LINKAGE ANALYSIS OF FAMILIES WITH INHERITED MENTAL RETARDATIONS

By

Muhammad Nawaz

Department of Biochemistry

Quaid-i-Azam University

Islamabad, Pakistan.

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CERTIFICATE

This thesis, submitted by **Mr. Muhammad Nawaz** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor: _____
Dr. Muhammad Ansar

External Examinar: _____
Dr. M. Ramzan Khan

Chairman: _____
Dr. Salman Akbar Malik

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Declaration

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

Muhammad Nawaz

In the name of Allah, Most Gracious, Most Merciful

Read! In the name of your Lord, who created; Created man, out of a clot of congealed blood: Proclaim! And your Lord is most bountiful, He who taught you to write, Taught man that which he knew not. No, but man transgresses all bounds, In that he looks upon himself as self-sufficient. Truly, to your Lord is the return. Do you see one who forbids, A votary when he prays? Do you see if he is guided? Or enjoins righteousness? Do you see if he denies and turns away? Knows he not that Allah sees? Let him beware! If he desists not, we will drag him by the forelock, A lying, sinful forelock! Then, let him call to his council: We will call on the angels of punishment! No, heed him not, but bow down in adoration, and bring yourself closer!

(SURAH 96: AL 'ALAQ or IQRA)

Dedicated

To my,

Loving parents whose earnest prayers made me able to achieve my goals.

And my fiancée Farah Nawaz, who always advocated me to step forward.

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Muhammad Nawaz

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LIST OF ABBREVIATIONS

µg	Micro gram
µl	Micro litre
aa	Amino acid
AAIDD	American Association on Intellectual and Developmental Disabilities
AAMR	American Association on Mental Retardation
AD	Alzheimer's disease
ADSLAP	Adenylosuccinate lyase
AP	Ammonium Persulphate
ARID	Autosomal Recessive Intellectual Disability
ARNSMR	Autosomal Recessive Non-Syndromic Mental Retardation
AS	Angelman syndrome
ATP	Adenosine TriPhosphate
ATP7A	ATPase, Cu(2+)-Transporting, Alpha Polypeptide
ATPase	Adenine Triphosphatase
ATRX	Alpha-Thalassemia/Mental Retardation Syndrome, type, X-linked
CC2D1A	Coiled-Coil and c2 Domains-containing protein 1a
cDNA	Complementary Deoxyribo Nucleic Acid
CGH	Comparative Genomic Hybridization
CHLC	Cooperative Human Linkage Centre
cM	centi Morgan
CNS	Central Nervous System
CNV	Copy Number Variations
CpG	Cytosine and Guanine separated by a Phosphate
CRBN	Cereblon
CSF	Cerebro-Spinal Fluid
DD	Developmental Delay
DNA	DeoxiriboNucleic Acid

DNMT	DNA Methyltransferase
dNTP	Deoxiribo Nucleotide Triphosphate
DPD	Dihydro-Pyrimidine Dehydrogenase
EDTA	Ethylene-Diamine-Tetra-Acetic acid
ER	Endoplasmic Reticulum
FMR1	Fragile X Mental Retardation1
GAMT	Guanidinoacetate Methyl-Transferase
GEF	Guanine nucleotide Exchange Factor
GK	Glycerol Kinase
GLUT1	Glucose Transporter-1
GRIK2	Glutamate Receptor, Ionotropic, Kainate 2
GTPase	Guanosine Triphosphatase
GWAS	Genome-Wide Association Studies
HD	Huntington's Disease
HMT	Histone Methyl-Transferases
ID	Intellectual Disability
IQ	Intelligence Quotience
K^+	Potassium ion
Kb	Kilobases
KCl	Potassium Chloride
KPK	Khyber Pakhtunkhwa
LOD	Logarithm of Odd
M	Molarity
Ma	Milliampere
Mb	Megabases
MECP2	Methyl-CpG-Binding Protein 2
ml	Millilitre
mM	Millimolar
MR	Mental Retardation
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA

mtDNA	Mitochondrial DNA
NDD	Neurodevelopmental Delay
NIBP	NF-kB Signalling Pathway
NF-kB	Nuclear Factor Kappa B
NS-MR	Non Syndromic Mental Retardation
NS-XLMR	Non-Syndromic X Linked Mental Retardations
ng	Nanogram
OD	Optical Density
OST	Oligo-Saccharyl Transferase
PAGE	Polyacrylamide Gel Electrophoresis
PAKs	p21-Activated Kinases
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
pH	Negative log of Hydrogen ion concentrations
PKU	Phenylketonuria
Pol	Polymerase
PPPC1A	Phospho-Protein (Serine/Threonine) Phosphatase 1
PQBP1	Polyglutamine-Binding Protein 1
PRPS1	Phospho-Ribosyl Pyrophosphate Synthetase
PRSS12	Protease Serine 12
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PWS	Prader–Willi Syndrome
RBCs	Red Blood Cells
RNA	Ribonucleic Acid
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
S-MR	Syndromic Mental Retardation
SNP	Single Nucleotide Polymorphism
SSADH	Succinic Semialdehyde Dehydrogenase
SWIP	Strumpellin and WASH Interacting Protein

S-XLMR	Syndromic X Linked Mental Retardation
SYNGAP1	Synaptic Ras GTPase Activating Protein 1
Taq	Thermus aquaticus
TBE	Tris Borate Ethylene-Diamine-Tetra Acetate
TE	Tris, Ethylene Diamine Tetra Acetate
TECR	Trans-2,3-Enoyl-CoA Reductase
TEMED	Tetra Methyl Ethylene Diamine
TRAPPC9	Trafficking Protein Particle Complex Subunit 9
TUSC3	Tumor Suppressor Candidate 3
UCSC	University of California Santa Cruz
UV	Ultravoilet
WHO	World Health Organization
XLMR	X-Linked Mental Retardation

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ABSTRACT

ABSTRACT

Mental Retardation or Intellectual Disability (MR/ID) refers significantly sub-averaged intellectual functioning with onset before age 18. The etiologies of MR are extremely heterogeneous and include both genetic and environmental factors. The mode of inheritance of MR can be autosomal or X-linked, dominant or recessive. The current estimates of X-linked genes associated with MR are high but 8 genes (*PRSS12, CC2D1A, CRBN, GRIK2, TUSC3, TRAPPC9, TECR* and *SWIP)* and 33 loci have been discovered so far. Mutation in these genes may lead to abnormal function of the brain and nervous system, which may result in mental retardation.

Present study involves two consanguineous Pakistani families (A and B), from Khyber Pakhtunkha (KPK) province, demonstrating autosomal recessive non syndromic mental retardation (ARNMR). Affected individuals were initially examined for the presence of malformations and history of metabolic disorders. To identify the causative genes, homozygosity mapping was carried out in both families A and B, by genotyping microsatellite markers linked to currently known ARNSMR loci. Analysis of genotyped results for all known loci showed no disease associated homozygosity. Data for all the genotyped loci in both families was analyzed using easyLINKAGE plus version 5.02, which failed to yield significant LOD score, indicating the exclusion of known loci. Thus linkage to all known loci was conclusively excluded in both families, indicating the involvement of potentially novel loci. The genes responsible for MR in these families can be identified by genome wide scanning using microsatellite markers or by whole genome SNP microarrays. From this study we can conclude that genetic causes of autosomal recessive non syndromic mental retardation are still not fully known and require further investigations.

INTRODUCTION

INTRODUCTION

The nervous system has evolved over millions of years, generating a wide variety of species-specific brains and behavioral capacities. It seems obvious that only anatomically humans are capable of creating symbolic objects (Felipe, 2011). The modern neuroscience has also contributed in this field by addressing the issue of mental processes from a biological point of view. A key issue in neuroscience is the understanding of the coexistence of local specialization and long distance integration in the complex structure of the brain (Palva, 2010).

The brain consists of soft, fragile, non-replaceable neural tissue and is supported and protected by the surrounding skin, skull, meninges and cerebrospinal fluid. The major regions of the brain are the cerebral hemispheres, diencephalon, brain stem and cerebellum. The cerebral hemispheres make up approximately 83% of total brain mass and collectively are referred as cerebrum that is associated with higher brain function such as thought and action, emotions, memory, speech and movement. Nerve fibers of cerebrum carry signals between the nerve cells and other parts of the brain and body and control muscular movement (Pasternak, 2005).

