR amplicons. [199]

For the multiplex design approach presented here, we used publicly available software tools like GenBank, BLAST, BCM sequence alignment, Arlequin Ver 3.0. This upfront utilization of publicly available informatics reduced our labor-intensive empirical studies common to multiplex PCR optimisation. An effective and working multiplex PCR primer mixture can be constructed quickly, so this multiplex design strategy is currently being used to expand the Y STR multiplex to simultaneously amplify 12 or more loci in a single reaction.

As the allele designation for alleles were typed using the Y-PLEX^{TM} 12 amplification system and respective allelic ladder, the allele designation for the haplotypes was concordant and consistent with the published nomenclature and the ISFG recommendations for STR analysis. So, the database for the twelve Y-STR loci for Punjabi population, was used for haplotype frequency calculation, loci. The data comprised of the analysis of 108 unrelated healthy individuals belonging to multiple groups of Punjabi population, gathered from the 35 districts of province of the Punjab. These were the suspects related to the sexual assault cases. Appropriate informed consent was obtained from all the participants. Protocol for PCR was the same as mentioned under the materials and methods. The amplified products were detected using the ABI PRISM^{TM} 310 Genetic Analyzer (Applied Bio system) The results were analysed using Arlequin version 3.0 software. The gene diversity at each locus, the number of haplotypes and haplotype diversity was calculated using a program developed by Chakraborty and

Lee who followed the Nei calculation method.[153] In a total of 108 individuals produced 68 haplotypes as shown in table 46 of which 50 were unique, 13 were found in 2 individuals, 2 in 3 individuals, 1 in 5 individuals, 1 in 8 individuals, and 1 in 13 individuals. The most common haplotype is shown in table, and was found in 13 individuals.

Table 45: Common Haplotype found in Punjabi Population, in our study.

Allele	**15**	**9**	**16**	**13**	**29**	**22**	**10**	**14**	**12**	**11**	**11**
Locus	DYS 19	DYS 385a	DYS 385b	DYS 389I	DYS 389II	DYS 390	DYS 391	DYS 392	DYS 393	DYS 438	DYS 439

The data obtained was compared with the studies performed on other population groups such as:

1. **Caucasians, African Americans, Hispanics, Native Americans**.[200]
2. **12 ethnic groups from Pakistan**, **except Punjab.** [201]
3. **Shaanxi Province of China.** [202)
4. **South Africa.** [203]
5. **Sao Paulo State, Southeastern Brazil**. [204]
6. **Japan**. [205]

The comparative study made between Punjabi group, 12 ethnic groups of Pakistani and other global populations have shown the greater genetic similarity of Punjabi population to those in Africa followed by the West than to the Eastern groups.[206] The genetic studies indicate such a similarity being more marked with African population, Western Asia and Europe but not with China and Japan. The work

carried out by Qamar and associates [206,207] also shows the same pattern that 4 out of 5 haplogroups in Pakistan (1,2,3,9 which together make up 79% of the total population) are also frequent in West Asia and Europe. Conversely, the haplogroups (4,5,10,13,20) that are frequent in East Asia are rare or absent in Pakistan forming 2.5% of the total. It is also mentioned in their study that the fifth haplogroup, haplogroup 28 is common in our population making up 14% of the total. Outside Pakistan and the nearby countries, it has been reported as: India (30%), Tajikistan (10%), Uzbekistan (3%), Russia (0.4%), Caucasus (1.4%) but has not been found in China and Mongolia. Batwing's [208] estimates the Pakistani haplogroup 28, chromosomes according to TMRCA (Time from the Most Recent Common Ancestor) were ~7,000 (4,000–14,000) years. Thus, within this time period, the Pakistani populations diverged from a common ancestral population. Since the estimated age corresponds to the early Neolithic period, the spread of this lineage might be associated with the local expansion of farmers.

Humans not only transmit genes from one generation to the next, they also transmit cultural traits. Some of these are extremely conservative, being transmitted quite faithfully from parents to offsprings. Foremost amongst these is language; children almost invariably acquire their mother tongue from their parents and other relatives. Language and other conservative traits such as wedding traditions, ceremonies, rituals, songs, foods, dresses, practices relating to disposal of the dead are therefore excellent devices to trace historical changes. If this be so, linguistic, cultural traits and genetic divergence ought to go hand in hand.

