Homozygosity Mapping of Candidate genes involved in inherited Non Syndromic Autosomal Recessive Mental Retardation (NSARMR) Disorder in Pakistani Kindred

Researchers

Inayat Ullah

3/FBAS/MSBT/S11

Supervisor

Dr. Asif Mir

Assistant Professor

Co-Supervisor

Dr. Imran Shabbir

Assistant Professor

Department of Biotechnology & Bioinformatics

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

(2011-2013)

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SUBMITTED BY

Inayat Ullah
3-FBAS/MSBT/S11

SUPERVISED BY

Dr. Asif Mir

Department of Biotechnology & Bioinformatics
Faculty of Basic and Applied Sciences
International Islamic University, Islamabad

(2011-2013)

Declaration

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 31/01/2013

Inayat Ullah

Department of Bioinformatics & Biotechnology
International Islamic University Islamabad

Dated: 31-1-2013

FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms. Inayatullah and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Bioinformatics

COMMITTEE

Supervisor

Dr. Asif Mir

Assistant Professor
Department of bioinformatics and Biotechnology
International Islamic University, Islamabad

External Examiner

Dr. Abdul Hameed

Principal Scientific Officer
IBGE, Islamabad.

Internal Examiner

Dr. Jabar Zaman Khan Khattak

Assistant Professor
Department of bioinformatics and Biotechnology
International Islamic University, Islamabad

Co-Supervisor

Dr. Imran Shabir

Assistant Professor
Department of bioinformatics and Biotechnology
International Islamic University, Islamabad

Chairman

Department of BI & BT

Dr. Jabar Zaman Khan Khattak

International Islamic University, Islamabad.

Dean

Faculty of Basic & Applied Sciences

Dr. Muhammad Sher

International Islamic University, Islamabad.

A thesis submitted to Department of Bioinformatics & Biotechnology

International Islamic University, Islamabad as a partial

fulfillment of requirement for the award of the degree of

MS Biotechnology.

Dedication

To My Family

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Inayat Ullah Mehsud

LIST OF ABBREVIATIONS

α	Alpha (12, regular)
β	Beta
µg	Microgram
µl	Microliter
µm	Micrometer
°C	Celsius/Centigrade
ADMR	Autosomal Dominant Mental Retardation
AMPA	Alpha-amino-3 hydroxy-5-methyl-4-isoxazole proprionic acid
ARMR	Autosomal Recessive Mental Retardation
ARNSMR	Autosomal Recessive Non Syndromic Mental Retardation
ATP	Adenosine triphosphate
CC2D1A	Coiled-coil and C2 domain containing 1A
cM	Centimorgan
CRBN	Cerebron
dd H2O	Double distilled H2O
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

EDTA	Ethylenediaminetetraacetic acid
Freud-1	Five' repressor element under dual repression bining protein-1
KARs	Kainate receptors
mA	Milliampere
$MgCl_2$	Magnesium chloride
mM	Millimolar
MR	Mental Retardation
mRNA	Messenger ribonucleic acid
ng	Nanogram
NMDA N-	methyl-D-aspartic acid
NS-ARMR	Non Syndromic-Autosomal Recessive Mental Retardation
NSMR	Non Syndromic Mental Retardation
OST	Oligosaccharyltransferase
PCR	Polymerase Chain Reaction
PRSS12	Protease serine 12
RNA	Ribonucleic acid
Taq	Thermus aquaticus
TBE	Tris/Borate/EDTA

TE	Tris EDTA
UV	Ultraviolet
XLMR	X-linked Mental Retardation

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ABSTRACT

Pakistan is one of the most suitable areas for the study of genetic disorders because of large family, stable community and consanguineous marriages. This study consists of two consanguineous families (A and B) to elucidate the genetic defects; linkage analysis via homozygosity mapping was performed in Family A and B.

In family 'A', the affected individuals shared the typical clinical findings reported in autosomal recessive non syndromic mental retardation families. To test the linkage via homozygosity mapping, the affected members and normal members were genotyped for the known loci for exclusion study. Linkage results clearly excluded the possibility of any of known reported loci. This suggested the involvement of new gene in family 'A'. After exclusion mapping, family members were genotyped using affymetrix 500K single-nucleotide polymorphism (SNP) microarrays. This approach allowed us to identify homozygous-by-descent (HBD) locus at 16q12.2-q21 and has not been reported previously. Whole exome sequencing is required to discover disease causing mutations in this family.

Family 'B' manifests the Autosomal Recessive Non-Syndromic Mental Retardation (ARNSMR) phenotype and genotyped with microsatellite markers for exclusion mapping. None of the microsatellite markers produced the required linkage results and thus exclusion of known ARNSMR loci is confirmed in family 'B'. This again is an indication of new genes involved in Autosomal Recessive Non-Syndromic Mental Retardation in family 'B'.

These exclusion findings in both families support that MR is heterogenous and that there are many more genes to be discovered which are involved in brain development and functioning. Microarray analysis of family A and B will helpful for the localization of causative gene for non-

syndromic MR. Identification of these causative genes are involved in many biological processes and will offer insight into the genetic basis of human cognitive function.

INTRODUCTION

1.1 GENETIC DISORDER

Alternation of a gene leads to a disease condition called genetic disorder. The genetically transmitted diseases are rapidly increasing in Pakistan which is regulated by the high ratio of consanguinity, unawareness of the people and lack of social and cultural trends. Mentally Retardation, Deafness, Bardet-Biedl syndrome, Muscular Dystrophy and Cone Dystrophy are highly prevalent genetic disorders in Pakistan. In Pakistan around 60% of the people are bound to consanguineous marriages, in which 80% of the marriages are between first cousins (Hussain *et al.*, 1998).

Individuals whose parents are closely relative are expected to have an increased proportion of their autosomal genome that is homozygous. In such cases the effect of this disorder will be so high (Smith, 1974). If the parents of the individuals are second cousin then their genomic homozygousity will be 1/64, if parents are first cousin then it will be 1/16, and when the parents are double first cousin then it will be 1/8 and when the parents are in incestuous mating then it will be 1/4(Lander and Botstein, 1987). The clinical effects occurrence of the autosomal recessive disorder in the consanguineous population is very high. That's why the presences of the genetic disorder are higher in the individuals whose parents are closely relative than non-consanguineous marriages (Jaber *et al.*, 1992; Ober *et al.*, 1999).

1.2 MENTAL RETARDATION

Mental retardation is a disorder causing cognitive impairment which is generally based on the intelligence quotient level of 70 or less and it can also cause deficiency in at least two adaptive functional behavior diagnosed at the age of 18 year (American Psychiatric Association 2000). American Association on mentally and Developmental Disabilities defined mental retardation as "The limitation on intellectual mechanics and adaptation of behavior" (AAIDD, 2002). American association on mental retardation also define MR as "affliction of neuro development process causing deficiency in at least two out of ten skill areas and mental disturbance of IQ level less than 70 are referred as limitations" (AAMR., 2002).

The adaptive functioning behaviors mean communication, Social skills, use of community resources, self-direction, leisure, health, safety, functional academic skills, home living, self-care and work. Its frequency is 1 to 3% throughout the world and it affects every social and culture class (Roeleveld *et al.*, 1997; Leonard an Wen 2002). Mental Retardation cases have high frequency particularly in the areas where the people living style is average or below average (Drews *et al.*, 1995; Roeleveld *et al.*, 1997; Durkin *et al.*, 1998; Durkin 2002; Emerson 2007).

1.3. CAUSES OF MENTAL RETARDATION

Genetic as well as environmental factors can cause mental retardation but more than 60% of cases having no particular reason (Rauch et al. 2006). MR can be caused by the environmental exposures such as teratogens, viruses and radiation. Severe head injury which is causing oxygen deficiency to the brain can also cause mental retardation. Although these factors make clear

some cases of NSMR, but it is also essential to think about its genetic reason (Chelly *et al.*, 2006).

Approximately 25-50% of the mental retardation cases are due to genetic causes. But this value may be boost up with severity (McLaren., and Bryson., 1987). Chromosomal abnormalities can also cause mental retardation with high ratio of prevalence, because several unusual kinds of aberrations have been reported (Rauch *et al.*, 2006). Autosomal trisomies can cause some degree of mental retardation which is commonly present in human with syndrome feature such as Down syndrome (Rauch *et al.*, 2006). Aneuploidies of the X-chromosome can cause sub-syndromic or syndromic forms of MR such as Ring X Turner syndrome. Further that the pathogenic copy number variation are also linked with mental retardation, while in the future this copy number variation will be having significant role in the invention of many new mental retardation genes (Ropers., and Hamel., 2005; Zahir., and Friedman., 2007).

Over the past 15 years many single genes causing NSMR have been identified. Majority of these genes may also cause Syndromic mental retardation or other neuro developmental and autism. There is a possibility that the other environmental as well genetics factors are also involved in the prevalence of this disorder. The genes causing NSMR emphasized on the significant of the particular genotype and phenotype association, which are often complicated to explain. There are also possibilities that NSMR genes in some cases are controlling by several factors, with possible involvement of environmental factors and multiple instances of genetic variation causing disease in a person but this has not been well deliberate. Most of the identified NSMR genes are present on the X-chromosome but the number of autosomal genes linked with NSMR is very few which is expectedly high (Kaufman *et al.*, 2010).

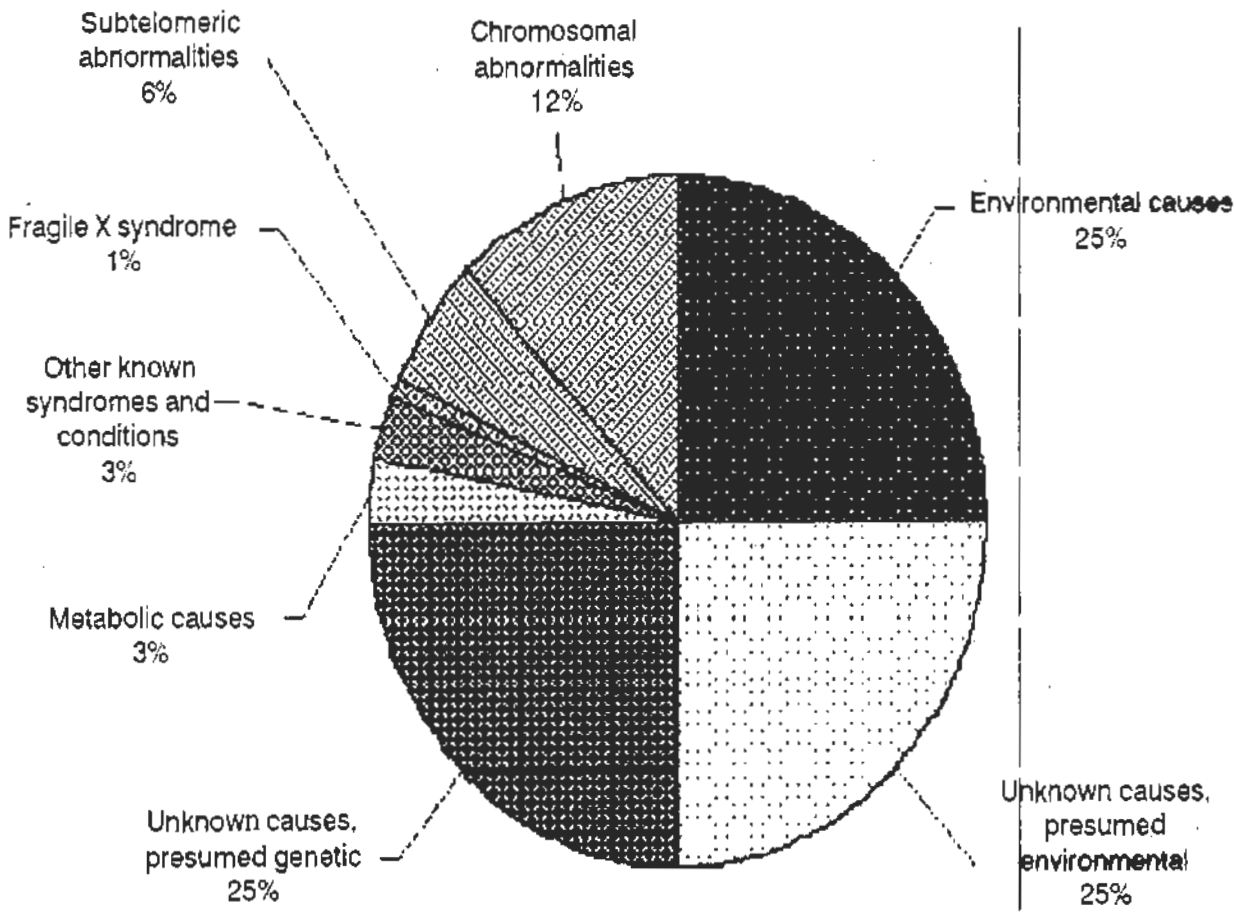


Fig. 1.1 Overview of causes of mental retardation (winnepennink *et al.*, 2003).

1.4. FORMS OF MENTAL RETARDATION

Mental retardation is classified into five categories on the bases of IQ level. These forms are mild, moderate, severe, profound and unable to classify (DSM IV; Table 1.1). More than that a person having IQ in between70-84 is consider as borderline form of MR but this is not officially documented in DSM-IV.

Epidemiological studies simply classify the mental retardation into mild and severe forms of mental retardation (Ropers and Hamel 2005). The frequency of mental retardation in both severe and mild forms are quite different because it is comparatively constant in severe form while its frequency in mild form is uneven and often depends on external environmental factors such as level of maternal education and access to healthcare and education (Leonard and Wen 2002; Drews *et al.*, 1995; Roeleveld *et al.*, 1997). Recent studies show that the catchment population for the studies and the age of the subjects can cause variability in the prevalence of mild form of mental retardation (Leonard., and Wen., 2002; Drews *et al.*, 1995; Roeleveld *et al.*, 1997).

Table 1.1 MR categorization by IQ and ability to function in society , Diagnostic and Statistical Manual of MR (DSM-IV-TR).