The diencephalon is located centrally within the forebrain, associated with grouping and relay station for sensory inputs, motor activities, cortical arousal and memories, controlling the autonomic (involuntary) nervous system and maintaining the body's homeostatic balance (Pasternak, 2005). The brain stem has structural similarly with spinal cord and it consists of grey matter which provides fiber pathways between higher and lower brain centers, visual and auditory reflex and sub-cortical motor centers, maintains body homeostasis, conduction and regulation of respiration and cranial nerves. The parts of brain stem also behave as autonomic reflex centre. The cerebellum accounts for about 11% of total brain mass which processes impulses received from the cerebral motor cortex, various brain stem nuclei and sensory receptors in order to control skeletal muscle contraction, thus giving smooth, coordinated movements (Pasternak, 2005).

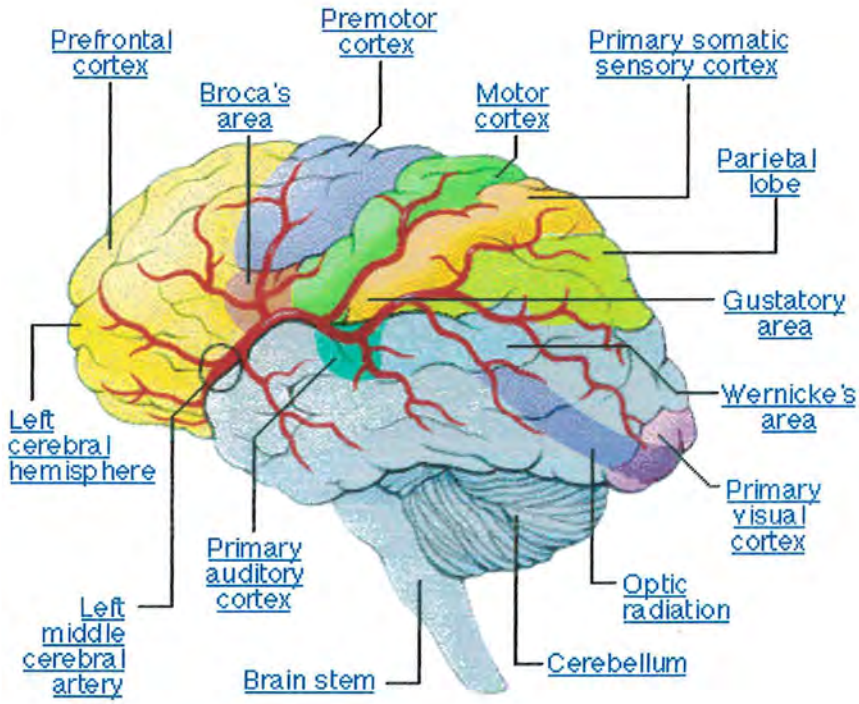


Figure 1.1: Major regions of the brain (Adopted from www.medem.com)

The peripheral and central nervous system (CNS) consist of numerous neurons and precise coordination and positioning of neurons is required for subsequent wiring of neurons into functional circuits. Impairment of these processes results in structural defects that are accompanied by severe mental abnormalities (Khodosevich and Monyer, 2011). Similarly the disruption of biological and molecular processes in the nervous system such as neuronal differentiation and synaptic plasticity, synaptic vesicles cycling and gene expression can cause intellectual disability or mental retardation (Basel-Vanagaite, 2007).

Neurological Disorders

Neurological disorders are diseases of the central and peripheral nervous system which include epilepsy, Alzheimer disease and other dementias, cerebrovascular diseases including stroke, migraine and other headache disorders, multiple sclerosis, Parkinson's disease, neuroinfections, brain tumors and traumatic disorders of the nervous system (World Health Organization, 2007). These disorders are mostly identified in sporadic cases and have been analyzed by genome-wide association. Genome-wide association studies (GWAS) have been extremely successful in associating SNPs with susceptibility to common hereditary diseases, but published SNP associations account for only a fraction of the genetic component of most common diseases and there has been considerable assumption about the 'missing heritability' (Manolio *et al.*, 2009). Numerous CNVs are also associated with ID and developmental delay (Stankiewicz and Beaudet, 2007; Zahir and Friedman, 2007; Shaffer *et al.*, 2007; Emanuel and Saitta, 2007; Baris *et al.*, 2007) or with related conditions such as autism (Ullmann *et al.*, 2007; Marshall *et al.*, 2008; Weiss *et al.*, 2008; Kumar *et al.*, 2008) while rare CNVs, either individually or in aggregate play role in susceptibility of neurodevelopmental diseases (Stefansson *et al.*, 2008).

Neurological disorders resulting due to changes in mitochondrial DNA (mtDNA) vary significantly between individuals. Different mtDNA mutations can cause similar phenotypes and different diseases features can be observed in patients with similar mtDNA defect. Mitochondrial dysfunction and oxidative stress are associated with development of common neurodegenerative disorders, including Alzheimer's disease

(AD), Parkinson's disease (PD) and Huntington's disease (HD) (Enns, 2003; Catherine, 2005; Mandemakers *et al.*, 2007; Sas *et al.*, 2007; Gogvadze *et al.*, 2009; Jellinger, 2009).

The ion channels in neurons and muscle also play very important role for selective transport of sodium, potassium, calcium and chloride ions across the membranes. Disorders caused by these channel proteins are known as channelopathies. Features of neuronal channelopathies include intermittent loss of brain functions (seizures, convulsions and epilepsy), uncontrolled muscle movement and severe headaches with vomiting nausea and extreme sensitivity to light (Catherine, 2005).

Cognition and Cognitive Abilities

Cognition is the scientific term for "the process of thought" and refers to a sense for the processing of information, applying knowledge, and changing preferences. The concept of intelligence (mental ability, cognitive ability) involves the capacity to learn from one's own experiences, be able to reason, plan and adjust to the environment in which one lives. Intelligence is hence practically useful and is not in the first place linked to school and abstract thinking. Today science has reached an era where clinical and basic research have converged and provided insight into the molecular basis of human cognition (Edwin *et al.*, 2002). Studies have reported direct link between the genes and development of human cognitive abilities (Paterson, 1999). The identification of genes responsible for human learning disorders will help to understand molecular mechanism that underlies human mental retardation (Edwin *et al.*, 2002).

Mental Retardation

Mental retardation (MR) or intellectual disability (ID) is defined as non-progressive cognitive impairment characterized by sub-average intellectual functioning and deficiency in at least two of self-survival skills diagnosed during childhood. MR display large clinical and genetic heterogeneity and involves 1–8% of the population (Ropers *et al.*, 2010). MR can be divided into syndromic forms (S-MR) characterized by additional malformations and/or neurological abnormalities and non-syndromic forms (NS-MR)

without any additional complications. It is among the most common disabilities in children, affecting 1-3% of the population (Chelly *et al.*, 2005).

MR has some defined features which include intellectual functioning level (IQ) below 70, significant limitations in two or more adaptive skill and the condition present before the age of 18. The ratio of mentally retarded males to females is as high as 1.4-1.6 mainly because of a large number of X-linked MR genes (Vanagaite, 2008). The American Association on Intellectual and Developmental Disabilities (AAIDD, 2002) has defined MR as significant limitations both in intellectual functioning and in adaptive behavior, which covers many everyday social and practical skills. Intellectual limitations refer to an Intelligence Quotient (IQ) which falls two standard deviations below the population mean of 100 (<70) and adaptive functioning limitations refer to impairments in at least two out of ten adaptive skill areas (AAMR, 2000).

Aetiology of Mental Retardation

The causes of MR/ID are extremely heterogeneous and although a cause for mental retardation has been diagnosed in half of the cases, it has been estimated that half of all cases are due to environmental factors and half due to genetic factors (Winnepenninckx, 2003). It is also believed that behavioral or societal factors such as poverty, malnutrition, maternal drug and alcohol use, as well as severe stimulus deprivation can contribute to MR (Armatas, 2009). Approximately in 30 to 50 percent of cases, the etiology is not identified even after thorough diagnostic evaluation (Cury *et al.*, 1997). High-throughput sequencing has greatly facilitated the elucidation of genetic disorders but compared with X-linked MR, the search for genetic defects underlying autosomal recessive MR still lags behind (Ropers *et al.*, 2011).

Neurological Disorders at Different Levels

Neurological disorders that are thought to be caused by different factors can result in abnormalities at various levels which are discussed below.

1. Epigenetic Level

Epigenetic changes are defined as alterations in gene expression that are self-perpetuating in the absence of the original signal that cause them. A major class of epigenetic mechanism is thought to involve persistent changes in chromatin structure (Berger *et al.*, 2009). Most transcriptional regulatory events cause changes to chromatin structure and composition, lead to either silencing or inappropriate expression of specific sets of genes manifesting certain diseases. Most genes associated with mental retardation (learning disability) affect chromatin-remodeling processes and are involved in the process of neuronal plasticity and in long lasting changes in brain function (Kramer *et al.*, 2009). Genetic defects in the epigenetic machinery can lead to severe disorders of brain development such as the Rett syndrome, Rubinstein Taybi syndrome, Fragile X syndrome, Alzheimer's disease, Huntington's disease and psychiatric disorders such as autism, schizophrenia, addiction and depression (Grafodatskaya *et al.*, 2010). Epigenetic changes mainly involve DNA methylation and Histon modifications.