As might have been predicted, the various communities in Punjab displayed distinct allele distribution, it is because this sub-continent is remarkable for its diversities

both biological and human.[209] The biological diversity owes itself to the country's position at the trijunction of the African, the northern Eurasian and the Oriental realm; its great variety of environmental regimes, and its relative stability of biological production. It is this biological wealth that has attracted to the subcontinent, many streams of people at different times, from different directions; bringing together a great diversity of human genes and human cultures. On the other hand in other lands the dominant human cultures have tended to absorb or eliminate others, in Indian sub-continent the tendency has been to isolate and subjugate the subordinated cultures, thereby augmenting cultural diversity. This tendency to nurture diversity has been favoured by the diversity of our country's ecological regimen.[210]

The people of Punjab are mainly descendants of the so-called **Aryan** tribes that entered India from the northwest during the 2nd millennium BC, as well as the pre-Aryan population, probably Dravidians, who had a highly developed civilization. This prosperous and flourishing civilization (Indus Valley) was brought to an end by the savage invasions of the Aryans about 1,500 B.C. These warlike nomads had encountered a very sophisticated civilization of the Indus valley. The Aryan advent was in fact the arrival of Barbarians into a region already highly organized into an empire based on a long established tradition of literate urban culture.[211,212]

However, the Aryans during their stay picked up much from the Indus Civilization which stood them in good stead during their settled life in India. "Aryans entered and Aryanized the middle country of the Ganges Doab after picking up ideas of craftsmen in the Indus Valley and the Baluch borderland." (Ibid)

The Aryan tribes occupied Pakistan have been identified as Sivas, Parsas, Kayayas,

Vrichivants, Yadus, Anus, Turvasas, Dratyus and Nichyas. The Sivas Aryans had their capital at Sivistan which is supposed to be modern Sehwan.
It may be of interest to mention here that so long as the Aryans stayed in Pakistan, they did not evolve that particular religion called 'Hinduism' with its caste system and other taboos. It was only when they crossed the Sutlej and settled in the Gangetic valley that this abominable system was evolved. To the east of Sutlej the Indo-Aryans were usually safe from foreign invasions and free to work out their own rule of life undisturbed. This also explains the absence of Hindu holy cities and temples in Pakistan.[213]

The Punjab region, due to its location near Central Asia and the Middle East has been prone to numerous invasions that have left imprints upon the local Punjabi population that remain present in the numerous sub-groups. The Punjabi people are a heterogenous group and can be subdivided into a number of tribal groups in Pakistan called ‘qaums’ while they adhere to caste identities in India, each having it’s own subtle differences.

In terms of ancestry, the majority of Punjabis share many similar genes with other northern India populations, but also show a significant relationship with west Eurasian groups. In 2004, a Stanford study conducted with a wide sampling from India, including 112 Punjabis, and selected other countries, displayed the following:

Results showed that Indian tribal and caste populations derive largely from the same genetic heritage of Pleistocene southern and western Asians and have received limited gene flow from external regions since the Halocene.[214]

The study by Parikh and associates also found that roughly 60% of genetic markers in the Punjab were of West Asian origin, the highest amongst the sampled group

of South Asians.[215] Another study also showed that there has been limited gene flow in and out of north India, but the highest amount of genetic inflow from the west showed up in the Punjab region. Broadly, the average proportion of mtDNAs from West Eurasia among Indian caste populations is 17% . In the northern states of India their share is greater, reaching over 30% in Kashmir and Gujarat and nearly 60% in Indian Punjab.[215] Some preliminary conclusions from these varying tests support a largely north Indian genetic base for most Punjabis accompanied by some of the highest degrees of west Asian admixture found in north India.

The Punjabis found in Pakistan are composed of clans and tribes (the aforementioned *qaums*) often with a correspondence with traditional occupations. Pre-Islamic Punjabi Shaikhs, Kambohs, Gujjars, Jats and Rajputs (such as the Janjuas) predominate with the Gakhars, Awans, and Arains, comprising the main tribes in the north, while Gilanis, Gardezis, Syeds and Quraishis are found in the south. There are Pashtun tribes like the Niazis and the Shaikhs, which are very much integrated into Punjabi village life. Especially the members of the Niazi tribe, who see themselves as Punjabis first. They have big communities in Mianwali, Bakkar, Lahore, Faisalabad, Sahiwal and Toba Tek Singh. Punjabis in Pakistan, especially in major urban cities have diverse origins, with many post Islamic settlers tracing their origin to Afghanistan, Persia, Arabia, Kashmir and Central Asia. Many Punjabis may contain some physical traces of Central Asians due to the invasions.[216]