Severity	Proportion Formally Diagnosed MR	Functional Level	IQ
Borderline	Not formal diagnoses	Equivalent to the general or minimal support.	74-84
Mild	85%	Can often live independent with social support.	50-69
Moderate	10%	Acquire some communication and self-help skill, require moderate supervision.	35-49
Severe	3-4%	Acquire only basic self-help and communication skill, require supervision.	20-34
Profound	1-2%	Require highly structural and supervised living condition.	<20

1.5. CELLULAR PATHWAYS INVOLVED IN MENTAL RETARDATION

Genes linked with mental retardation are being identified increasingly in the last few years which shows that the phenotype can appear as the final common pathway of many different types for abnormal cellular processes. Intellectual processing in human is complexed due to these cellular processes. Monogenic forms of mental retardation are particularly taking into deliberation that what is presently acknowledged about mental retardation associated genes? The common thought about this disease is that it can stem from two broad mechanistic themes: alterations in synaptic organization and dysfunction of neurodevelopmental programs (Vaillend *et al.*, 2008; Kramer and van Bokhoven, 2009).

1.5.1. Neurogenesis

Neurogenesis and cell relocation happens in a strongly measured spatiotemporal way through growth in which neurons form complex axonal and dendritic networks. Equally the efficient cell-to-cell association and basic genetic uniqueness display the accuracy of this procedure. Throughout growth minor disturbances in some of these procedures can reason the intellectual disability in offspring. Throughout embryonic growth the major neurons originate up after two dissimilar descendant cells. After these two cells one provide ascend neuron that will transfer about to the cortex and the cell increase as a stem cell. To allowing the current study which proposes that throughout the main partial of development the majority of neurogenesis is current in the intellectual hemisphere. Neurogenesis also exists in the sub-ventricular region, olfactory bulb and hippocampus in grown person (Diaz and Gleeson, 2009).

Upsurge or reduction in the germinal epithelial propagation can central to syndromes in which the neuronal quantity does not in regulator called macro or microcephaly. Familial or *denovo* is heterogeneous collection of conditions that is related with regularity of reasoning injuries (Adachi *et al.*, 2011).

1.5.2. Neuronal migration

A main reason of the epileptic confiscations, growing aberration and intellectuality in infantile origin by a syndrome of neuronal relocation comprise a varied assembly of ailments of the nervous coordination (Verrotti *et al.*, 2010). The upright mitotic neurons are controlled in pilasters when neurogenesis proceeds and then transfer away from the ventricular region in a circular way to their last goal highbrow cortex (Diaz and Gleeson, 2009). Neuronal relocation monitored by the firm instruction of the design and judgment which is valuable for appropriate intellectuality and growth. Irregular relocation subtle tiesare important to allocate the neurons in dissimilar style in the cerebral cortex is the reason of numerous syndromes categorized by cortical dysgenesis such as lissencephaly. Cerebra lob struction and epilepsy are produced as of the mind superficial containing no *sulciorgyri.* Alterations in LIS1and DCX genes can produce typical sort 1 lissencephaly. LIS1gene is an autosomal which encrypts the LIS1 protein which is a microtubule related protein (Verrotti *et al.*, 2010).

1.5.3. Synaptic function

The electrical message inside neurons controlled by biochemical synapses which show part in the transmission of material from presynaptic axon terminals area to postsynaptic dendritic sections. Dendritic backs are the small dendritic projections which are the best excitatory synapses in the wits (Hotulainen and Hoogenraad 2010). Synaptic cleft distinct the presynaptic

and postsynaptic locations and grip them firmly at the correct aloofness by diversity of cell adhesion molecules (CAMs). Neuroligins and neurexins are the cell linkage fragments which show part in the growth of effective pre synaptic areas particular in precise discharge of the neuro transmitters to the synaptic cleft and vesicle combination to the plasma membrane (Price *et al.*, 2006).

Receptors are presynaptic neurexins and neuroligins are neurexins ligands that are present in the under development side. Mental postsynaptic and autism spectrum disorder (ASD) are caused due to mutations in NLGN3/4 gene (Vaillend et al, 2008). Dynamic areas made by NLGN4 presynaptic terminals in the genes through contacts with β-receptor presynaptic neurexin. Protein interaction, as APBA2 or CNTNAP2 belongs to the species of neurexin involved in schizophrenia and autism spectrum disorders. The weakness of synaptic may be a common cause between these disorders (Ropers, 2008). It is now known that Ephrinsis a wonderful molecule is involved in axon, exploratory play a role in stabilizing the synapse (Shen., and Cowan., 2010).

1.5.4. Transcription regulation

Gene expression and protein synthesis occurs through the regulatory process, which is determined by the dynamics of connection and gene, copying and organizing connectivity and export of RNA degradation. Cause fluctuations in gene expression as a result of the changes in this mechanism. But if this abnormal gene expression is in the critical stages of development and will prime to faulty brain development. Neurons are highly specialized cells in which few specific aspects of RNAs metabolism perform critical role especially for their functions, development process, trafficking of mRNAs to growth cones(dendritic and axonal) regulates the neuronal growth (Hengst and Jafari, 2007). After the formation of synapses the mRNAs continue

to be transported to the hub and nerve ramifications. These processes play a significant role in synaptic plasticity and also serve as memory link diversity, fluctuations could play a role in cognition (Martin and Zukin, 2006).

1.6. CLASSIFIACTION OF MENTAL RETARDATION

Mental Retardation is generally classified into two main classes

1.6.1. Syndromic Mental Retardation

In this form of mental retardation not only intellectual disability is present but is associated with physical disability such as imaging findings, organ anomalies, biochemical parameters dysmorphisms (Jamra *et al.*, 2011). Flaws in a particular gene or chromosomal aberrations can cause mental retardation syndrome. About 15% of all cases of syndromic MR are due to the Down syndrome and other cytogenetically visible chromosomal abnormalities (Leonard Wen, 2002). According to the recent studies which suggest that another 15% may be due to submicroscopic deletions and duplications spanning 100,000 to several million DNA base pairs (de Vries *et al.*,2005; KirchhoV *et al.*, 2005; Shaw-Smith *et al.*, 2004; R. Ullmann; M. KirchhoV *et al.*, unpublished observations).

1.6.2. Non Syndromic Mental Retardation

In non-syndromic mental retardation only mental disability occurs while the individual do not have major physical abnormalities, dysmorphism or neurological abnormalities (L Basel-Vanagaite *et al.*, 2003). While other symptoms are not defined as behavioral disorders, ataxia, microcephaly and light epilepsy still exist in the form of non-syndromic. Mutations in a single gene, it may disrupt its function and may cause mental retardation or a variety of the phenotypes

PRSS12 gene was first exposed in the Algerian family that is associated ARNMR. *PRSS12* encode neurotrypsin which is any enzyme essential in synaptic reorganization throughout the advanced phases of neurodevelopment and adult synaptic plasticity. Expression of neurotrypsin is very high in the hippocampus, amygdala and cerebral cortex. Neurotrysin neurons are found in the presynaptic membrane and presynaptic active zone, with in the neural cell (Molinari *et al.*, 2002).

B. *TUSC3* (MIM 601385)

TUSC3 gene encodes a protein that plays an important role in the transfer14-sugar oligosaccharide from dolichol to a nascent protein which is necessary step in the N-linked glycosylation of protein (Molinari et al, 2008; Garshasbi *et al.*, 2008). Mutations of this gene have recessive inheritance model appears broken exonic in affected individuals from two families, and two members of the family affected and seven family members affected in the other (Garshasbi *et al.*, 2008; Molinari *et al.*, 2008). A recent study showed that it play an important role in MG^{+2} regulation and biallelic knockdown of this gene causes decreased total and free intracellular MG^{+2} in human cell lines as well abnormmal development in zebrafish embryo (Zhou and Clapham 2009).

C. *CRBN* (MIM 609262)

CRBN genes encodes Lon protease depends on ATP which directly involved in the expression and assembly of surface disposal of large amount of Ca^{+2} that activates K^+ channels which have an important role in the control of the transmitter and release nerve cell excitability (Higgins *et al*, 2004; Jo *et al* 2005). Significant increase disposal Ca^{2+}activated K^+ channels upset due to

mutations in the gene *CRBN* which make it slower and more rapid activation kinetics disruption due to increased sensitivity of Ca^{2+} between cells and cause of this boom. This causes mild mental retardation by homozygous nonsense mutations in this gene (Higgins *et al*, 2008).

D.*CC2D1A* (MIM: 610055)

A transcriptional repressor protein is Coded from this gene, which is regulated by calcium, it is considered putative candidate to regulate NF-κ B pathway (Basel-Vanagaite *et al*., 2006; Matsuda *et al*., 2003). *CC2DIA* gene is known for regulation of transcriptional repression of *HTR1A* gene, the serotonin 1 A receptor, and the *DRD2* gene dopamine receptor , it is also called as five prime repressor under dual repression binding protein-1 (Frued-1), (Ou *et al*., 2003; Rogaeva *et al*., 2007).

E. *TRAPPC9* (MIM: 611966)

TRAPPC9 are complex encoding protein particles stands for traffickining protein particle complex-9 also called NIBP. TRAPC9 genes contain amino acids 1148-1246 encoded by 23 exon gene. Due to a breakdown path NF-κ B to post CC2D1A that are relevant to the most important causes to give MR TRAPC9 genes because of their involvement in the same path. The deletion of four base pairs in exon 14 due to R475X truncating mutation in exon sevent of TRAPC9 was identified at q 24 in Iranian and Pakistani families, truncating the protein and segregate with phenotype. Mode of inheritance for this mutation is autosomal recessive, mean parents of affected individuals are heterozygous and homozygous members of family express phenotype (Mir *et al*., 2009).

F. *TECR* (MIM:610057)

Trans-2,3-enoyl-CoA reductase (TECR), also known as synaptic glycoprotein 2 (GPSN2), a type of synaptic glycoprotein which is known to be involved in synthesis of long chain fatty acids (VLCFA) in reducing elongation step procedure of microsomal fatty acyl (Moon et al, 2003). *TECR* genemouse orthologueis extremely articulated in the anxiousorganization (Zhang *et al.*,2004).Diseases involving defects in the installation of ordinary VLCFA degradation have neurological consequences. For example, a mutation in FACL4, a long-chain acyl-CoA synthetase, which is vital in the degradation of VLCFA and manufacture of intermediates main connection of fat complex, causes X-linked NSMR (Meloni *et al.*, 2002; Piccini *et al.*, 1998; Wong *et al.*, 2003). The role of genes in crete also play a role in fatty acyl elongation and high expression in the nervous system causing mutation Pro-182-Leu that can disrupt routes and result in similar NSMR. Besides this, it is also possible to Crete code as synaptic diabetes, is not yet known, could have a particular purpose in the nervous system that affects the statement between nerve cells or synaptic plasticity (Smalla *et al*, 2000; Kleene *et al*, 2004; Martin *et al.*, 2002).

G. *GRIK2* (MIM: 138244)

GRIK2 cause different forms, such as mild to severe NSARMR that six people in the family according reported (Motazacker *et al.*, 2007). It encodes a protein that is a receptor subunit called kinates GLuR6. Success is expressed in the hippocampus in the mossy fibers of the brain where it was found the protein GLuR6 to convert long-term potentiation (LTP) in mice models (Bortolotto *et al.*, 1999; Contractor *et al.*, 2001). Decreased LTP in fiber Mossy shown mice knockout GLuR6 a phenotype can be delivered through the use of small levels of K^+, which signifies KARs triggered by depolarization of station LTP presynaptic (Contractor *et al.*, 2001;

Schmitz *et al.*, 2003). In the hippocampus part of the brain LTP is used the development of learning and memory (Contractor *et al.*, 2001; Schmitz *et al.*, 2003).

Although there are only seven genes that have been identified with that segregated with NSARMR as well as the mapping by identifying at positions of HBD 30 to be essential in the discovery of new genes in the future (Najmabadi *et al.*, 2007; Garshasbi *et al.*, 2009 Uyguner *et al.*, 2007; Rafiq *et al.*, 2010; Abou Jamra *et al.*, 2010; Kuss *et al.*, 2010). In recent meetings it is recommended three additional genes as NSARMR genes, but so far has not been published. Two of these were known to be involved in missense mutations, which would represent the first event of missense somatic mutations causing NSMR (Najmabadi *et al.*, 2009; Moheb *et al.*, 2009). *ZNF526* gene is one of these genes, while C2H2 finger protein that is prearranged by zinc *ZNF526* show a role in the brain (Moheb *et al.*, 2009).

ST3GAL3 is additional gene in which a missense alteration occurs. It translates a glycosyltranferase which purpose in the alteration of the sialic acid to galactose comprising substrate (Najmabadi *et al.*, 2009; Grahn *et al.*, 2002). Homozygosity at the similar location 1p34 on chromosome has been recognized in two extra dissimilar relatives (Najmabadi *et al.*, 2009).

ZC3H14 gene lastly described in two diverse relations in which border move and nonsense transformation takes place. It is a newly defined as CCCH-type zinc finger gene which co-localizes with the joining aspect SC35 and a poly adenosine RNA obligatory field. It also recommended that it show conceivable purpose in mRNA dispensation, though more learning is essential to withstand this prerogative (Garshasbi *et al.*, 2009; Leung *et al.*, 2009).

MATERIALS AND METHODS

2.1 FAMILY VISIT

In this study we represented two families A and B with clinical phenotype of autosomal receesive mental retardation. Family A belongs to Khairpur district of Sindh province and the other family B belongs to Jhelum district of Punjab province.

We visited the families at their local town where we met with the elders of the family and got the information about the disease and then draw the pedigrees. The information we got about the disease from these families including numbers of the affected individual, family history, numbers of generation involved etc.

2.2 PEDIGREE ANALYSIS

The standard method for drawing the genetics pedigrees were used as previously used by Bennet *et al.*,95 (Bennet *et al.*,95).The pedigrees of both the families were drawn according the above stated method. Male are represented by square and females are represented by circle. The filled circles and squares representing the affected individuals while the unfilled circles and square showing the normal individuals. The dead individuals are representing by a slanting line on the circle or square. Each generation is symbolizing by the Roman numerals while generation with in a generation is symbolize by the Arabic numerals. The consanguineous marriages are represented by the double lines. From the pedigree of the families we can study the mode of the inheritance of the disorder. The pedigree was designed according to the information collected from the elders of the family.