DNA methylation regulates gene expression (Suzuki and Bird, 2008) by controlling activity of intragenic non-coding RNAs which could play key roles in neural development and in neuropsychiatric disorders (Qureshi and Mehler, 2010). The modification of CpG dinucleotides is abundant in human and mouse brains and increases during aging in mouse hippocampus and cerebellum (Song *et al.*, 2011). Some tissue-specific genes are affected by age-associated DNA methylation changes, such as genes involved in DNA binding and regulation of transcription in various brain regions (Hernandez *et al.*, 2011). The importance of DNA methylation in assisting essential gene regulatory events that are associated with brain function and neurological disorders was revealed when the gene encoding MECP2 was identified as the target of mutations that cause Rett syndrome (Amir *et al.*, 2003), while the modification in histone marks, affect the neuronal activity and is associated with memory formation (Murgatroyd and Spengler, 2011).

2. Inherited Metabolic Diseases in Neurodevelopmental Disorders

The inborn errors of metabolism are the cause for a subset of developmental disabilities as mental retardation, autism spectrum disorders, movement disorders, and cerebral palsy. Several inborn errors of metabolism include features of autism and/or neurodevelopmental delay (Kayser *et al.*, 2008). Inherited metabolic neurodevelopmental disorders include, disorders of creatine biosynthesis, gamma-aminobutyric acid catabolism, purine and pyrimidine metabolism, and glucose transport across the blood-brain barrier which are discussed below.

Guanidinoacetate methyltransferase deficiency is an autosomal recessive inborn error of creatine biosynthesis characterized by delay in the first few months of life with epilepsy and variable neurologic signs (Stockler *et al.*, 1997; Francois *et al.*, 2006). Succinic semialdehyde dehydrogenase deficiency is an autosomal recessive inborn error of metabolism (Jakobs *et al.*, 1981, 2001) that causes mild to severe impairment of motor skills, language, speech and intellect. Adenylosuccinate lyase deficiency is an autosomal recessive disorder of purine metabolism which results in psychomotor delay, autism, and seizures (Berghe *et al*,. 1984). Posphoribosylpyrophosphate synthetase super activity is an X linked inborn error of purine metabolism with neurodevelopmental abnormalities, including sensory neural deafness along with features of gout and hyperuricemia (Duran *et al.*, 1997). Dihydropyrimidine dehydrogenase deficiency is an autosomal recessive inborn disorder of pyrimidine catabolism known as uraciluria thyminuria. Glucose transport defect of the blood-brain barrier (GLUT1 deficiency syndrome) is an autosomal dominant inborn error of glucose transport across the blood-brain barrier which results in mental retardation and learning disabilities along with ataxia, dystonia, seizures and acquired microcephaly.

3. Chromosomal Abnormalities

Chromosomal abnormalities are responsible for up to 28% of all mental retardation cases (Curry *et al.*, 1997). Moderate to severe ID is found in about 3% of patients carrying de novo balanced chromosome rearrangements (Bugge *et al.*, 2000) and may cause particular rare diseases and syndromes (Zhang *at al.*, 2009). Chromosomal abnormalities

include numerical chromosome abnormalities, partial chromosome abnormalities, deletions including microdeletions and sub telomeric deletions.

A numerical chromosome abnormality is caused by additional (polyploidy) or missing (monosomy) chromosomes from the normal set of 46. Deletions, insertions, inversions and translocations on any part of chromosome result in different disorders with variable phenotypes. A well known example is the Robertsonian translocation, which results from the breakage of two acrocentric chromosomes (13, 14, 15, 21 or 22) at or close to their centromeres followed by a fusion of the long arms (Winnepenninckx *et al.*, 2003). Depending on size and location, the deletion may cause diverse phenotypes including mental retardation. Examples of such cytogenetically visible deletions include cri-du-chat syndrome, characterised by mental retardation and cat-like crying in childhood (Winnepenninckx *et al.*, 2003). 10% of patients with mental retardation carry deletions or duplications (Vissers *et al.*, 2003; Smith *et al.*, 2004). Interstitial deletion involving a segment on chromosome 7q11.23 causes Williams-Beuren syndrome, associated with mental retardation in combination with cognition. Some of the genomic imbalances are quite common, such as microdeletion involving the 1p36.1 region that is found in 1% of the patients with idiopathic ID (Battaglia *et al.*, 2008). Chromosomal rearrangements at telomeric regions are the significant causes of idiopathic (Flint *et al.*, 1995) as well as familial mental retardation (Feder *et al.*, 2000). The telomeric regions are extremely gene rich which explains why the deletions of subtelomeric sequences frequently cause mental retardation (Winnepenninckx *et al.*, 2003).

4. Genetic Level

The genetic etiology of NSMR has been characterized in only approximately 10% of reported cases, with just 19 X-linked and eight autosomal genes reported to date (Vanagaite *et al.*, 2007; Motazacker *et al.*, 2007; Lisik *et al.*, 2008; Garshasbi *et al.*, 2008; Molinari *et al.*, 2008; Hamdan *et al.*, 2009; Ropers *et al.*, 2011). It has been suggested that genes implicated in NSMR may also harbor mutations that are associated with other developmental abnormalities, such as autism (Kirov *et al.*, 2008) and schizophrenia (Friedman *et al.*, 2008).

Inheritance Patterns of Mental Retardation

Based on the inheritance pattern, mental retardation is classified into X linked Mental Retardation (XLMR) and Autosomal Recessive Mental Retardation (ARMR). Both XLMR and ARMR are further subdivided into syndromic and non syndromic forms based specifically on presence and absence of clinically recognizable features (AAMR, 2000).

X Linked Mental Retardation

X-linked gene defects have long been considered to be important causes of mental retardation, on the basis of the observation that mental retardation is significantly more common in males than in females. Clinical observations and linkage studies revealed that X-linked mental retardation (XLMR) is a highly heterogeneous condition (Ropers and Hamel, 2005). The most common form of XLMR, the Fragile X (Fra(X)) mental retardation syndrome is associated with FMR1 gene (Verkerk *et al.*, 1991; Lina, 2008). Since then, the number of cloned XLMR genes has been increasing exponentially. X-linked gene defects are thought to be responsible for 10% of the ID found in males, which means that there must be other factors to explain why cognitive impairment is far more common in males than females (Ropers, *et al*, 2005; Nguyen *et al.*, 2006; Skuse *et al.*, 2007). So far, >80 genes have been implicated in X-linked ID, largely owing to coordinated efforts of international consortia, and mutations in these genes account for>50% of the families with this condition (Ropers *et al.*, 2008).

Syndromic and Nonsyndromic XLMR

XLMR is subdivided into syndromic (S-XLMR) and non-syndromic (NS-XLMR) forms, depending on whether further abnormalities (in addition to mental retardation) are found on physical examination, laboratory investigation and brain imaging. Roughly two thirds of XLMR cases are thought to be non syndromic; however, as the possibilities for classifying families through molecular studies improve, and as patients are examined in more detail, it is likely that the proportion of syndromic cases that are diagnosed will increase, with a concomitant decrease in the non-syndromic cases. This is illustrated by

the Fra(X) syndrome, which was initially described as non syndromic and is now considered to be the most frequent example of S-XLMR (Ropers and Hamel, 2005). Up till now 98 syndromic genes have been discovered, while the count of nonsyndromic mental retardation causing genes is 66 (Ropers and Hamel, 2005).

Fragile X Syndrome (FXS) - the most Common form of Mental Retardation

Fragile X syndrome is caused by the loss of functional FMRP which belongs to a small family of highly conserved proteins referred to as the fragile X–related proteins (Zhang *et al.*, 1995; Siomi *et al.*, 1996). FMRP binds with RNA homopolymers and mRNAs and regulates the RNA metabolism (Zalfa *et al.*, 2003; Stefani *et al.*, 2004). In neurons, FMRP is localized within and at the base of dendritic spines in association with polyribosomes (Feng *et al.*, 1997). FXS is exclusively caused by an expansion of a CGG repeat in the 5′ untranslated region of the FMR1 gene and was the first example of a trinucleotide repeat expansion mutation (Oostra and Willemsen, 2009). In the normal population, the CGG repeat ranges from 5-50 CGGs with an average length of 30 CGG units, while Fragile X patients have >200 CGG units that are usually hypermethylated and the methylation extend to the adjacent promoter region of the FMR1 gene. The gene is transcriptionally silenced and the gene product (fragile X mental retardation protein (FMRP) is absent. The lack of FMRP in neurons is the cause of the mental retardation in fragile X patients (Verkerk *et al.*, 1991; Lina *et al.*, 2008). Recent studies have linked the miRNA pathway to fragile X syndrome (Qurashi *et al.*, 2007).