Punjabis are traditionally farmers and warriors having transferred into modern times with a dominance of agriculture and the military in Pakistan. In addition, Punjabis in Pakistan have been quite prominent politically having had many elected Members of

Parliament. As the most ardent supporters of a Pakistani state, the Punjabis in Pakistan have shown a strong predilection towards the adoption of the Urdu language, while still identifying themselves as ethnic Punjabis for the most part. Religious homogeneity remains elusive as a Sunni-Shia divide and a Christian minority have not completely wiped out diversity since the partition of British India. A variety of related sub-groups exist in Pakistan and are often considered by many Pakistani Punjabis to be simply regional Punjabis including the Seraikis (who overlap and are often considered transitional with the Sindhis) and Punjabi Pathans (which publications like *Encyclopædia Britannica* consider a transitional group between Punjabis and Pathans).[217]

Therefore, it is observed that haplogroup distribution in Punjab despite of some notable linguistic differences is strikingly similar to one another. Although the observed Allele frequencies indicated greater shared genetic identity between the various groups of Punjabi population than with the other global groups, significant intercommunity differences were observed at all loci confirming that they effectively behave as discrete breeding units. In the Awan community, the degree of genetic isolation may have been exacerbated by the fact that they traditionally speak Hindko, akin to the Pushto language used in the neighbouring N.W.F.Province rather than Punjabi.[218]

The notably diverse pattern of Y-Chromosome haplotypes within the Rajpoot community{traditional Rajput clans called Hindu warrior super class} is because they are tribal in origin or may have migrated to Indian sub-continent from central Asia.[208] In the present context, and given their large numbers in Punjab, it seems probable that there was some amalgamation of Rajput clans following their conversion from Hinduism to Islam, which largely occurred from the 16th century onwards. There also is the possibility that

non-Rajput individuals may have elected to self-identify as a member of the Rajput community after conversion to Islam, thus further enhancing the range of Y-haplotypes.

The population that is genetically most distinct, the Hazaras, claims descent from Genghis Khan's army; their name is derived from the Persian word "hazar," meaning "thousand," because troops were left behind in detachments of a thousand. Toward the end of the 19th century, some Hazaras moved from Afghanistan to the Khurram valley in Pakistan, the source of the samples investigated here. Thus, their oral history identifies an origin in Mongolia and population bottlenecks ~ 800 and ~ 100 years ago.

Among the two predominant Y haplogroups present in this group of population, haplogroup 1 is widespread in Pakistan, much of Asia, Europe, and the Americas, and so provides little information about the place of origin. Haplogroup 10, in contrast, is rare in most Pakistani populations (1.4%, when the Hazaras are excluded) but is common in East Asia, including Mongolia, where it makes up over half of the population (unpublished results). BATWING analysis, suggested TMRCAs of 400 (120–1,200) and 100 (6–600) years respectively. Thus, the genetic evidence is consistent with the oral tradition and, in view of its independent nature, provides strong support for it.[219]

Therefore, the failure to find a Y link with a suggested population of origin does not disprove a historical association, but it does demonstrate that the Y-chromosomes derived from such historical events have been lost or replaced. Analyses of mitochondrial DNA and other loci can help to elucidate the population histories and could be particularly interesting in population groups in which there is a contrast between the phenotype and the typical Y haplotypes of the same population. In DNA analysis, the application of mitochondrial (mt) DNA sequencing and haplotype frequency calculation

is a novel approach used in human identification, population studies, evolutionary biology and anthropology. Single nucleotide polymorphic differences within functional genes are detected easily by single stranded conformational polymorphism (SSCP)[142] Forensic science is bound to be immensely benefited by the success of microchip technology. DNA typing of biological material will then be a matter of a few minutes with highest degree of perfection in results, but some inadvertent bias in sampling may complicate the hypothesis. For this reason the evidence samples should be typically processed through a forensic DNA laboratory prior to the suspect reference samples to avoid any possibility of contaminating the evidence, as the DNA testing techniques have become so sensitive that biological evidence too small to be seen by the naked eye and even one that is degraded can link the suspect to the crime scene. Automation of the STR typing methods have enabled scientists to quickly collect the data from these markers. Computerised interpretation of discrete alleles data has made the results easier and highly discriminating between unrelated and even closely related individuals.

While, we cannot predict the future with certainty. Short tandem repeats DNA markers have had and will continue to have an important role to play in forensic DNA typing in major crimes like "**Sexual assault on female victims".**

CONCLUSIONs.