2.3 BLOOD SAMPLING

We collected the blood samples from both the families in clean 5ml sterilized syringes while butterflies were used for children less than 2 year age then injected in EDTA vacutainer which is a standard potassium tubes (BD, USA). Then we stored the collected blood samples at 4°C before using for DNA extraction.

2.4 HUMAN GENOMIC DNA EXTRACTION

Two methods for the genomic DNA extraction from the blood are commonly used, Phenol extraction method commonly called organic method and the invitrogen Kit Method.

2.4.1 DNA Extraction by commercially Available Kit

DNA extraction was also carried out by using Genomic Isolation Kit (Invitrogen).

I. In 1.5 ml micro-centrifuge tube, 300µl of blood was taken along with 900 µl of RBC lysis solution.

II. Incubated the mixture at room temperature for one minute and then at 13000 rpm centrifuged for 20 seconds. 300 µl of cell lysis solution was added after centrifugation and vigorously vortexed for 3 minutes, then quickly placed on ice for 1 minute.

III. 100 µl of protein precipitation solution was added to the cell lysate after cooling and centrifuged for 1 minute at 13,000 rpm.

IV. In a clean tube the supernatant having DNA was isolated. Then we added 300 µl of 100% isopropanol and then mixed the samples smoothly by inverting the tubes, centrifuged for 1 minute at 13000 rpm to precipitate the DNA.

V. To remove Isopropanol 70 % ethanol was used to wash the precipitated DNA.

VI. DNA was dissolved in 50 µl of DNA hydration solution after evaporation of residual ethanol, and then incubated it for 5 minutes at 65°C .

2.4.2 Organic Preparation For DNA Extraction

Genomic DNA extraction from blood samples was also performed by using standard phenol-chloroform procedure.

I. In a 1.5 ml micro-centrifuge tube 0.75 ml of blood was taken and then mixed with equal volume of solution A and was kept at room temperature for 5-10 minutes. The tubes were centrifuged in a micro-centrifuge for 1 minute at 13,000 rpm.

II. The supernatant was discarded and the pellet was again re-suspended in 400 µl of solution A, and was centrifuged again for 1 minute at 13,000 rpm.

III. Again the supernatant was discarded and the nuclear pellet was re-suspended in 400 µl of solution B and incubated at 37°C overnight or at 65°C for 3 hours in incubator by adding 25 µl of proteinase K and 12 µl of 20% SDS solution.

IV. On the next day or after 3 hours respectively, then in samples fresh mixture with equal volume of solution C and solution D of 0.5 ml was added, mixed and centrifuged at 13,000 rpm for 10 minutes.

V. The upper layer commonly called aqueous phase was replaced into a new microcentrifuge tube and equal volume of solution D was added, Centrifuged for 10 minutes at13, 000 rpm.

VI. The clear aqueous phase was transferred into a new tube. DNA was precipitated by adding 55 µl of 3M sodium acetate (pH 6) and equal volume of chilled isopropanol. Then precipitated DNA was centrifuged so that the DNA get settled in the pellet.

VII. 70% ethanol was used to wash the DNA pellet and then dried in the incubator for 8-10 minutes or by leaving the eppendorf tubes in inverted position on tissue paper for 15 minutes.

VIII. DNA was dissolved in proper amount (150-200 µl) of Tris-EDTA buffer (TE), after evaporation of remnants ethanol, and then stored in refrigerator.

Once DNA extracted then 1% agarose gel was used to picture the integrity of obtained DNA samples, and then diluted for PCR amplification to 40-50 ng/µl.

Table 2.1 Composition of Solutions

Solution A	**Solution B**	**Solution C**
5 mM $MgCl_2$ 0.32 M Sucrose 10 mM Tris pH 7.5 1% Triton X-100	2 mM EDTA pH 8.0 10 mM Tris pH 7.5 400 mM NaCl	Phenol
Solution D	**TE Buffer**	**10XTBE**
24 ml Chloroform 1 ml Isoamyl-alcohol	0.1 mM EDTA 10 mM Tris pH 8.0	0.025 M Borate 0.89 M Tris EDTA pH 8.3

2.5 AGAROSE GEL ELECTROPHORESIS

DNA and amplified PCR products were analyzed on 1- 2% agarose gel prepared by melting 0.3-0.6g of agarose in 30 ml 1 X TBE in a microwave oven for one minutes. 0.5 µl of ethidium bromide (0.5 µg/ml final concentrations) visualize DNA. DNA or PCR product were mixed with the bromophenol blue dye (0.25% bromophenol blue with 40% sucrose) and then loaded in the wells. For half an hour electrophoresis was performed at 120 volts in 1 X TBE buffer. Then the amplified products were seen by placing the gel on UV transilluminator (BioRad, Italy).

2.6. GENOTYPING

Genotyping was done by using standard methods as follows

2.6.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed in 0.2 ml tubes (Axygen, USA) containing 20 µl total reaction mixture in (Table 2.3 for PCR mixture).

The reaction mixture was centrifuged for few seconds for careful mixing. The reaction mixture was taken through thermocycling conditions which are mentioned in the table 2.3. The PCR was performed by the use of thermal cyclers provided by Perkin Elmer (veriti system, USA).

Table 2.2 PCR Reaction Mixture for 1X.

S#	Composition	Reagents
1.	2 µl	DNA
2.	2 µl	$MgCl_2$
3.	2 µl	PCR Buffer 10X
4.	0.2 µl	dNTP
5a.	1.5 µl	Primer (f)
5b.	1.5 µl	Primer (r)
6.	0.2 µl	Taq
7.	10.6 µl	dH_2O

Table 2.3 PCR Reaction program

Steps	Cycle	Time	Temperature
Initial denaturation	1 x	05 minutes	95°C
Denaturation	35 x	30 seconds	94°C
Annealing		45 seconds	50-60°C
Extension		30 seconds	72°C
Final Extension	1 x	07 minutes	72°C

2.6.2. Vertical Gel Electrophoresis

The amplified PCR products were resolved on 8% non-denaturing polyacrylamide gel (For gel composition see table 2.4) for allele separation. Conical flask of 250 ml was used for gel solution. When the solution prepared then it was poured in the space between the two glass plates alienated at a distance of 1.5 mm. The open side of these two plates was used for comb insertion, after that it was allowed to polymerize for 45-60 minutes at room temperature. Before loading the PCR products into the wells these were mixed with loading dye of 5 µl (0.25% bromophenol blue with 40% sucrose) and then loaded into the wells. A vertical gel tank model V16-2 (Life Technologies, USA) was used for electrophoresis at 130 volts (60 mA) for 90-100 minutes depending upon the size of amplified length. Ethidium bromide solution (10 mg/ml) was used to stain the gel and visualized on Gel Doc system (BioRad, Italy).

Table 2.4 PCR Reaction program

Composition	Chemical
13.5 ml	30% Acryl amide solution
20 µl	TEMED
05ml	10 XTBE
350 µl	Ammonium per sulphate (25%)
31.5 ml	dH_2O

2.6.3 Primer Database Analysis and Genotyping

The study of microsatelliite markers was realized by PCR, as 8% standard non-denaturing polyacrylamide gel was used to determine the PCR products as mention above. Microsatellite markers were seen by placing the ethidium bromide stained gel on UV transilluminator and genotypes were allowed by visual assessment of the gels. Cooperative Human Linkage Centre mapped the microsatellite markers, were got from Research Genetics, Inc. (USA). Heterozygosity and repeat polymorphism played important role in the selection of the marker. Their hetrozygosity as well as the length of the PCR products and the cytogenetic locations of these markers were obtained from genome database homepage (*www.gdb.org*) and Rutger's map (Kong *et al.*, 2004)

Genotyping tested the linkage to each candidate loci three or four markers per locus. The autosomal recessive nonsyndromic Mental Retardation loci considered in the current study are shown in table 2.5 on page 5.

2.7 LINKAGE STUDIES

The unbalanced autosomal or X-chromosome anomalies were excluded by the initial cytogenetic analysis in all the affected members of each family. Karyotyping was used to expellee the Chromosomal abnormalities.

2.7.1 Exclusion mapping of known MR loci

To arrange the linkage approach for the present study, the genetic databases were used to screen and to select which genes are already identified to link with related diseases in Pakistani population. Both the families A and B were initially screened for linkage to known loci which is

totally based on the results of these searches. Microsatellite markers were summarized (Table2.5) to be found in the region of these known MR loci. These were used at first pass analysis for genetic linkage in families with autosomal recessive nonsyndromic mental retardation. Genotyping of these markers was performed as described above.

2.7.2 Linkage and Haplotype Analysis

After genotyping with microsattelite markers summarized in table 2.5 of the families, software (Synergy, USA) were used for alleles to score for each of them. Then marker files were created for each microsattelite marker in easy LINKAGE plus Version 5.0 format (Linder and Hoffmann, 2005) pedigree files were created in linkage format and the data was checked for genotyping errors and Mendelian inconsistencies using the PEDCHECK software (Connel *et al.*, 1998) incorporated in easy LINKAGEPLUS Version 5.0.

No.	Locus	Markers	Cytogenetic Location (cM)*
1.	MRT-I	D4S3024	124.45 (cM)*
		D4S191	121.08 (cM)*
2.	MRT-2	D3S3050	10.31 (cM)*
		D3S1620	10.52 (cM)*
		D3S3630	6.08 (cM)*
3.	MRT-3	D19S558	33.33 (cM)*
		D19S564	37.94 (cM)*
		D19S840	37.94 (cM)*
		D19S892	42.28 (cM)*
		D19S226	36.35 (cM)*
		D19D385	42.28 (cM)*
4.	MRT-4	D1S255	58.66 (cM)*
		D1S2892	70.41 (cM)*
		D1S2645	73.21 (cM)*
		D1S447	73.81 (cM)*
		D1S451	75.66 (cM)*
5.	MRT-5	D5S464	14.3 (cM)*
		D5S635	14.91 (cM)*
		D5S676	16.72 (cM)*
		D5S2064	19.67 (cM)*
		D5S432	22.88 (cM)*
6.	MRT-6	D6S1682	109.19 (cM)*
		D6S249	104.71 (cM)*
		D6S1717	107.25 (cM)*
		D6S2418	

TH-1652

		D6S1679	111.17 (cM)*
		D6S1563	13.61 (cM)*
7.	MTRT-7	D8S1021	60.87 (cM)*
		D8S1810	60.34 (cM)*
		D8S499	60.34 (cM)*
		D8S259	60.87 (cM)*
		D8S283	
		D8S379	
8.	MRT-8	D10S560	90.01 (cM)*
		D10S1650	92.81 (cM)*
		D10S188	93.92 (cM)*
		D10S1699	97.29 (cM)*
		D10S607	99.52 (cM)*
9.	MRT-9	D14S1431	
		D14S80	26.59 (cM)*
		D14S608	28.01 (cM)*
		D14S975	31.13 (cM)*
		D14S54	31.75 (cM)*
		D14S1034	31.75 (cM)*
		D14S121	34.43 (cM)*
10.	MRT-10	D16S3056	35.44 (cM)*
		D16S3045	40.65 (cM)*
		D16S417	43.89 (cM)*
		D16S690	57.79 (cM)*
		D16S685	57.79 (cM)*
		D16S3044	58.46 (cM)*

		D16S3080	59.86 (cM)*
		D16S3035	62.11 (cM)*
11.	MRT-11	D19S423	65.77 (cM)*
		D19S211	66.3 (cM)*
		D19S538	
		D19S574	69.5 (cM)*
		D19S908	69.5 (cM)*
		D19S219	70.14 (cM)*
		D19S562	70.17 (cM)*
12.	MTR-12	D1S429	13.88 (cM)*
		D1S2688	139.02 (cM)*
		D1S3723	140.39 (cM)*
		D1S2778	141.48 (cM)*
		D1S2651	142.24 (cM)*
		D1S221	142.24 (cM)*
		D1S2726	144.38 (cM)*
		D1S2789	145.86 (cM)*
13.	MRT-13	D8S256	148.12 (cM)*
		D8S1837	156.59 (cM)*
		D8S1743	162.94 (cM)*
		D8S1704	164.25 (cM)*

Table 2.5: List of Microsatellite Markers Used For Linkage of Known Loci During Study. *Average sex distance is cM according to Rutgers combined linkage-Physical human genome Map (Kong *et al.*, 2004).

RESULTS

3.1 Analysis of Pedigree:

In the present study two families (A & B) have been studied from local population, where due to socio-ethnic reason the consanguineous marriages are common and thus are suitable for locating the defective genes by genetic linkage method. Affected family members were evaluated with the help of standard questionnaire (amended version of Wechsler Intelligence Scale) for severity of disease and IQ assessments. Head circumference and height of affected individual were measured in inches. Clinical evaluation was performed by the doctor of local hospital. Photographs of affected and normal individuals were taken. Furthermore; parents were interviewed about the prenatal and neonatal medical history of the proband and mother during pregnancy.

3.2 Family A

Family A belongs to Khairpur City. The normal family members are engaged in regular farming and cattle herding. Inter-marriages are a common practice in the area. After discussing with the elders of the family, the pedigree was drawn (Figure 3.1); it indicates four generations with three affected males (IV:8, IV:9, IV:10) and three female (IV:3, IV:4, IV:5) individuals. The pedigree analysis shows that affected individuals were produced by the unaffected parents in all two loops and the affected status was independent of the sex suggesting that the trait is transmitted in autosomal recessive manner. The consanguineous parents III:1 and III:2 , III:6 and III:7 are normal phenotypically, but resulted in six affected individuals. Mental retardation is congenital in all the affected individuals. The individuals have no other associated abnormality. Blood

samples were collected from ten family members including five affected (IV: 3, IV:4,IV:5,IV:8, IV:9, IV:10) and five normal (III:1, III:2, III:6, III:7,IV:6) and DNA was isolated by organic preparation using 1.5 ml microcentrifuge tubes. One of the affected female (IV:5) has died as she was heart patient.