Major Genes for XLMR

IL1RAPL1 (interleukin 1 receptor accessory protein-like 1) was first identified as a candidate gene for mental retardation after the finding of deletions in families with mental retardation, adrenal hypoplasia, Duchenne muscular dystrophy, and glycerol kinase deficiency. Initially, a mutation was identified in 1/20 small XLMR families (Carrie *et al.*, 1999), but latter complex rearrangement of this gene has been described (Wheway *et al.*, 2003).

Tetraspanins, so named for the presence of four transmembrane domains, are present in nearly all mammalian cells (Todd *et al.*, 1998). The TM4SF2 gene is expressed in a range of human tissues, including fetal and adult brain. Its encoded protein is a member of the tetraspanin family members which are known to participate in molecular complexes such as b-integrins. Such complexes are accepted to be involved in the regulation of actin cytoskeleton organization, suggesting that the primary defect resulting from mutations in the TM4SF2 gene would be an impaired ability of the actin cytoskeleton to drive neutrite outgrowth leading to an abnormal neuronal function (Castellvi and Mila, 2000).

PAKs are serine/threonine kinases activated by small GTPases of the Rho-family, namely Rac1 and Cdc42. The mutations in the PAK3 gene have been associated with non syndromic MR cases (Allen *et al.*, 1998; Bienvenu *et al.*, 2000). Interestingly, their contribution to synapse formation and plasticity has been clearly demonstrated and are involved in Signal transduction (Humeau *et al.*, 2009).

Autosomal Mental Retardation

Autosomal mental retardation can be either syndromic or non-syndromic on the basis of phenotypic appearance and dominant or recessive, on the basis of mode of inheritance. Anautosomal recessive mode of inheritance may account for nearly a quarter of all individuals with non-syndromic mental retardation (NSMR). In non-syndromic ID, cognitive impairment is the single clinical feature among the patients.

Genes Involved in ARNSMR

Until 2011, eight gene defects have been clearly linked to non syndromic autosomal recessive mental retardations, i.e. PRSS12 (neurotrypsin; MIM 606709), CC2D1A (MIM 610055), CRBN (cereblon; MIM609262), GRIK2 (glutamate receptor 6; MIM#138244), TUSC3 (MIM #601385), TRAPPC9 (MIM#611966), TECR and most recently SWIP (Ropers *et al.*, 2011). Studies on very large consanguineous families have yielded numerous additional loci for MR (kuss *et al.*, 2011) and are shown in table 1.1

Table: 1.1: Genes and loci involved in ARNSMR

Locus	Chromosomal Location	Gene	Symptoms
MRT1	4q25-q26	PRSS12	Severe
MRT2A	3p26.3	CRBN	Mild
MRT3	19p13.12-p13.2	CC2D1A	Severe
		TECR	Severe
MRT4	1p33-p34.3	Unknown	Mild
MRT5	5p15.2-p15.32	Unknown	Moderate-Severe
MRT6	6q16.1q21	GRIK2	Mild to severe
MRT7	8p12	TUSC3	Moderate
MRT8	10q21.3-q22.3	Unknown	Mild -moderate
MRT9	14q12-q13.1	Unknown	Mild
MRT10	16p12.1-q12.1	Unknown	Moderate
MRT11	19q13.2-q13.32	Unknown	Moderate
MRT12	1p34-p33	Unknown	Severe
MRT13	8q24.3	TRAPPC9	Moderate
MRT14	2p25.3-p25.2	Unknown	-

Continued:

Locus	Chromosomal Location	Gene	Symptoms
MRT15*	9q34.3	Unknown	-
MRT16*	9p23-p13.3	Unknown	-
MRT17*	11p15.5-p15.4	Unknown	-
Not allocated	12q23-q24	SWIP	Moderate-Severe
MRT*	Chr1	Unknown	Moderate
MRT*	Chr1	Unknown	Moderate
MRT*	Chr4	Unknown	Moderate–severe
MRT*	Chr4	Unknown	Severe
MRT*	Chr4	Unknown	Moderate
MRT*	Chr5	Unknown	Moderate
MRT*	Chr9	Unknown	Mild–moderate
MRT*	Chr9	Unknown	Moderate
MRT*	Chr14	Unknown	Mild–moderate
MRT*	Chr15	Unknown	Moderate
MRT*	Chr17	Unknown	Moderate
MRT*	Chr19	Unknown	Moderate
MRT*	Chr19	Unknown	Mild–moderate

* Linkage regions for NS-ARMR, published by (kuss *et al.*, 2011; Ropers *et al.*, 2011 and Rehman *et al.*, 2011).

***PRSS12* Gene**

Neurotrypsin (*PRSS12*) gene encodes a secreted protein of 875 amino which belongs to the subfamily of trypsin-like serine proteases (Molinari *et al.*, 2002). The *PRSS12* gene is most likely involved in synapse maturation and neural plasticity (Wolfer *et al.*, 2001). Molinari *et al.*, (2002) demonstrated that neurotrypsin is highly expressed in brain and involved in learning and memory.

Recently, Reif *et al.*, (2007) explained that this protein specifically cleaves agrin, which is involved in the formation of filopodia on neuronal axons and dendrites (McCroskery *et al.*, 2006; Annies *et al.*, 2006). Agrin cleavage generates an inactivating ligand of the Na+/K+ - ATPase at CNS synapses, previously identified as the neuronal receptor for agrin (Hilgenberg *et al.*, 2006). Neurotrypsin is highly expressed in the cerebral cortex, the hippocampus and the amygdala. Within neuronal cells, it is localized in the pre-synaptic membrane and the pre-synaptic active zone of both excitatory and inhibitory synapses (Vanagaite *et al.*, 2007). Recent studies on the expression of neurotrypsin in hippocampal neurons argue for an essential role of Neurotrypsin in activity-dependent synapse remodelling (Frischknecht *et al.*, 2008).

***CRBN* Gene**

The second gene causing ARNSMR, *CRBN*, was identified in a family originating from Germany with 10 affected individuals, is located at 3p26.2 (Higgins *et al.*, 2004). A nonsense mutation R419X in the *CRBN* gene results in truncated protein (Joseph *et al.*, 2008). The cereblon protein encoded by the *CRBN* gene, belongs to an ATP dependent Lon protease gene family represented by multi-domain enzymes associated with diverse functions from proteolysis to membrane trafficking (Jo *et al.*, 2005). Cereblon was identified as being directly associated with large conductance Ca21-activated K1 (BKCa) channels (Rotanova *et al.*, 2006). Recently it was found that BKCa channel overexpression causes impairment of learning and memory in hippocampal-dependent tasks, but does not alter basal synaptic transmission or pre-synaptic release mechanisms (Hammond *et al.*, 2006). CRBN is also involved in regulating the surface expression and electrical properties of BKCa channels (Jo *et al.*, 2005). It was reported that endogenous

CRBN was expressed in the juxtanuclear region and cytoplasm, but not in the nucleus (Sooyeon *et al.*, 2005). CRBN is a cytosolic protein and regulates the assembly and neuronal surface expression of large conductance Ca2+-activated K+ channels (BKCa) in brain regions involved in memory and learning. Such alterations may contribute to cognitive impairments in patients with mild ARNSMR (Joseph *et al.*, 2008).

CC2D1A Gene

Vanagaite *et al.*, (2005) identified a protein-truncating mutation in the gene *CC2D1A* in nine consanguineous families with severe autosomal recessive NSMR. *CC2D1A* is a member of a previously uncharacterized gene family that carries two conserved motifs, a C2 domain and a DM14 domain. The C2 domain is found in proteins which function in calcium-dependent phospholipid binding. The DM14 domain is unique to the CC2D1A protein family, and its role is unknown. CC2D1A (Freud-1) is a putative signal transducer participating in the positive regulation of the I-kB kinase/NF-kB cascade, which plays a role in neurotrophin regulated signaling pathways that control many aspects of survival, development, and function of neurons (Reichardt *et al.*, 2006). CC2D1A and CC2D2A (Vanagaite *et al.*, 2007) may have similar functions and may be components of the same or parallel pathways that are important components for neuronal development, disruption of which leads to developmental delay (Noor *et al.*, 2008). Phylogenetic analysis shows similarity to many potential orthologues in mammals, in other vertebrates and in invertebrates, but no candidate orthologue was found in the yeast genome (Vanagaite *et al.*, 2006).