- In a total of 930 cases analysed for biological evidence of spermatozoa in the submitted specimens, 300(32.25%) were found to be negative, and they were further analysed for Y-STR. Among them 108 cases (36%) provided proof of sexual contact and was used to corroborate the testimony of the sexual assault female victims. This was also very useful for the cases of non-penetration, as well as for non-ejaculation.
- Y-STR analysis was of great help in sexual assault cases where a very small amount of male DNA was present. In vasectomised, azoospermic cases, and in cases where no sperms were detected, due to use of condoms, digital penetration or douching after intercourse.
- The longer interval between the assault and the examination handicaps forensic experts who are not in a position to take suitable samples, as a few or no sperm cells are detectable. Our study lay stress that swabs ought to be taken from the victims of sexual assault for Y-STR DNA typing after long delays of sexual assault, as Y-STR are detectable even in old and degraded samples.
- The pattern observed during the study regarding the sensitivity of Y-STR markers is that sensitivity was indirectly proportional to the number of base pairs, DYS 393 with a smaller number of base pairs was the most sensitive, and DYS 389II with a large number of base pairs was least sensitive.
- Majority of the rape victims were young i.e in the age group of 11 – 20 yrs. They were assaulted when they were alone, nearly 2/3rd of the cases occurred at the residence of either victim or assailant or a common friend.

- Majority of them were married, belonging to rural area with low socio-economic background. Poverty played a major role in making their lives more dangerous by increasing their vulnerability to sexual victimisation. It was observed that the absence of male has been a provocative factor in cases of widow and divorce victims.
- Nearly all cases of gang rape were a show of power, aggression and revenge, and most of them were kidnapped prior to assault. They were kept in lock & chain and were raped repeatedly, resulting in pregnancy in some of them.
- Signs of struggle were present and mostly seen over the breasts, cheeks and genitalia in the form of abrasions, contusions, lacerations, oedema and haematoma. Presence of bite marks, 67% over the breasts and 33% over the cheeks supported the concept of rape being a method of violent offence rather being a sexual enjoyment.
- It is also concluded that rape victims have no typical profile, portrayed to be as young, attractive, sexually provocative females. It may be a contributory factor, whereas elderly and even pregnant females have been raped. Therefore, victims are not chosen for their attire but more for their availability, opportunity and vulnerability.
- Y-STR analysis showed the feasibility of haplotype determination on swabs initially characterized as "negative", which can be further simplified by multiplex amplification.
- The Y-plex ^{TM} 12 system enabled simultaneous amplification and analysis of the 11 Y-STR loci recommended by SWGDAM, as well as the Amelogenin locus.

Amelogenin served as an internal control for PCR and as a useful marker while investigating mixture samples containing male and female DNA.

- It is also concluded that Y chromosomes STRs provide valuable tools for the construction of human evolutionary trees. During the last decade the availability of a large number of STRs has greatly assisted these genome based investigations. However, today there are a few studies specifically conducted on highly inbred Punjabi population. Therefore, an additional step was taken to investigate the population genetic characteristics of Y STR polymorphism in Punjabi population. The study indicated a marked genetic similarity with African, West Asian and European population rather with China and Japan. We observed that the haplogroups found commonly in Punjab were also frequently seen in Africa, West Asia and Europe, and conversely the haplogroup that are frequent in East Asia are rare or absent in Pakistan, forming only 2.5% of the total.
- It is the need of the time, that the construction of haplotypes by reference to the pedigrees, with an increased density of STRs anchored in the region of early development genes and other known functional genes, and the investigation of polymorphism within the genes themselves, should help to further provide more information towards the resolution of new vistas in the field of genomic studies of the population.
- It will not be out of place to mention here that youth is the custodian of future generations and we should respect their views and galvanize them in accordance with the modern and national requirements. Hence we lay stress that cost

effective projects should be designed to promote the highest standards of academics, research, and fact finding results in the field of Forensic sciences.

- The project should aim at the provision of authentic results, best productivity, increased innovation, quality controlled services by the Forensic experts to the public, helpless people who are physically, emotionally, and economically tormented. They should get their rights.
- Services of a well oriented and highly qualified experts involved in different aspects of DNA research should be made available for the benefit of the public.
- Keeping in mind the highly sensitive nature of the rape cases as well as that of the DNA technique it will be of crucial importance that such a facility be centralised, should be easily and freely available to the public. By doing so it will be cost effective and one can ensure the quality assurance of the investigations.

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