3.2.1 Linkage Analysis in family A

Linkage studies were carried out in both families. Linkage in families were searched using microsattelite markers (Table 2.5) corresponding to candidate genes involved in elated autosomal recessive non syndromic mental retardation, it is clear that at least some candidate interval should be tested for linkage or exclusion prior to embarking on genome wide search . Table 2.5 summarizes the microsattelite markers, which were used in present study for candidate gene analysis. Reported average hetrozygosity for the selected markers is > 70%. Analysis of microsattelite, markers was carried out using a standard PCR reaction and electrophoresis in 8% non-denaturing polyacrylamide gel as discussed in materials and methods, amplified microsattelite markers were visualized by staining the gel with ethedium bromide and the genotypes were analyzed and assigned by GEL doc (Dolphin gel Doc system) and by visual inspection.

In family A ten DNA samples (III:1, III:2, III:6, III:7, IV: 3, IV:4, IV:5, IV:6, IV:8, IV:9, IV:10) including normal and affected individuals were selected for genotyping the markers linked to the candidate genes. Three to four markers per locus were used to test the linkage. From the analysis of the results obtained with polymorphic microsatellite markers specific for known MR loci (figure 3.2-3.48), it was evident that all the affected individuals were heterozygous for the different combinations of the parental alleles, thus excluding the linkage in family A to the

known MR loci. Further analysis with Genome-wide-search by homozygosity mapping was decided for genetic linkage to novel loci. The Commercially available microarray analysis was done on the four affected and one normal individual to identify the Homozygous region between affected persons. The snapshot of microarray analysis with dChip software is given in figure 3.49. Family members were genotyped using Affymetrix 500K single-nucleotide polymorphism (SNP) microarrays. This approach allowed us to identify homozygous-by-descent (HBD) locus at 16q12.2-q21 and has not been reported previously.

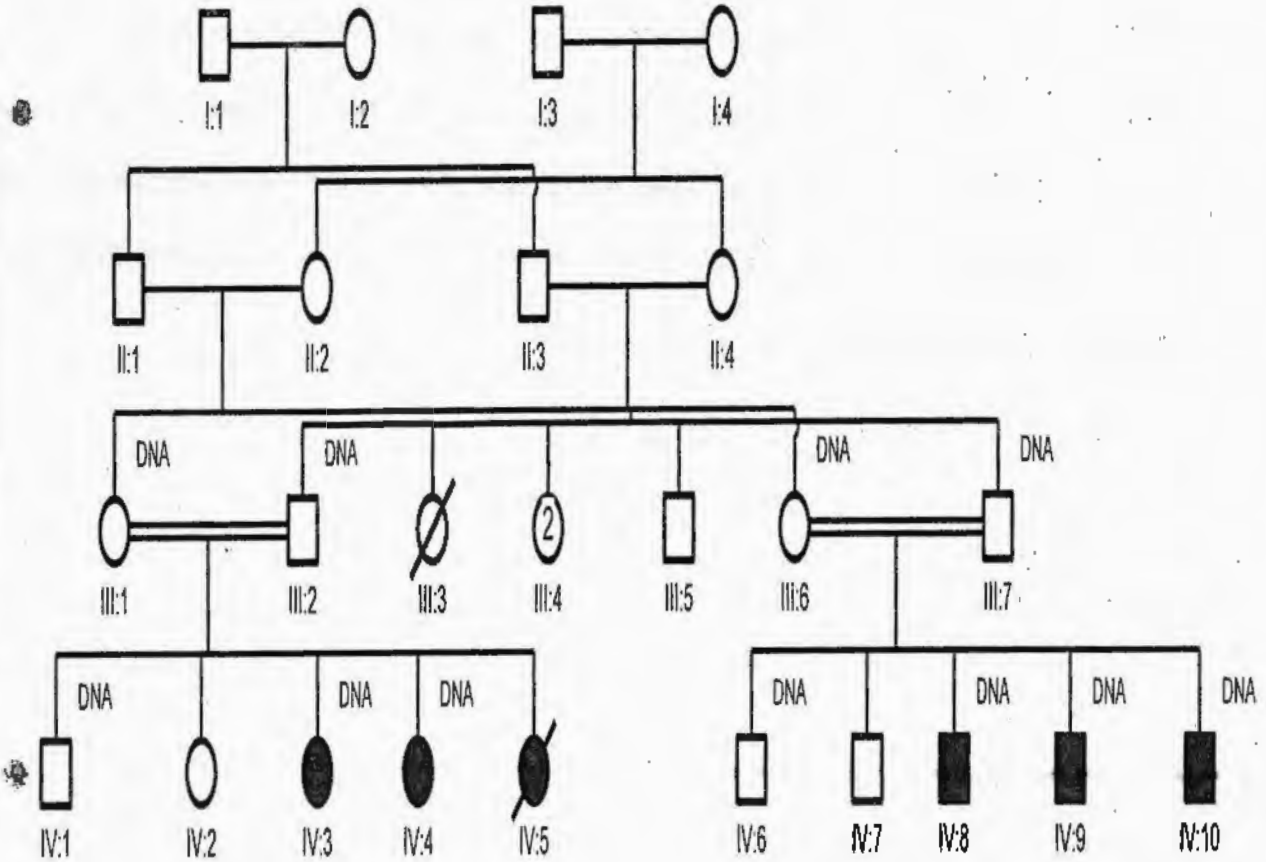
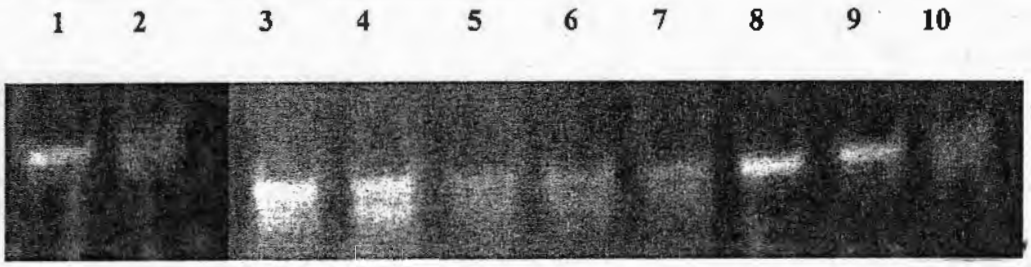


Figure 3.1: Pedigree of family A with Autosomal Recessive non syndromic Mental Retardation in which square represents males while circles represent females. Affected individuals have been represented by filled circles and squares. Double line represents inter family marriages.



Family A

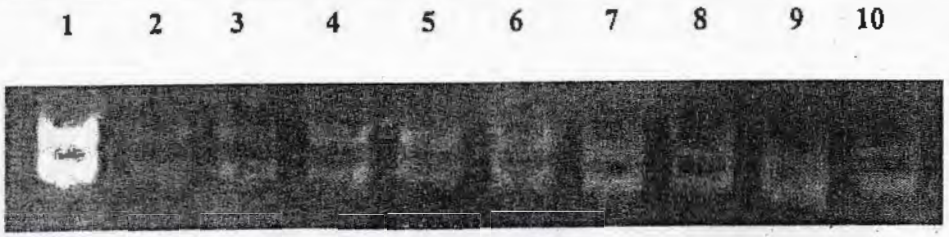
1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

Figure 3.2: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D4S191 at 121.08 cM, on chromosome 4. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

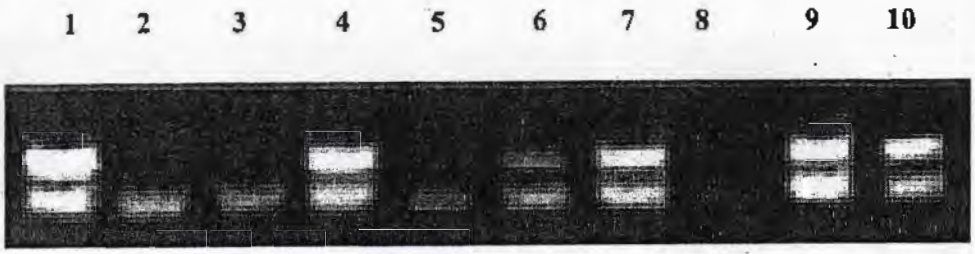
Figure 3.3: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D4S3024 at 124.45 cM, on chromosome 4. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

Figure 3.4: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D3S3630 at 6.08 cM, on chromosome 3. The Roman with Arabic numerals refers to the individuals in pedigree



Family A

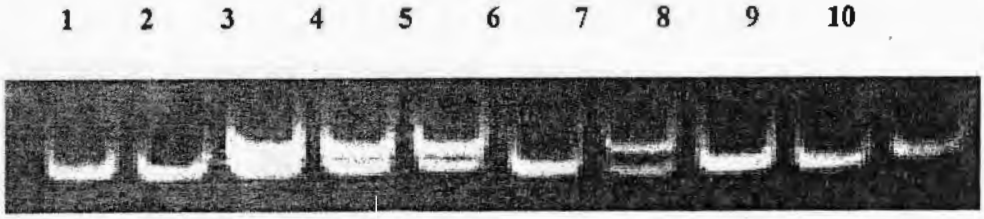
1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

Figure 3.5: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D3S3050 at 10.31 cM, on chromosome 3. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

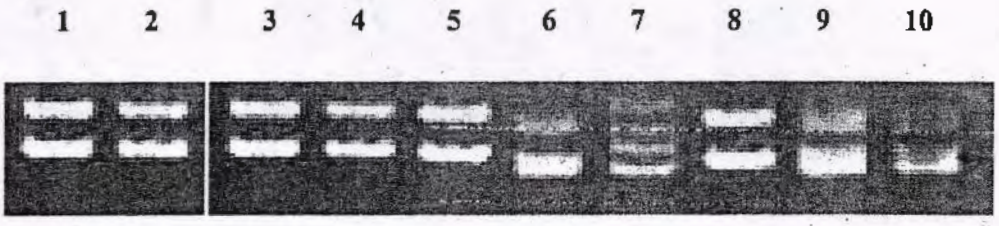
Figure 3.6: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D3S1620 at 10.52 cM, on chromosome 3. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

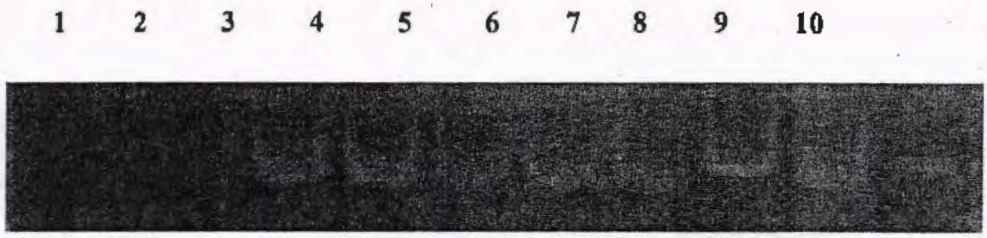
Figure 3.7: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S558 at 33.33 cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

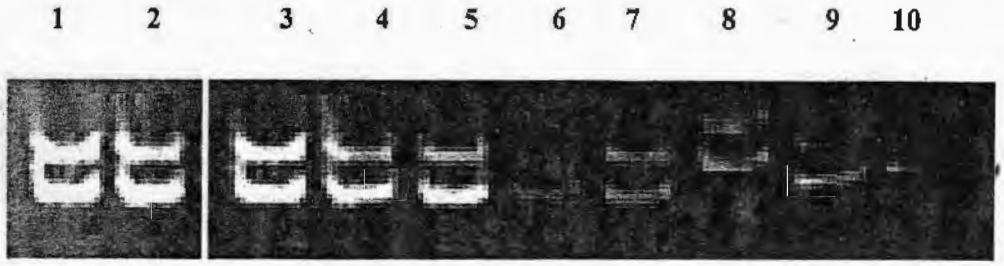
Figure 3.8: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S226at 33.35cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.9: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S840 at 37.94 cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.10: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S385 at 42.28 cM, on chromosome 19.The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

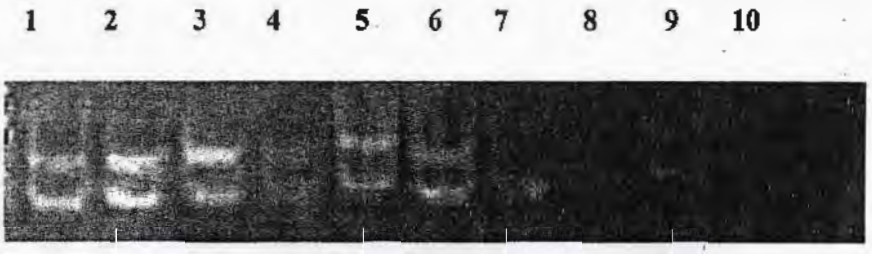
1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.11: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S255 at 58.66 cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

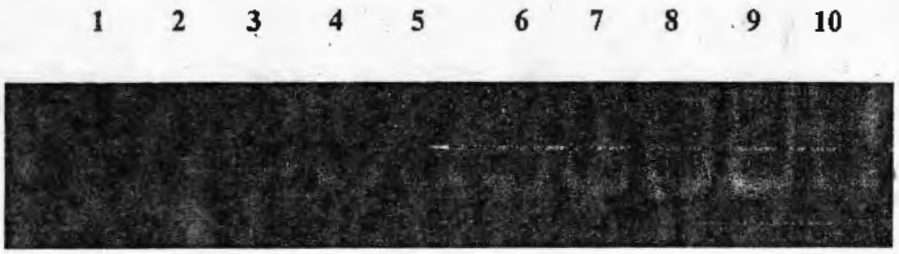
Figure 3.12: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S2645 at 73.21 cM, on chromosome 1. The Roman with Arabic numerals refers to th individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

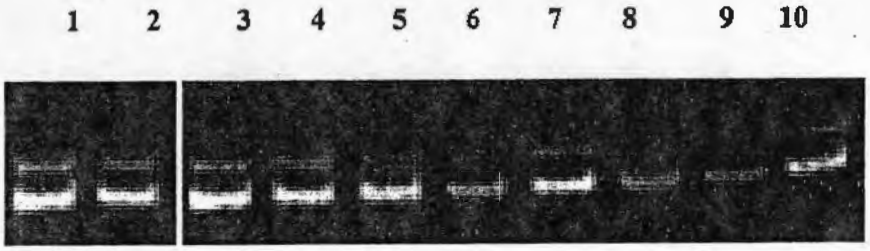
Figure 3.13: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S447 at 73.81 cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