GRIK2 Gene

Ionotropic glutamate receptor 6 gene (*GRIK2*, also called "*GLUR6*") was first time identified in consanguineous Iranian family (Motazacker *et al.*, 2007) with moderate-to-severe nonsyndromic autosomal recessive mental retardation. The predicted gene product lacks the first ligand-binding domain, the adjacent transmembrane domain and the putative pore loop, suggesting a complete loss of function of the GLUK6 protein. *GRIK2* encodes GLUK6, which is highly expressed in the brain. GLUK6-containing receptors are present at the presynaptic level (Barrett and Edgar, 2006) as well as at the

postsynaptic level (Bureau *et al.*, 2000) and the primary involvement of GLUK6 in excitatory synaptic activity suggests that defective GLUK6 might cause cognitive impairment through alterations in local brain circuitry (Motazacker *et al.*, 2007).

TUSC3 Gene

TUSC3 is the fifth gene that has been implicated in NS-ARMR and has 11 exons spanning ~224 Kbp of the genomic DNA on chromosome 8p22. It encodes a predicted 348 amino acid protein with five potential transmembrane domains and seems to be involved in protein glycosylation pathway. *TUSC3* is assumed to encode a subunit of the ER-bound oligosaccharyltransferase (OST) complex that catalyzes a fundamental step in the protein N-glycosylation process (MacGrogan *et al.*, 1996) and interacts with the alpha isoform of the catalytic subunit of protein phosphatase 1 (PPPC1A) (Munton *et al.*, 2004). It remains to be seen whether TUSC3 has a direct role in glycosylation and why in patients with TUSC3 deficiency clinical signs are confined to the brain (Garshasbi *et al.*, 2008). The findings of Calıskan *et al.*, (2011) indicated that affected individuals show no aberrant glycosylation of serum, assuming a loss of function of TUSC3 resulting in nonsyndromic MR by homozygous frameshift mutation in the *TUSC3* gene (Molinari *et al.*, 2008). The study of Caliskan *et al.*, (2011) as well as the report by Molinari *et al.*, (2008) indicate that mutations leading to loss of function of TUSC3 give rise to NS-MR. Khan *et al.*, (2011) identified and reported a novel deletion mutation in TUSC3 gene in a family with autosomal recessive NS-ASRM from Pakistan.

TRAPPC9 Gene

It is a 23 exon gene that encodes the 1148–1246 amino acid protein, NIBP, which is highly conserved across evolution. In humans, multiple NIBP isoforms are expressed at high levels in the muscle and kidney and to a lesser extent in the brain, heart, and placenta. Only isoform 1 is present in the brain, where it is expressed in the cell bodies and processes of neurons, while Knockdown of NIBP has been shown to reduce TNFa-induced NF-kB activation, prevent nerve growth factor-induced neuronal differentiation (Hu *et al.*, 2005). DNA Sequence analysis revealed a nonsense variant (c.1708C>T [p.R570X]) within exon 9 of this gene that is responsible for an undetectable level of

TRAPPC9 protein in patient skin fibroblasts. Moreover, stimulation of TNF-a causes defect in IkBa degradation, suggesting impaired NF-kB signaling in patient cells that results in MR (Philippe *et al.*, 2009). Loss of NIBP function through truncating mutations might cause disruption of neuronal differentiation, which could result in the cerebral white matter hypoplasia observed through MRI (Mir *et al.*, 2009).

***TECR* Gene**

TECR (trans-2,3-enoyl-CoA reductase), also referred to as GPSN2 (synaptic glycoprotein2) identified by Calıskan *et al.*, (2011) on 19p13 in families with non syndromic autosomal mental retardatioms. TECR is a synaptic glycoprotein that is involved in the synthesis of very long chain fatty acids (VLCFA) in a reduction step of the microsomal fatty acyl elongation process (Moon *et al.*, 2003). The mouse orthologue of *TECR* is highly expressed in the nervous system (Zhang *et al.*, 2004). The role of *TECR* in fatty acyl elongation and its high expression in nervous system suggest that it act as a synaptic glycoprotein and may have a specialized function in the nervous system that affects communication between neurons (Martin *et al.*, 2002) or synaptic plasticity (Smalla *et al.*, 2000; kleene *et al.*, 2004). Although the precise mechanism through which the mutations in *TECR* leads to mental retardation is not known, the role of this gene in fatty acid synthesis and the phenotypes of the affected individuals suggest that NSMR indicate inborn error of metabolism.

***SWIP (K1AA1033)* Gene**

SWIP (**S**trumpellin and **W**ASH **I**nteracting **P**rotein) gene is a member of the recently discovered WASH complex, which is involved in actin polymerization and multiple endosomal transport processes. The mutation in this gene was identified by Ropers *et al.*, (2011) in a large consanguineous family with autosomal recessive intellectual disability (ARID). This mutation is thought to reduce SWIP levels and to destabilize the entire WASH complex. The potentially disease-causing mutations, c.3056C.G was identified, which, results in a Pro1019Arg which destabilizes the SWIP. SWIP is a large ubiquitously expressed protein of 1173 amino acids, with no identifiable domain. Recently it has been described (Derivery *et al.*, 2009; Rottner *et al.*, 2010) as a subunit of

the WASH complex which involves a core of five subunits, namely SWIP, FAM21, strumpellin, Ccdc53 and WASH and the recruitment of the heterodimer of Capping Protein. These trafficking pathways are very important in neurons, where cargoes have to travel long distances. Alterations in these pathways may lead to several forms of neuropathies (Dion *et al.*, 2009).

Genes Involved in Autosomal Dominant NSMR

Identification of autosomal dominant genes causing MR is very difficult due to the lack of large families because the affected individuals rarely reproduce. Only a single gene causing autosomal dominant familial non-syndromic MR have been reported to date (Fadi *et al.*, 2009), although several genes assumed to be involved in autosomal dominant non syndromic MR have been identified by mapping of the chromosomal breakpoints in patients with balanced chromosomal aberrations (Guzauskas *et al.*, 2007).

Moreover, autosomal dominant genes have yet to be identified, mainly because mental retardation results in lower reproductive fitness, which in turn decreases the likelihood of identifying families that are amenable to linkage analysis. However a very recent study has reported the presence of de novo truncating mutations in the autosomal gene SYNGAP1 in individuals with autosomal dominant NSMR (Fadi *et al.*, 2009).

Mental Retardation in Pakistan

The prevalence of MR has been found to be higher in Pakistan than in developed countries. It also seemed to be related to poor socioeconomic conditions, as the prevalence in the upper-middle class is comparable to figures from developed countries, while the prevalence in children from poor population groups is much higher (Bashir *et al.*, 2007).

Few reports are available from Pakistani population on molecular genetics of XLMR (Ahmed *et al.*, 1999, Ahmed *et al.*, 2001) and syndromic autosomal recessive mental retardation (John *et al.*, 2006, Noor *et al.*, 2008). Some other studies have reported the presence of mental retardation with alopecia (John *et al.*, 2006; Wali *et al.*, 2006; Wali *et*

al., 2007). A new syndrome MORM (mental retardation, truncal obesity, retinal dystrophy and micropenis) has also been reported from a Northern Pakistani family (Hampshire *et al.*, 2006) with a mutation in INPP5E gene which causes primary cilium signaling defects, ciliary instability and ciliopathies in human and mouse (Monique *et al.*, 2008). Study on consanguineous Pakistani family having ARMR with Retinitis Pigmentosa (Noor *et al.*, 2008) showed a homozygous splice site mutation within a coiled-coil and C2 domain-containing gene, CC2D2A. Skipping of exon 19 resulted in frameshift and a truncated protein lacking the C2 domain. Mir *et al.*, (2009) identified TRAPPC9 gene for autosomal recessive mental retardation in a large consanguineous Pakistani family, with confirmation in an Iranian family. In the Pakistani family, although the phenotype appears to be nonsyndromic MR, mild cerebral white matter hypoplasia and small head circumference are present in most of the patients. Genetic study of more than 10 consanguineous Pakistani families with NS-ARMR showed linkage to three novel loci (MRT14, 15 and 16) in addition with already known NS-ARMR loci (Rafiq *et al.*, 2010). In another study a novel deletion mutation in the TUSC3 gene in a consanguineous Pakistani family with autosomal recessive nonsyndromic intellectual disability has been determined (Khan *et al.*, 2011). This deletion encompassed almost the entire TUSC3 and its downstream region. Recently autozygosity mapping of Pakistani families revealed a novel locus, MRT17 for NS-ARMR, in the telomeric 11p15 region (Rehman *et al.*, 2011).

The purpose of current research is to perform linkage analysis of Pakistani families with inherited ARNSMR. The collected families will be tested for linkage to known loci and efforts will be made to identify the candidate genes responsible for ARNSMR in these Pakistani families.

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Families Studied

In this study, two families represented as family 'A' and 'B' suffering from autosomal recessive non syndromic mental retardation were recruited from remote areas of Khyber Pakhtunkhwa (KPK) Province of Pakistan. The families were visited at their place of residence to collect relevant information to construct pedigrees by following the guidelines described by Bennett *et al* (1995) with software named Cyrillic 2.1. In the pedigrees of both families, males and females were symbolized by squares and circles respectively. Unfilled symbols designate normal individuals, while black filled ones represent affected individuals. Deceased individual are represented by crossed circles and squares. Roman numerals are given to successive generations and Arabic numbers are assigned to individuals within a generation. Double lines are drawn to represent consanguineous marriage. Autosomal recessive mode of inheritance was observed by segregation of disease within the family.

2.2 Clinical Examination and Diagnosis

The families under study were diagnosed on the basis of clinical data. MR was diagnosed by learning disabilities, cognitive impairment, I.Q and available clinical data. The prenatal and postnatal history of the affected members was collected by interviews in local language and by different tests. Photographs of each affected individual were taken to evaluate facial dysmorphism.

2.3 Blood Sampling

Blood samples from normal and affected individuals of both families, including their parents were drawn with sterile 10 ml syringes and immediately transferred into standard potassium ethylene diamine tetra acetate (EDTA) vacutainer tubes (BD Vacutainer K3 EDTA, Franklin Lakes USA) to prevent clotting. Standard procedure of blood sampling was used to ensure patients safety and the used syringes were immediately discarded in safe manner. The blood samples were transported to lab of Genomics (LOG), Department of Biochemistry, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad for storage at 4^0C till further processing for genomic DNA extraction.

2.4 Extraction of Genomic DNA from Human Blood

Total human genomic DNA was extracted and purified from peripheral blood samples of each individual from both families by using two methods:

- Chloroform-phenolic extraction
- Commercially available kit

2.4.1 Chloroform-Phenolic Extraction or Organic Preparation

0.75 ml blood was taken in a 1.5 ml microcentrifuge tube and mixed with an equal volume of solution A and incubated at room temperature for 10-15 minutes. The tubes containing equal volume of blood and solution A were then centrifuged in a microcentrifuge for 1 minute at a speed of 13,000 rpm (Eppendorf, Model 5417C, Germany). After discarding the supernatant, the pellet was resuspended in 400 µl of solution A and was centrifuged again at 13,000 rpm for 1 min. Supernatant of this centrifiguation was again discarded and nuclear pellet was resuspended by gentle shaking in equal volume of solution B and digested at 37^0C overnight by adding 12 µl of 20% SDS solution and 5 µl of proteinase K (20mg/ml). On the following day 0.5 ml of fresh mixture of equal volume of solution C and D was added in samples, mixed and centrifuged at 13,000 rpm for 10 minutes. The upper layer or aqueous phase was shifted to a new microcentrifuge tube carefully and equal volume of solution D was added. Centrifugation was then carried out again at 13,000 rpm for 10 minutes and aqueous phase (upper layer) was transferred to a new tube. After adding 55 µl of 3M sodium acetate (pH 6) and equal volume of isopropanol, the tubes were inverted gently for several times to precipitate DNA. Finally the DNA pellet was washed with chilled 70% ethanol and dried in the incubator B28 (Binder, Germany) for 10-15 minutes. After evaporation of ethanol, DNA was dissolved in appropriate amount (150-200 µl) of Tris-EDTA (T.E) buffer.

2.4.2 Composition of Solutions

Solution A

Sucrose 0.32 M

Tris (pH 7.5) 10 mM

$MgCl_2$ 5 mM

1% (v/v) Triton X-100

Solution B

Tris (pH 7.5) 10 mM

NaCl 400 mM

EDTA (pH 8.0) 2 mM

Solution C

Phenol

Solution D

Chloroform 24 volumes

Isoamyl alcohol 1 volume

DNA Dissolving Buffer (T.E.)

Tris (pH 8.0) 10 mM

EDTA 0.1 mM

2.4.3 DNA Extraction by Commercially Available Kit

DNA extraction was also carried out by using Genomic Isolation Kit (Pure Gene, Gentra USA). Three hundred microlitre of blood was taken in 1.5 ml microcentrifuge tube along with 900 µl of RBC lysis solution. The mixture was then kept in incubator for one minute at room temperature then centrifuged for 20 seconds at 13000 rpm. After centrifugation 300 µl of cell lysis solution was added, vortexed vigorously for 3 minutes and were placed in ice for 1 minute. After cooling, protein precipitation solution (100 µl) was added to the cell lysate and centrifuged for 1 minute at 13,000 rpm. The DNA suspended

in the supernatant was taken into a clean tube, and 300 µl of 100% isopropanol was added to precipitate genomic DNA. Samples were then centrifuged at 13000 rpm for 1 minute and isopropanol was removed, and then 70% ethanol was added to this precipitated DNA for thorough washing. After evaporation of residual ethanol, 50 µl of DNA hydration solution was added and incubated at 65°C for 5 minutes. After the extraction of genomic DNA, 1% agarose gel was used to visualize the integrity of obtained DNA samples.

2.5 Quantification of DNA

Extracted DNA was quantified by using nanodrop 1000 spectrophotometer (Thermo scientific, USA) to evaluate the quality and quantity of DNA. For this purpose, 1µL DNA sample was taken and absorbance at 260 and 280 was measured to find concentration of each DNA sample. Then each sample was further diluted to 40ng/µl for PCR amplification.

2.6 Linkage Analysis of Families with Mental Retardation

In order to find causative gene in two MR families, pedigrees were analyzed to infer the mode of inheritance. In both families, search for linkage was carried out by genotyping polymorphic microsatellite markers mapped within known autosomal recessive mental retardation (ARMR) loci. Table 2.1 summarizes microsatellite markers for 12 known ARNSMR loci, which were used for linkage analysis in the families A and B.

The microsatellite markers were amplified by using PCR, and PCR products were run on 8% standard non-denaturing polyacrylamide gel as described below. The ethidium bromide stained polyacrylamide gels were visualized on UV transilluminator and genotypes were assigned by visual inspection of images acquired by gel documentation system (SYNGENE, UK). Microsatellite markers mapped by Cooperative Human Linkage Centre (CHLC), were obtained from Gene link (USA). The cytogenetic locations of these markers were obtained from Rutger's map (Kong *et al.*, 2005).

2.6.1 Polymerase Chain Reaction (PCR)

PCR reaction was performed in 0.2 ml tubes (Axygen, USA) containing 25 µl total reaction mixture. The reaction was prepared by adding 1 µl sample DNA (~40 ng), 2.5 µl

10 X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTPs (10 mM), 0.3 µl of each forward and reverse primer (30 pmole/ul) and 0.2 µl Taq DNA polymerase (5 U/µl, Fermentas, UK) in 18.7 µl PCR water. The reaction mixture was centrifuged for few seconds for thorough mixing. The reaction mixture was taken through thermocycling conditions consisting: 5 minutes at 95°C for denaturation of template DNA followed by 40 cycles of amplification each consisting of 3 steps: one minute at 95°C for denaturing DNA into single strands; 1 minute at 55°C (variable) for primers to hybridize or "anneal" to their complementary sequences on either side of the target sequence: and one minute at 72°C for extension of complementary DNA strands from each primer, final 10 minutes at 72°C for Taq DNA polymerase to synthesize any unextended strands left. PCR was carried out in Gene Amp PCR system 9700 (Applied Biosystems, Singapore) or T1 thermocycler (Biometra, Germany).

PCR products of amplified DNA samples were analyzed on 2% agarose gel prepared by melting 1g of agarose in 50 ml 1 X TBE (0.89 M Tris-Borate, 0.032 M EDTA, pH 8.3). Ethidium bromide (5µl), (0.5 µg/ml final concentrations) was added to visualize DNA with UV transilluminator.