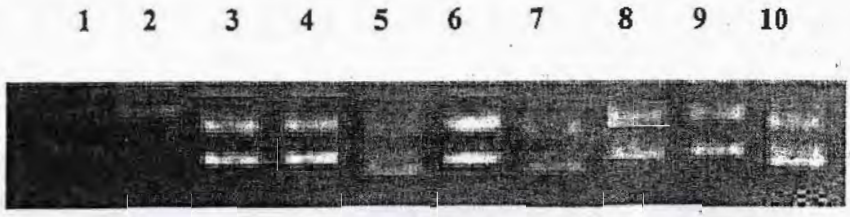
Figure 3.14: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S451 at 75.66 cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

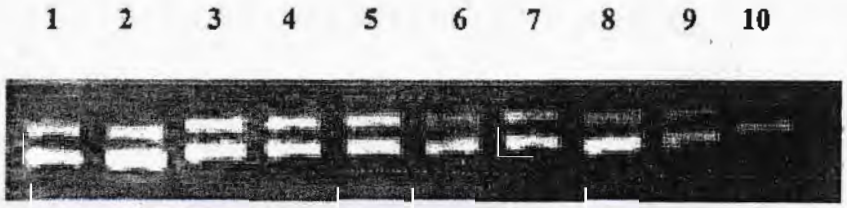
Figure 3.15: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D5S676 at 16.72 cM, on chromosome 5. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

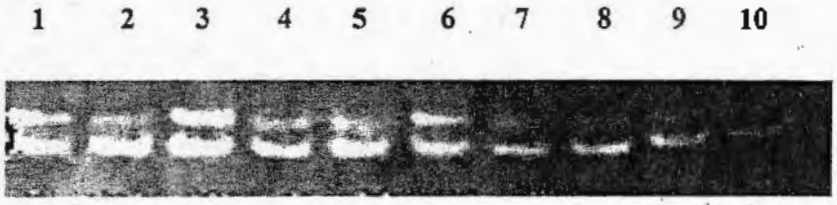
Figure 3.16: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D5S2064 at 19.67 cM, on chromosome 5. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

Figure 3.17: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D5S432 at 22.88 cM, on chromosome 5. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

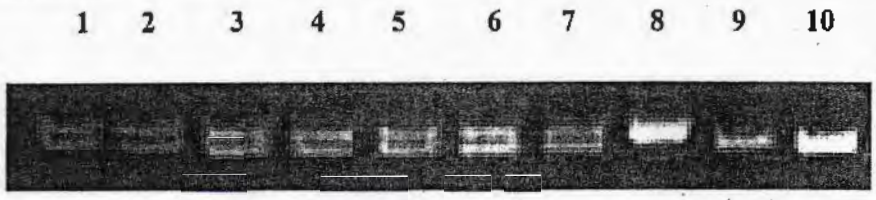
1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

Figure 3.18: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D6S1717 at 107.25 cM, on chromosome 6. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

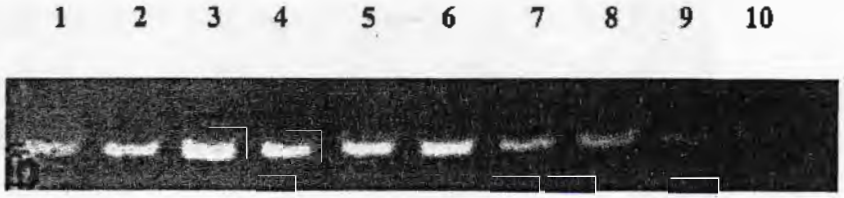
Figure 3.19: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D6S1682 at 109.19 cM, on chromosome 6. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

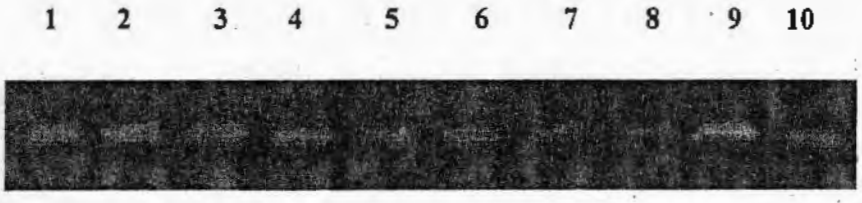
Figure 3.20: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D6S2418 at ____cM, on chromosome 6. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

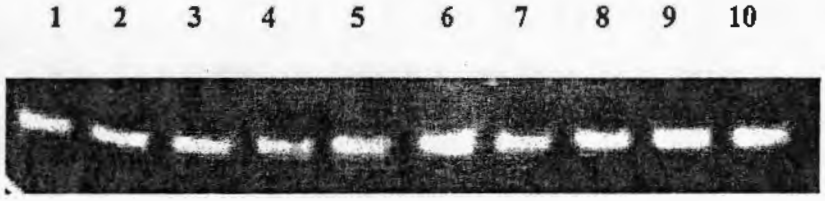
Figure 3.22: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D6S1679 at 111.17 cM, on chromosome 6. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

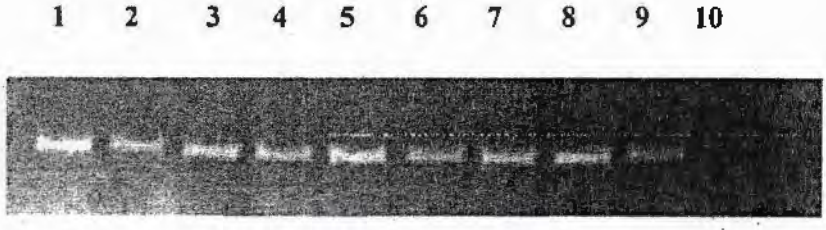
Figure 3.22: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D6S1563 at 13.61 cM, on chromosome 6. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

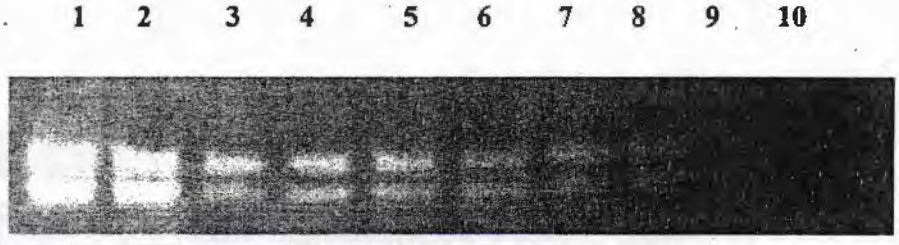
Figure 3.23: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D8S259 at 60.87 cM, on chromosome 8. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

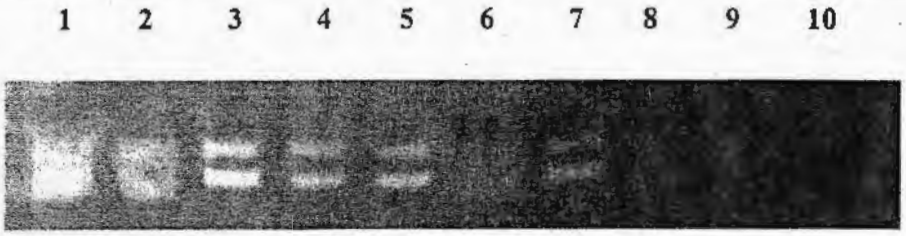
Figure 3.24: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel sho allele pattern obtained with marker D8S499 at 60.34 cM, on chromosome 8. The Roman with Arabic numerals ref the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

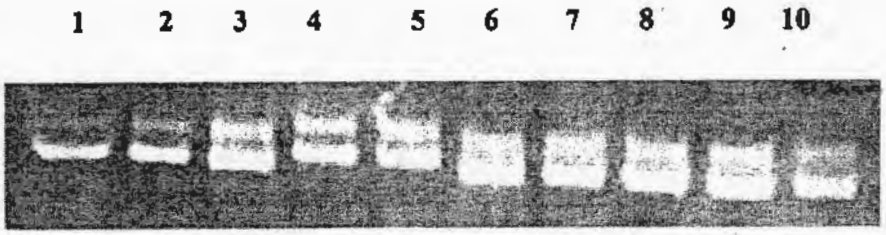
Figure 3.25: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel sh allele pattern obtained with marker D10S560 at 90.01 cM, on chromosome 10. The Roman with Arabic numerals to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

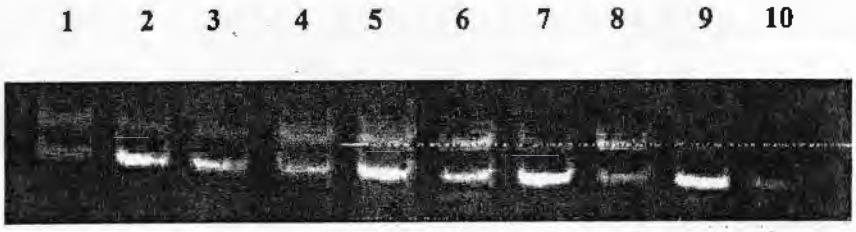
Figure 3.26: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D10S188 at 93.92 cM, on chromosome 10. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

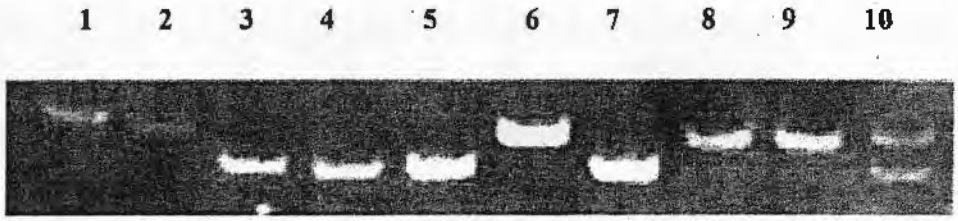
Figure 3.29: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D10S1699 at 97.29 cM, on chromosome 10. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

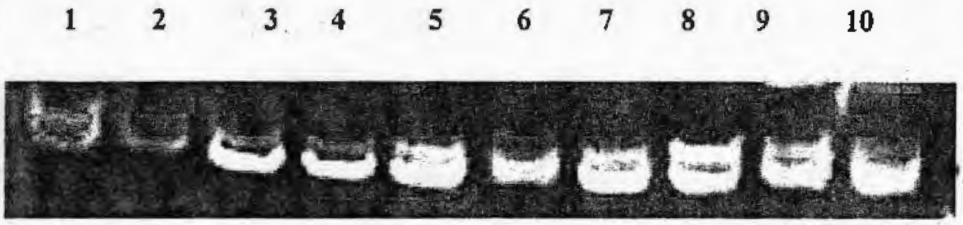
Figure 3.28: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D10S607 at 99.52 cM, on chromosome 10. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

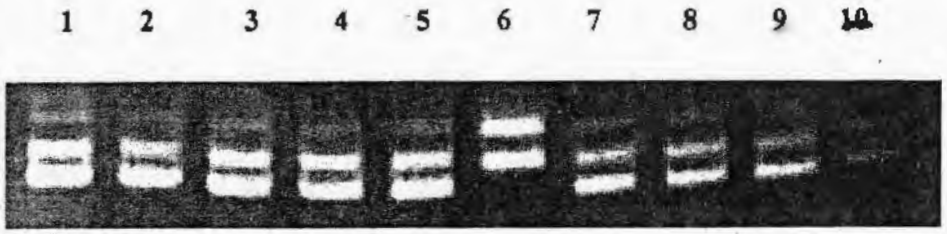
Figure 3.29: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D14S608 at 28.01 cM, on chromosome 14. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

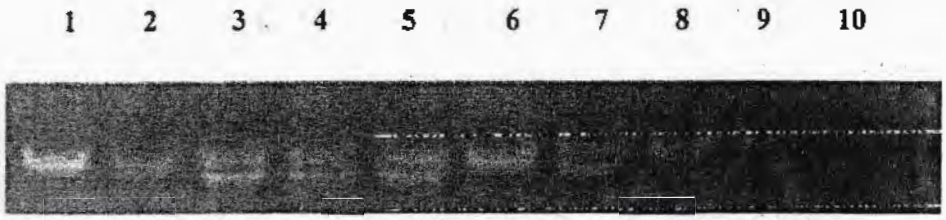
Figure 3.30: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D14S975 at 31.13 cM, on chromosome 14. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

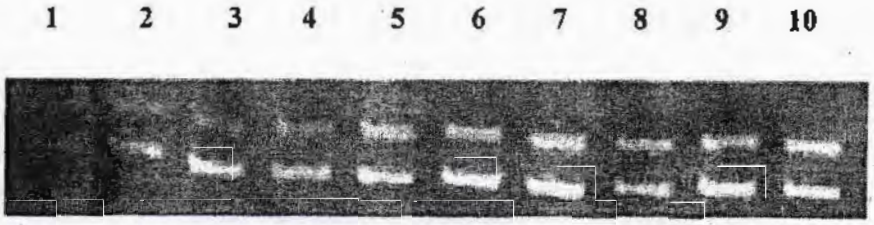
Figure 3.31: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D14S1040 at 31.75 cM, on chromosome 14. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

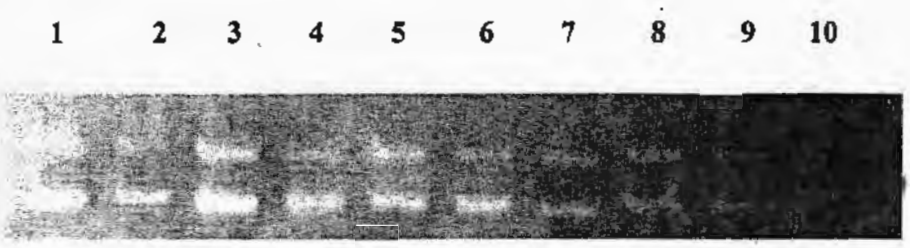
Figure 3.32: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D14S121 at 34.43cM, on chromosome 14. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

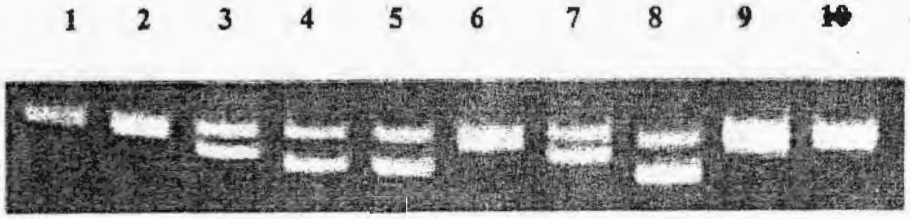
Figure 3.33: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D16S3056 at 35.44cM, on chromosome 16. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