2.6.2 Vertical Gel Electrophoresis

After amplification the PCR products were resolved on 8% non-denaturing polyacrylamide gel. Gel solution was made in a 250 ml conical flask, and was poured between the two glass plates separated at a distance of 1.5 mm with spacers. After placing the comb, gel was allowed to polymerize for 45-60 minutes at room temperature. After polymerization of gel the glass plates were fixed in vertical gel tanks in such a way that the smaller plate face inside the tank and larger plates face outside the tank. Samples were mixed with loading dye (0.25% bromophenol blue with 40% sucrose) and loaded into the wells. Electrophoresis was performed in vertical gel tank model V16-2 (Life Technologies, USA) at 100 volts (30 mA) electric current for 1-2 hours, depending upon the size of amplified product. The gel was stained by dipping it in ethidium bromide solution (0.5ug/ml) and visualized on Gel Doc system (SYNGENE, UK). Finally acquired images were analyzed to score allele pattern of every genotyped marker for each individual of both families.

2.6.2.1 Composition of 8% Polyacrylamide Gel (50 ml)

13.5 ml 30% Acrylamide solution (29g polyacrylamide, 1g NN'-Methylene-bisacrylamide)

5 ml 10 X TBE

350 µl 10% Ammonium persulphate (AP)

17.5 µl TEMED (N, N, N', N'-Tetra Methyl Ethylene Diamine)

31.13 ml distilled water

2.6.3 Calculation of LOD Score

LOD score was calculated using the Superlink v1.5 of easyLINKAGE plus version 5.02 (Linder and Hofman, 2005). For two point linkage analysis disease allele frequency of 0.001 was used. For analysis of both families an autosomal recessive disease model and equal marker allele frequencies were used.

Table 2.1: List of microsatellite markers used to test linkage in family A and B

Marker Name	Genetic Distance (cM)* Rutgers Map	Locus Name	Chromosomal region
D4S2297	123.65	MRT1	4q25-q26
D4S1522	125.35		
D4S191	125.89		
D4S3024	126.65		
D3S3630	5.95	MRT2A	3p26.2
D3S2358	9.44		
D3S4538	9.44		
D3S1620	10.31		
D3S1560	12.64		
D19S226	36.7	MRT3	19p13.12
D19S415	36.98		
D19S929	37.65		
D1S429	133.29	MRT4	1p21.1-p13.3
GATA133A08	133.63		
D1S3723	134.79		
D5S2505	17.59	MRT5	5p15.2-p15.32
D5S1390	18.87		
D5S208	23.66		
D6S962	109.74	MRT6	6q21
D6S1642	110.69		
D8S1125	55.67	MRT7	8p12-p22
D8S379	56.67		
D10S535	93.7	MRT8	10q22
D10S1648	88.19		
D14S80	20.53	MRT9	14q11.2-q12
D14S615	21.3		
D14S54	24.91		
D14S121	28.42		
D16S685	58.4	MRT10	16p12-q12
D16S409	59.27		
D19S198	67.72	MRT11	19q13.2-q13.3
D1S186	66.7	MRT12	1p34-p33
D1S421	95.27		

cM: Centi Morgan

RESULTS

RESULTS

Description of the Families

Family A

The pedigree drawing presented in figure 3.1 indicates four generations with 5 affected individuals (IV-1, IV-2, IV-5, IV-6 and IV-9). The pedigree analysis showed that affected individuals being produced by the unaffected parents. MR is only manifested in childrens suggesting that the trait is transmitted in autosomal recessive manner. The parents (III-1, III-2, III-3 and III-4) are phenotypically normal but both couples resulted in affected children IV-1, IV-2, IV-5, IV-6 and IV-9, respectively. Detailed clinical evaluation of the affected individuals of the family A reveal severe mental retardation without any additional abnormality, low I.Q and poor adaptive and social skills. All affected individuals can recognize their parents, relatives and can easily reach home from nearby areas. Affected individuals of this family can speak, show slightly depressive behavior and never attended school and lack the concept of currency and counting. None of the affected individuals experience epileptic fits since childhood. Clinical and morphological features of affected individuals of the family A are presented in table 3.1.

For linkage and molecular analysis the blood samples were collected from seven members of family A, including three affected (IV-1, IV-2 and IV-6) and four normal (III-1, III-2, IV-4 and IV-7) individuals.

Family B

Family B also demonstrates nonsyndromic MR. The pedigree drawing presented in figure 3.2 indicates four generations with three affected males (IV-1, IV-4 and IV-5) and no female was affected in this family. The pedigree analysis showed that affected individuals being produced by the unaffected parents suggesting that the trait is transmitted in autosomal recessive manner. The parents (III-1, III-2, III-3 and III-4) are phenotypically normal but all of these couples resulted in affected children (IV-1, IV-4 and IV-5). Careful clinical evaluation of the affected individuals of the family B, reveal

the presence of severe mental retardation, aggressive and self fighting and hand biting behavior, can recognize their whole family and reach home from nearby places. The affected individuals lack concept of currency and counting, quality and quantity and cannot speak complete statements with clarity. Two affected individuals (IV-4 and IV-5) started speaking at the age of 8 years; while in affected individual (IV-1) speech develops at the age of 2 years. None of the affected individuals attended school for formal education. There are no phenotypic features of microcephly. Head circumferences of affected individuals IV-1, IV-4 and IV-5 are 46.99 cm, 51 cm and 50 cm respectively. Clinical and morphological features of affected individuals of the family B are shown in table 3.1.

For linkage studies and molecular analysis, the blood samples were collected from seven members of family, including three affected (IV-1, IV-4 and IV-5) and four normal (III-1, III-2, IV-2 and IV-3) individuals.

Linkage Analysis

On the basis of genetic linkage studies in inherited autosomal recessive nonsyndromic MR, it is clear that at least some candidate intervals should be tested for linkage or exclusion before going to genome-wide scan. In the present study, the two families (A and B) were tested for linkage to 12 known loci by genotyping polymorphic microsatellite markers mapped within the candidate linkage intervals.

Table 3.1: Clinical and morphological features of affected individuals from both families A and B

Clinical Features	Family A		Family B	
	IV-1	IV-2	IV-4	IV-5
Mental Retardation	Severe	Severe	Severe	Severe
Behavior	Depressive	Depressive	Hyperactive	Hyperactive
Aggressiveness	Less	Less	No	Yes
Growth	Normal	Normal	Normal	Normal
Schooling	No	No	No	No
Eye contact	Normal	Normal	Normal	Normal
Epileptic Fitz	No	No	No	No
Speech disabilities	No	No	Yes	Yes
Learning disability	Yes	Yes	Yes	Yes
Facial dysmorphism	No	No	No	No
Muscular dystrophy	No	No	No	No
Self biting/beating	No	No	Yes	Yes
Repetitive behavior	No	No	No	No
Watery mouth	No	No	No	No
Concept of money	No	No	No	No
Can recognize home	Yes	Yes	Yes	Yes
Age	30 years	16 years	11 years	9 years
Sex	Male	Male	Male	Male

Family A

In family A, seven DNA samples including four normal (III-1, III-2, IV-4 and IV-7) and three affected (IV-1, IV-2 and IV-6) individuals were genotyped for polymorphic microsatellite markers specific for each ARNSMR locus mentioned in table 2.1. The genotyping results (Figure 3.3-3.24) were analyzed to score alleles to find stretch of homozygosity shared by all the affected individuals. Although the homozygosity stretch was shared by the affected individuals (IV-1, IV-2 and IV-6) along with a normal member (IV-7) at MRT2A locus for three markers D3S2358, D3S4538 and D3S1560 between 9.44cM to 12.64cM, but downstream marker D3S1515 showed no further homozygosity (Fig 3.6 - 3.9). Furthermore non significant LOD score 0.79 at D3S1560 ruled out the chances for linkage at this region. Haplotype of this homozygosity stretch is shown in figure 3.25. The data for all other genotyped loci was also analyzed using easy LINKAGE plus V 5.02 (Linder and Hofman, 2005), which yielded a maximum two-point LOD score of 1.46 at the markers D19S415 (Fig 3.26) which is insignificant. Haplotype of this region is shown in figure 3.27. Hence, on the basis of the genotyping results and LOD score calculations, linkage to known loci was excluded in the family A.

Family B

Linkage analysis was performed using seven DNA samples including four normal (III-1, III-2, IV-2 and IV-3) and three affected (IV-1, IV-4 and IV-5) individuals to identify the underlying gene in this family. All members were genotyped for polymorphic microsatellite markers specific for each ARNSMR locus presented in table 2.1. From the analysis of allelic patterns obtained from results, it was evident that all the affected individuals were heterozygous for the different combinations of parental alleles (Fig 3.28-3.41). The data for all the genotyped loci was analyzed using easyLINKAGE plus V 5.02 (Linder and Hofman, 2005), which yielded a maximum two-point LOD score of 1.72 at marker D3S1560 and 1.42 at marker D1S429 (Fig 3.43) suggesting that these regions are susceptible to disease. For this purpose downstream markers were used for further testing, but no homozygosity was observed. Since homozygosity stretch is partial and the obtained LOD score is insignificant, which ruled out the chances of linkage to these regions. Haplotype of this region is shown in figure 3.42. Hence the molecular

analysis and LOD score calculation of all genotyped markers flanking known loci (table 2.1) conclusively excluded linkage in this family.