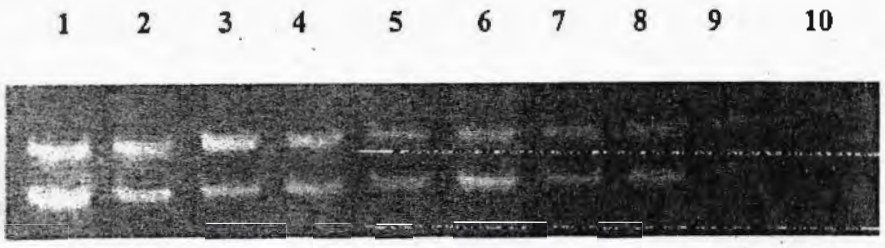
Figure 3.34: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D16S3045 at 40.65cM, on chromosome 16. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.35: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D16S690 at 57.79 cM, on chromosome 16. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

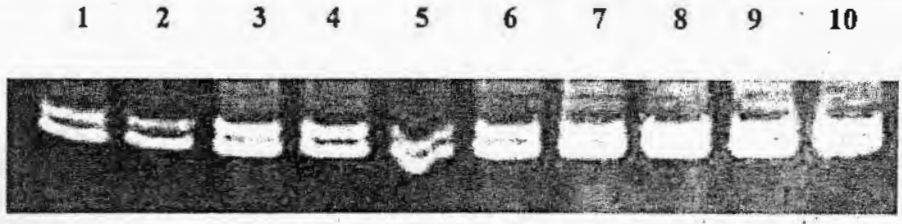
1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

Figure 3.36: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D16S3044 at 58.46 cM, on chromosome 16. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

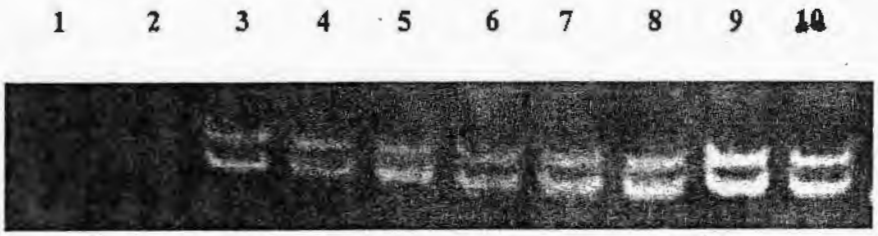
Figure 3.37: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D16S3080 at 59.68 cM, on chromosome 16. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

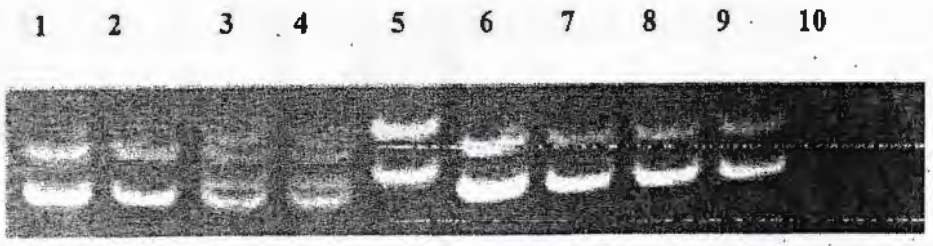
Figure 3.38: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D16S3035 at 62.11cM, on chromosome 16. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1. IV:3 Affected
2. IV: 4 Affected
3. IV: 8 Affected
4. IV: 9 Affected
5. IV: 10 Affected
6. III: 1 Normal
7. III: 2 Normal
8. III: 6 Normal
9. IV: 6 Normal
10. III: 7 Normal

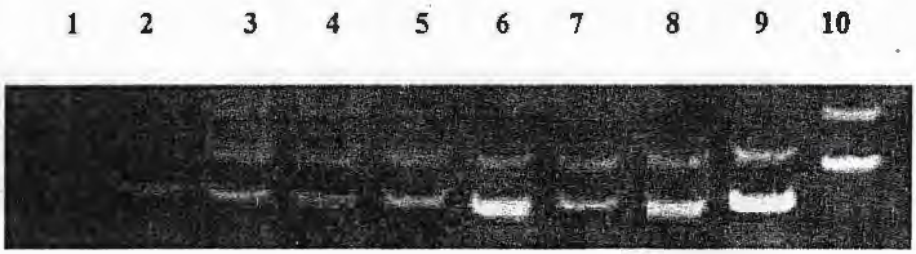
Figure 3.39: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S423 at 65.77 cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

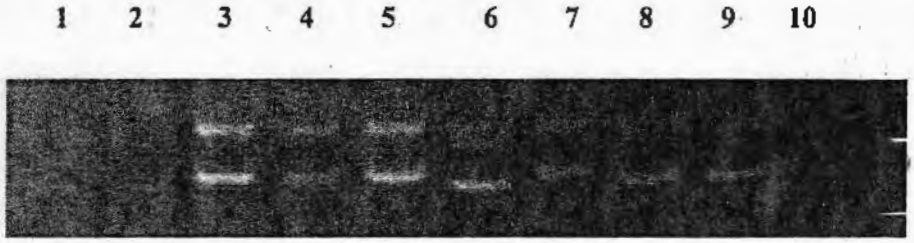
Figure 3.40: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S211 at 66.3 cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

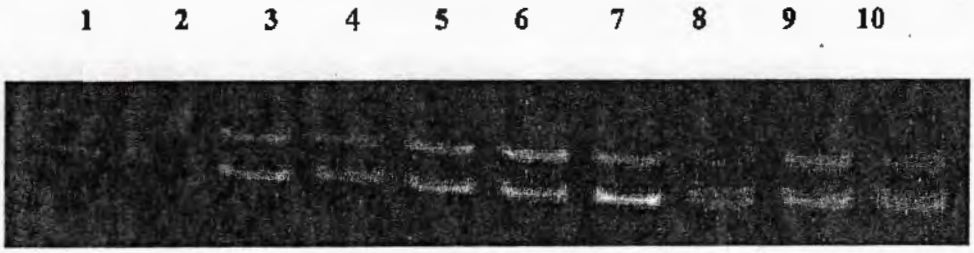
Figure 3.41: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S538 at 68.08 cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

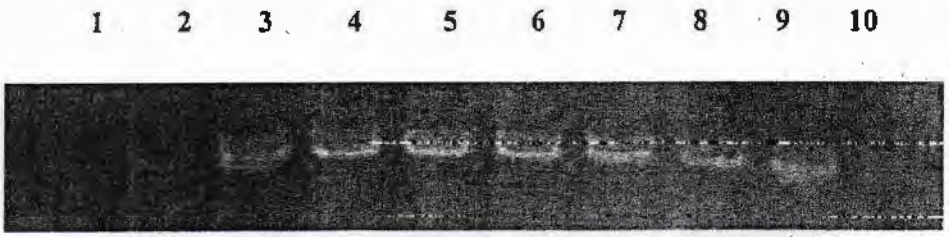
Figure 3.42: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D19S908 at 69.5cM, on chromosome 19. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

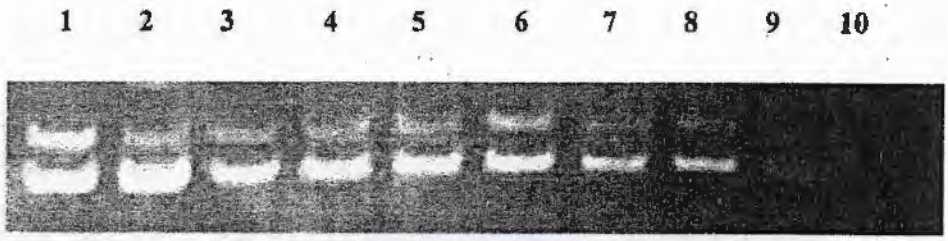
Figure 3.43: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S429 at 13.88 cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.44: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S2651 at 142.24cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.45: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S2726 at 144.38 cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

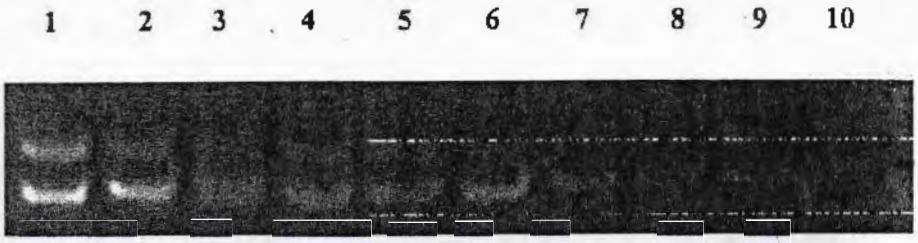
1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.46: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D1S2789 at 145.86 cM, on chromosome 1. The Roman with Arabic numerals refers to the individuals in pedigree.

Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.47: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D8S1704 at 164.25cM, on chromosome 8. The Roman with Arabic numerals refers to the individuals in pedigree.



Family A

1.	IV:3 Affected	6	III: 1 Normal
2.	IV: 4 Affected	7	III: 2 Normal
3.	IV: 8 Affected	8	III: 6 Normal
4.	IV: 9 Affected	9	IV: 6 Normal
5.	IV: 10 Affected	10	III: 7 Normal

Figure 3.48: Electropherogram of the ethedium bromide stained 8% non denaturing polyacrylamide gel showing allele pattern obtained with marker D8S1837 at 156.59 cM, on chromosome 8. The Roman with Arabic numerals refers to the individuals in pedigree.

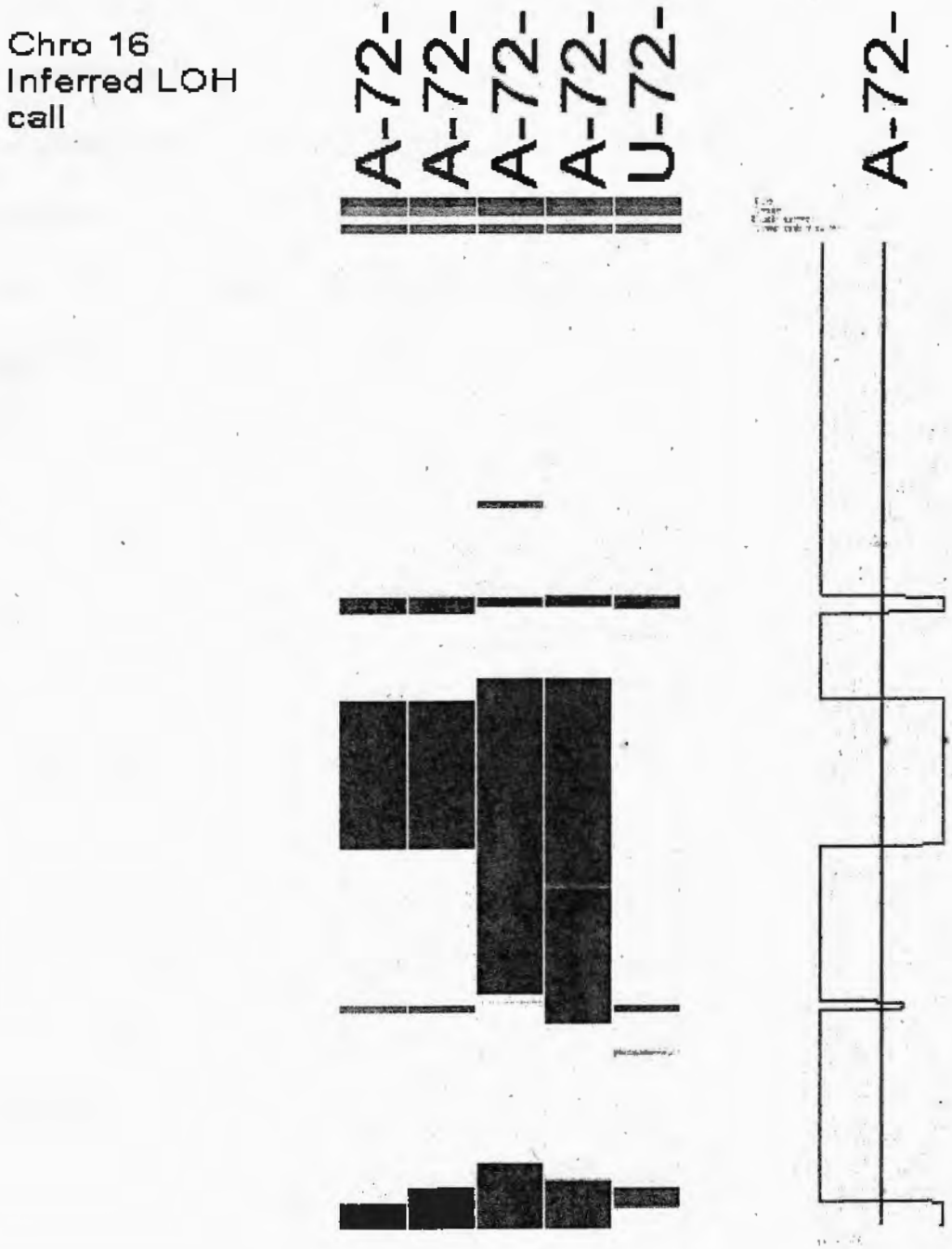


Figure 3.49: Snapshot of Microarray analysis through Dchip software showing homozygous region between affected individuals at chr16:54,850,716-65,477,189

3.3 Results of Family B

Family B is living in Jhelum City of Punjab. The pedigree was constructed after careful investigations with the family elders presented in Figure 3.50. The family members traditionally marry within the family and result in many consanguineous marriages. The family consists of four generations with seven affected individuals in three loops. Affected individuals have only mental retardation. As the disease controlling trait appears among both males and females, affected individuals are produced by normal parents. Affected members of the family depict mild to severe mental retardation since their birth.

Blood samples were collected from four affected family members (IV: 5, IV:6, IV:8, IV:10) and three normals (III: 3, III: 4, III:5) and DNA extraction was carried out as per protocol.

3.3.1 Linkage Analysis in Family B

Linkage studies were carried out in both families. Linkage in families were searched using microsattelite markers (Table 2.5) corresponding to candidate genes involved in elated autosomal recessive non syndromic mental retardation, it is clear that at least some candidate interval should be tested for linkage or exclusion prior to embarking on genome wide search . Table 2.5 summarizes the microsattelite markers, which were used in present study for candidate gene analysis. Reported average hetrozygosity for the selected markers is > 70%. Analysis of microsattelite, markers was carried out using a standard PCR reaction and electrophoresis in 8% non-denaturing polyacrylamide gel as discussed in materials and methods, amplified microsattelite markers were visualized by staining the gel with ethedium bromide and the genotypes were analyzed and assigned by GEL doc (BIO-RAD, Italy) and by visual inspection.

In family B seven DNA samples were used for genotyping the markers linked to the candidate genes. Three to four markers per locus were used to test the linkage (Table 2.5). Analysis of the results obtained with polymorphic microsatellite markers specific for known MR loci, it was evident that all the affected individuals were heterozygous for the different combinations of the parental alleles, thus excluding the linkage in family B to the known MR loci (figure 3.52-3.75). Further genotyping with SNP-microarray chip will identify the linkage by homozygosity mapping method to novel locus.

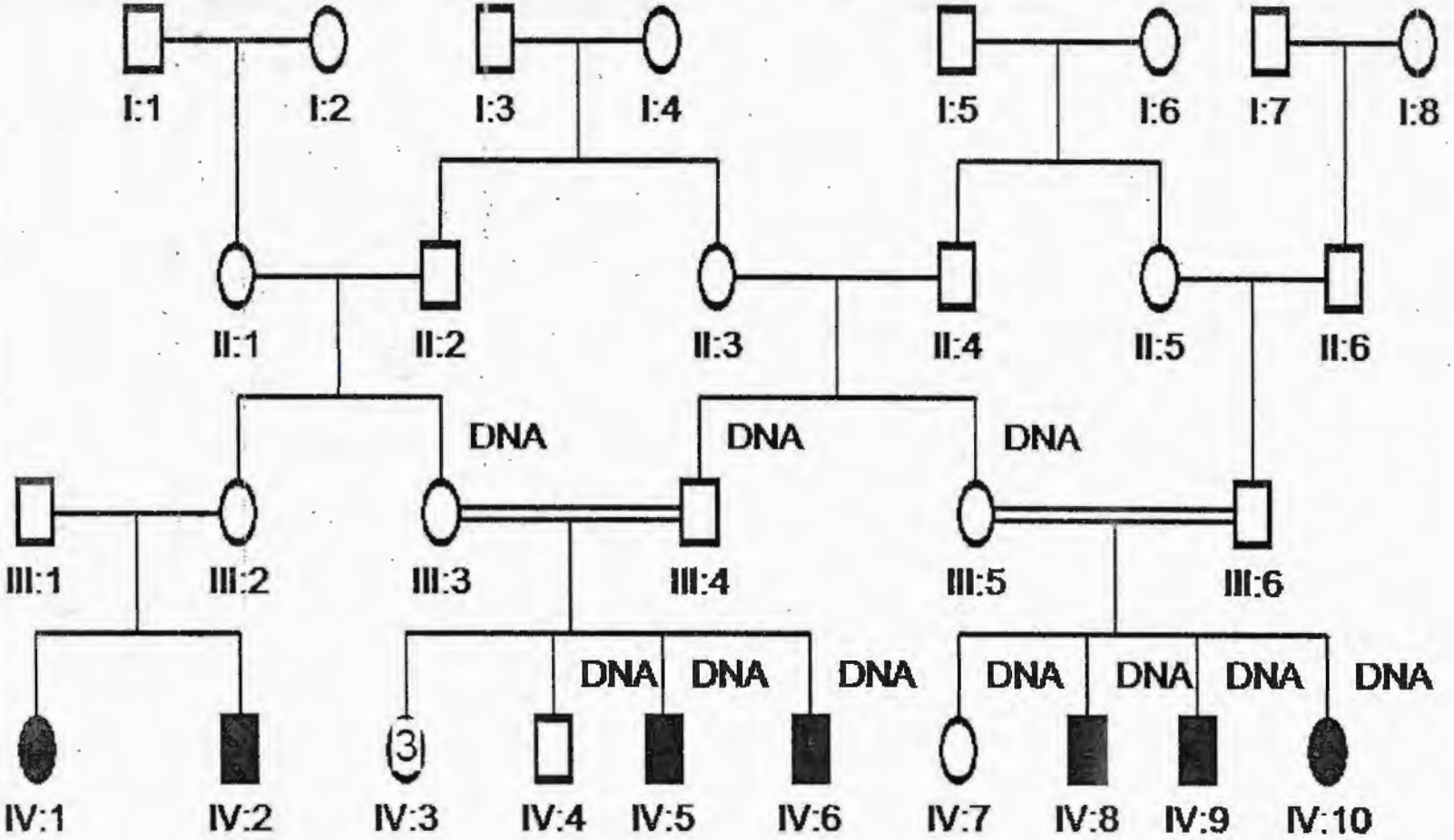


Figure 3.50: Pedigree of family B with Autosomal Recessive non syndromic Mental Retardation in which square represents males while circles represent females. Affected individuals have been represented by filled circles and squares. Double line represents inter family marriages

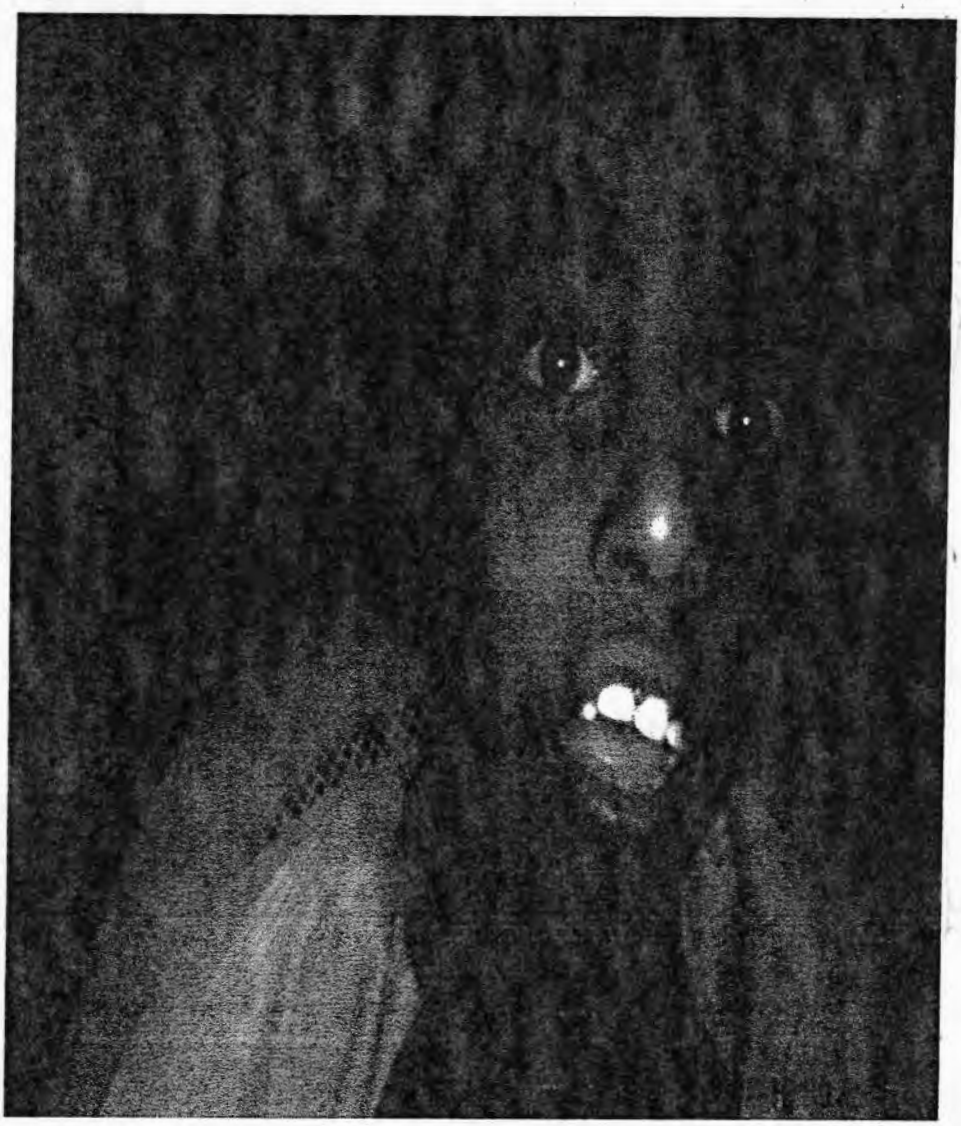
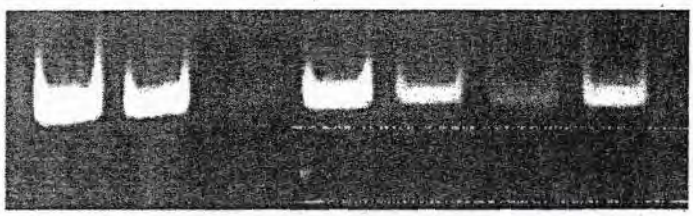


Fig. 3.51: Clinical presentation of affected individual (IV-8) in family B showing Mental Retardation MR phenotype

1 2 3 4 5 6 7

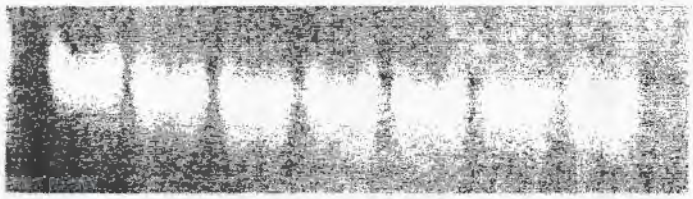


Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.52: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D4S3024 at 124.45 cM, on chromosome 4. The Roman and Arabic numerals refer to the individuals in the pedigree.

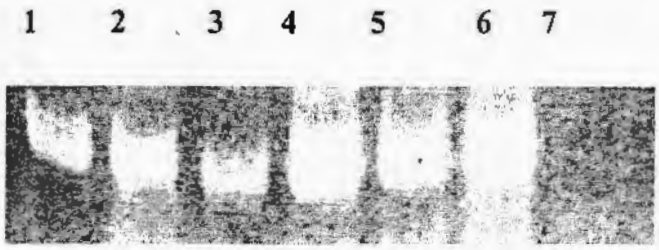
1 2 3 4 5 6 7



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

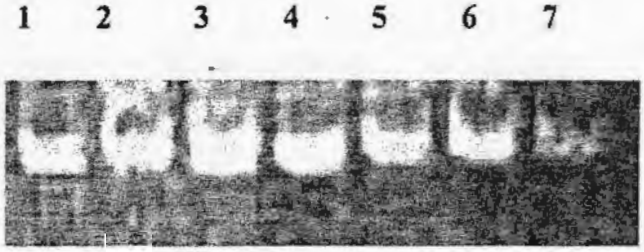
Figure 3.53: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D3S3050 at 10.31 cM, on chromosome 3. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

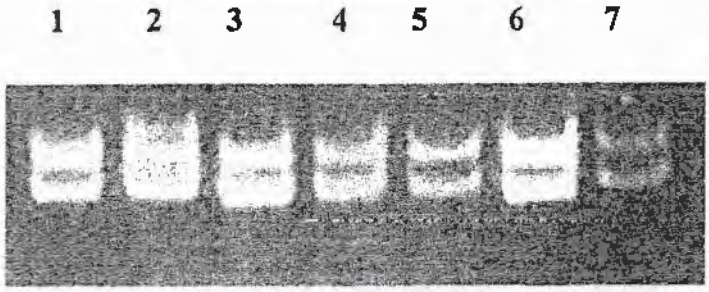
Figure 3.54: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D3S1620 at 10.52 cM, on chromosome 3. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

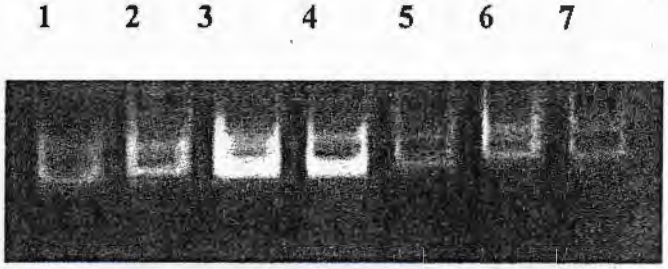
Figure 3.55: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D19S558 at 33.33 cM, on chromosome 19. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

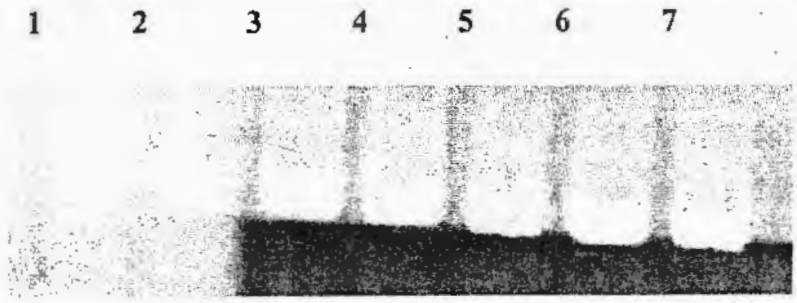
Figure 3.56: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D19S226 at 36.35 cM, on chromosome 19. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

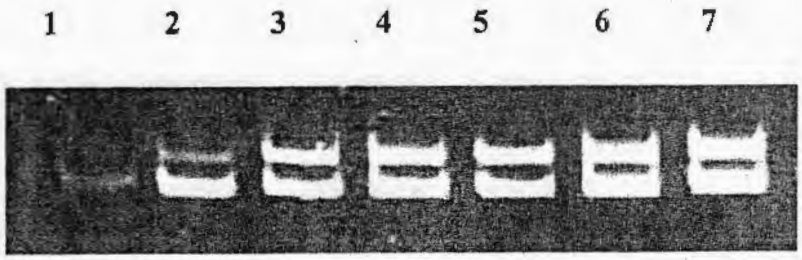
Figure 3.57: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D19S840 at 37.94 cM, on chromosome 19. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

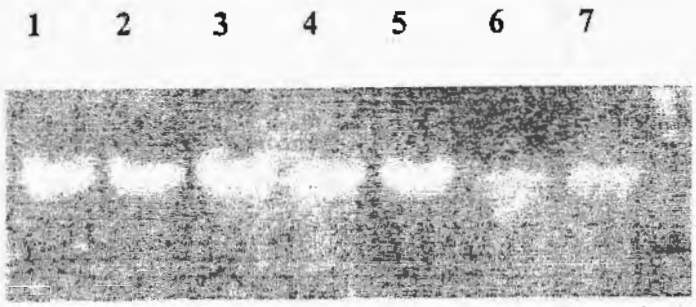
Figure 3.58: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D1S255 at 58.66 cM, on chromosome 1. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

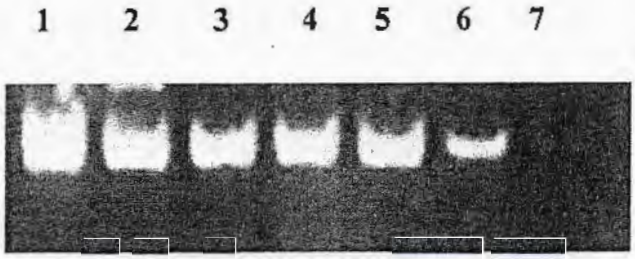
Figure 3.59: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D6S1682 at 109.19 cM, on chromosome 6. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.60: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D8S1810 at 60.34 cM, on chromosome 8. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.61: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D8S259 at 60.87 cM, on chromosome 8. The Roman and Arabic numerals refer to the individuals in the pedigree.

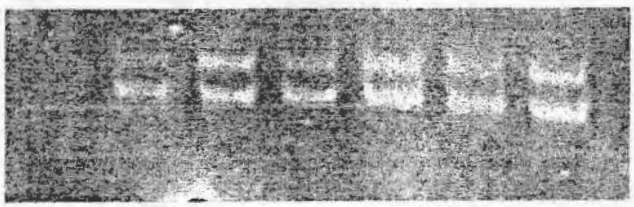
1 2 3 4 5 6 7

Family

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.62: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D10S1650 at 92.81 cM, on chromosome 8. The Roman and Arabic numerals refer to the individuals in the pedigree.

1 2 3 4 5 6 7

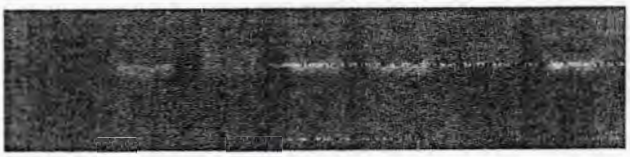


Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.63: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D10S607 at 99.52 cM, on chromosome 10. The Roman and Arabic numerals refer to the individuals in the pedigree.

1 2 3 4 5 6 7

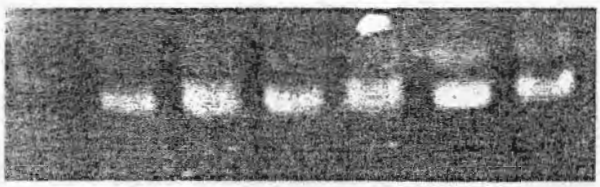


Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.64: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D14S80 at 26.59 cM, on chromosome 14. The Roman and Arabic numerals refer to the individuals in the pedigree.

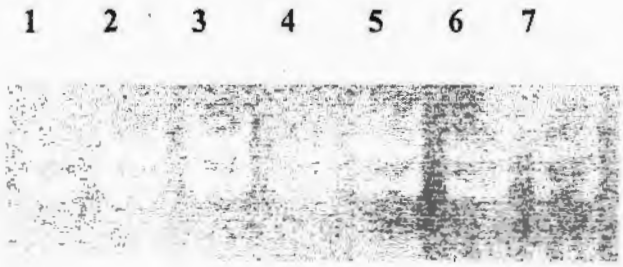
1 2 3 4 5 6 7



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

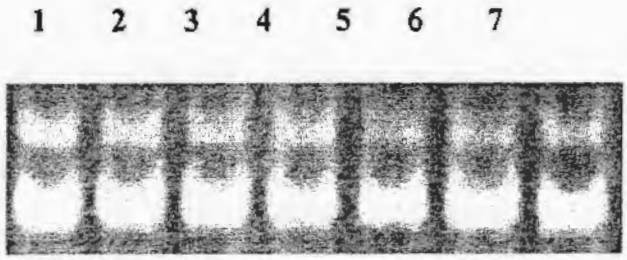
Figure 3.65: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D14S1034 at 31.75 cM, on chromosome 14. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.66: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D16S3045 at 40.65 cM, on chromosome 16. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

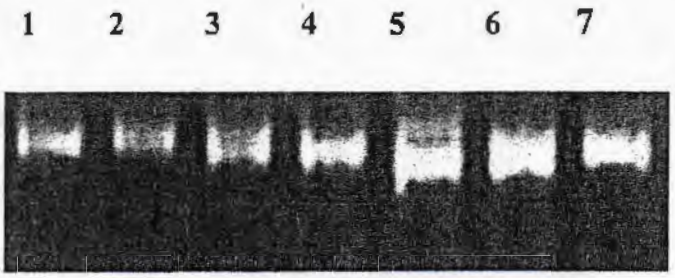
1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.67: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D16S690 at 57.79 cM, on chromosome 16. The Roman and Arabic numerals refer to the individuals in the pedigree.

Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.68: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D16S3044 at 58.46 cM, on chromosome 16. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.69: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D16S3080 at 59.68 cM, on chromosome 16. The Roman and Arabic numerals refer to the individuals in the pedigree.

Family B

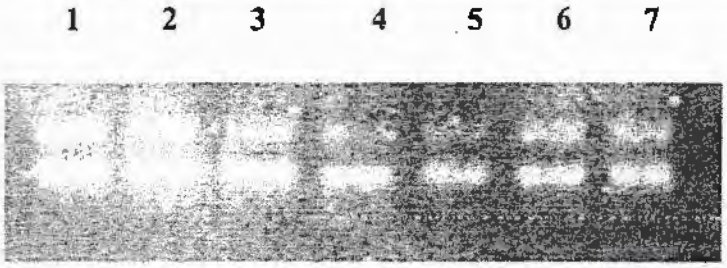
1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.70: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D19S423 at 65.77 cM, on chromosome 19. The Roman and Arabic numerals refer to the individuals in the pedigree.

Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

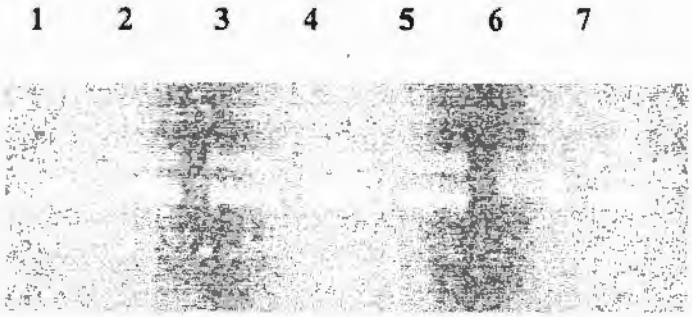
Figure 3.71: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D19S211 at 66.3 cM, on chromosome 19. The Roman and Arabic numerals refer to the individuals in the pedigree



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

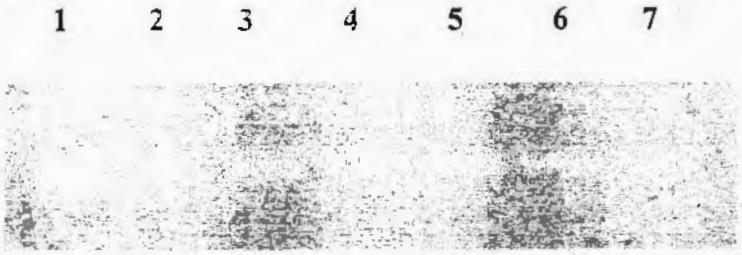
Figure 3.72: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D19S574 at 69.5 cM, on chromosome 19. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

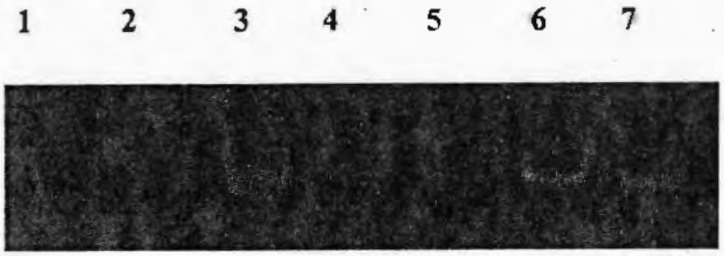
Figure 3.73: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D1S429 at 126.06 cM, on chromosome 1. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family B

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.74: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D1S2688 at 139.02 cM, on chromosome 1. The Roman and Arabic numerals refer to the individuals in the pedigree.



Family

1. III: 3 Normal
2. III: 4 Normal
3. IV: 5 Affected
4. IV: 10 Affected
5. IV: 6 Affected
6. III: 5 Normal
7. IV: 8 Affected

Figure 3.75: Electropherogram of ethidium bromide stained 8 % non-denaturing polyacrylamide gel showing allelic pattern obtained with marker D1S2651 at 142.24 cM, on chromosome 1. The Roman and Arabic numerals refer to the individuals in the pedigree.

DISCUSSION

Genetic disorders have been the centre of attention for scientists over the last few decades. A lot of effort is being put in the field of genetic disorders all around the world and it is estimated that about five new genetic disorders are being discovered every year. Genetic disorders are crucial in the sense that they have impact over generations. If a transmittable mutation occurs, it affects the following generation, rendering the whole family vulnerable to a certain disorder. At the same time, if a defect is somehow rectified, it will save the fore-coming generations from that defect. Most vital factor in the prevalence of genetic disorders is the consanguineous marriages. Individuals from the related families carry similar genes. If they are inter-married, impact of defected genes will be reinforced and the offspring will be more prone to the appearance of defect. On contrary, marriages outside the family result in the new gene combinations. This minimises the hazardous effects of defective genes and leads to the diversification of the gene pool. Unfortunately, people here in Pakistan prefer to marry within the close relatives because of social traditions and tribal constraints. This is the reason that a variety of genetic disorders is prevalent in Pakistan and it is a gold mine for the study of genetic disorders for the scientists all over the world.

When Mental Retardation is not accompanied by any other syndrome, it is called Autosomal Recessive Non-syndromic Mental Retardation (ARNSMR). The individual having Intelligence Quotient (IQ) less than 70% is referred as mentally retarded. Furthermore, sub-average general intellectual functioning and deficiency in at least two of self-survival skills that is diagnosed

during childhood is also a defining feature of MR (Raymond, 2006). To date, thirty loci and ten genes causing Mental Retardation have been reported. All of them encode brain proteins that ensure proper psychomotor development.

In the present study, two different families with consanguineous marriages family A and B, demonstrating autosomal recessive form of ARNSMR, respectively, were studied. The affected individuals showed typical Autosomal Recessive Non-Syndromic Mental Retardation (ARNSMR) phenotype during the initial clinical evaluation. The mode of inheritance in both families was autosomal recessive as per their pedigree analysis.

To search for the locus, carrying the candidate gene(s) responsible for ARNSMR in the families (A & B), linkage studies were performed by a method known as homozygosity mapping. We can map a recessive trait by using offspring of the consanguineous matings by homozygosity mapping. Because of genetic nature of the recessive inheritance, regions in close proximity of the disease causing locus are likely to be homozygous by descent in patients from the consanguineous families (Lander and Bostein, 1987; Sheffield *et al.*, 1998). The region of homozygosity would be expected to be random between different offspring of these matings, except a common disease locus shared by affected offspring. Thus homozygosity mapping is a suitable method for fishing out disease causing gene in this disease. To establish a linkage, four affected sibling are required in first cousin marriage and only three affected siblings are required in the second cousin marriage (Miano *et al.*, 2000).

The pedigree analysis of family "A" shows that affected individuals were produced by the unaffected parents in all two loops and the affected status was independent of the sex suggesting

that the trait is transmitted in autosomal recessive manner. The consanguineous parents III:1 and III:2 , III:6 and III:7 are normal phenotypically, but resulted in six affected individuals. Homozygosity analysis was performed with polymorphic microsatellite markers from candidate linkage intervals of known loci. Genotyping of ten family members including five affected (IV: 3, IV:4,IV:5,IV:8, IV:9, IV:10) and five normal (III:1, III:2, III:6, III:7,IV:6) in family 'A' revealed that the affected individuals were heterozygous for different combinations of parental alleles. Analysis of the genotyped markers ruled out the linkage of the family 'A' to any of known MR loci and indicates the possible involvement of novel gene.

In order to map the recessive disorder in the family "A", we performed homozygosity mapping using commercial facility at TCGA, Toronto, Canada. After exclusion mapping, Family members were genotyped using Affymetrix 500K single-nucleotide polymorphism (SNP) microarrays. This approach allowed us to identify homozygous-by-descent (HBD) locus at 16q12.2-q21 and has not been reported previously. Whole exome sequencing is required to discover disease causing mutations in this family.

To test the linkage in family 'B', four affected family members (IV: 5, IV:6, IV:8, IV:10) and three normal (III: 3, III: 4, III:5) were genotyped using polymorphic microsatellite markers from known ARNSMR loci. All affected individuals are mentally abnormal, cannot speak properly and do not show any social skills. Genotyping revealed that the affected individuals were heterozygous for different combinations of parental alleles. Analysis of these markers excluded the presence of known loci and predicted the involvement of novel loci. Affymetrix 500K single-nucleotide polymorphism (SNP) microarrays approach is required to identify homozygous-by-

descent (HBD) locus to elucidate the candidate locus carrying the gene involved in the disease in this family.

Identification of novel genes in both families (A and B) presented here and subsequent characterization of proteins they encode will further increase our knowledge of molecular mechanisms underlying the disease. This and further studies in this field will help getting rid of such genetic anomalies and securing human beings. Recent advances in the field of MR are quite promising about the discovery of new genes involved in these disorders in the near future.

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