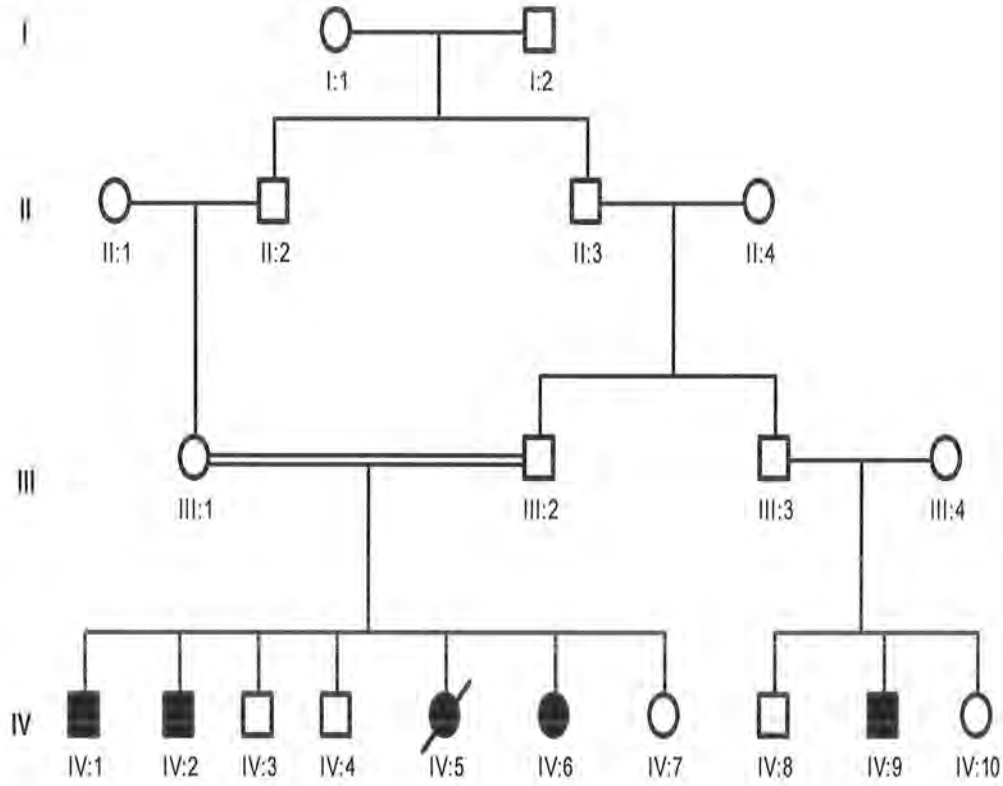


Figure 3.1: Pedigree of family A with autosomal recessive MR. The pedigree shows the recessive mode of inheritance. Circles represent females and squares represent males. Filled circles and squares represent affected individuals and hollow circles and square shows normal individuals. Double line indicates consanguineous marriages, while the crossed circle represents deceased individual.

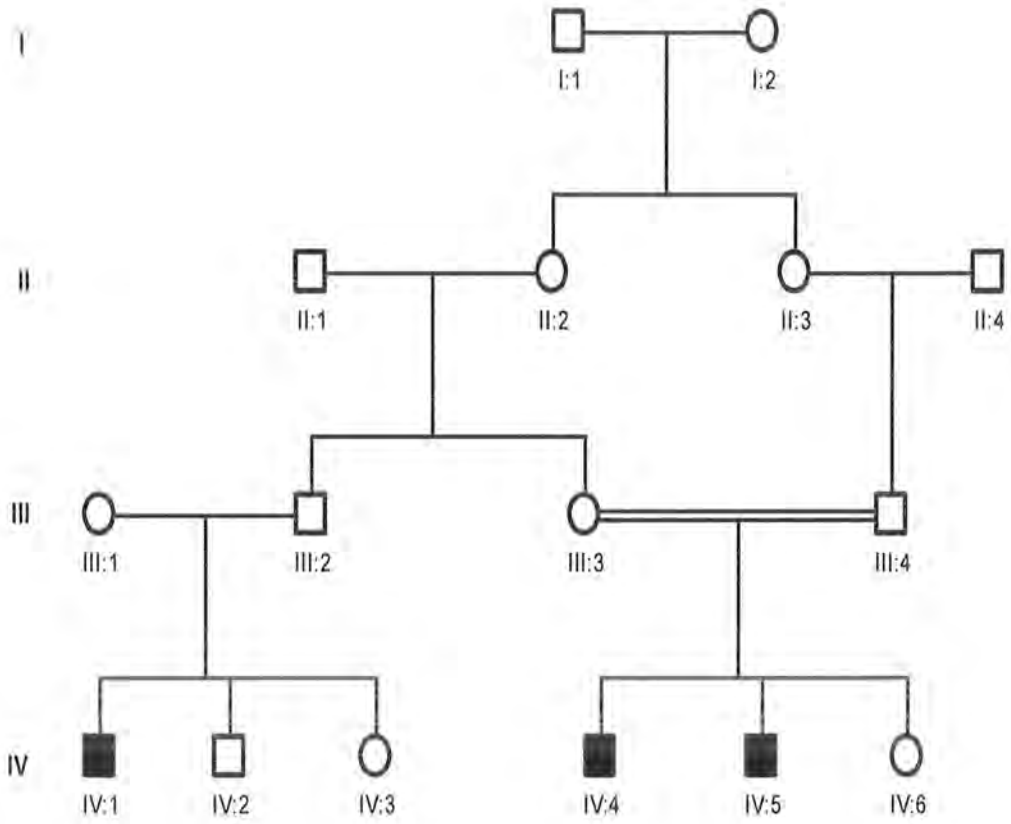
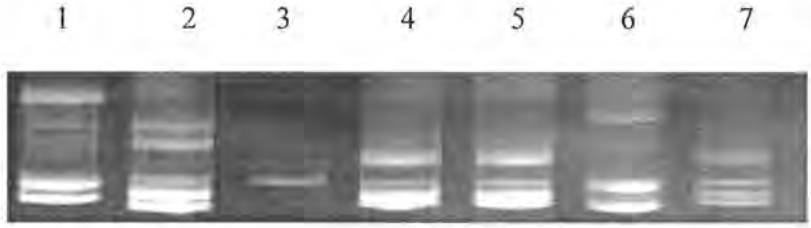


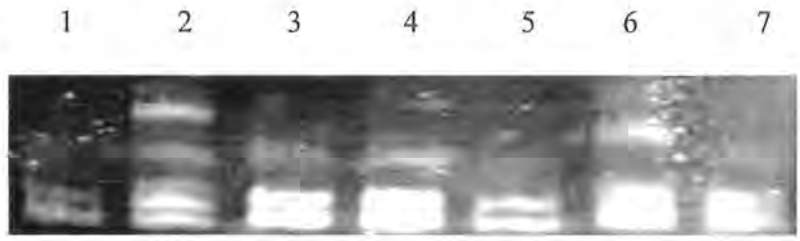
Figure 3.2: Pedigree of family B with autosomal recessive MR. The pedigree shows the recessive mode of inheritance. Circles represent females and squares represent males. Filled circles and squares represent affected individuals and hollow circles and square shows normal individuals. Double line indicates consanguineous marriages.

FAMILY A



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.3: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D4S2297 at 123.65 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.4: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D4S191 at 125.89 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.5: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D4S3024 at 126.65 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.6: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S3630 at 5.95 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

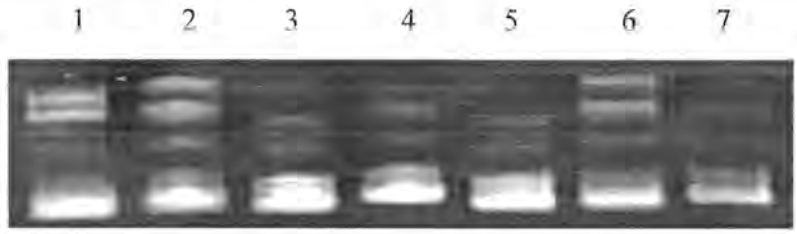
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.7: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S2358 at 9.44 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.\

7 6 5 4 3 2 1

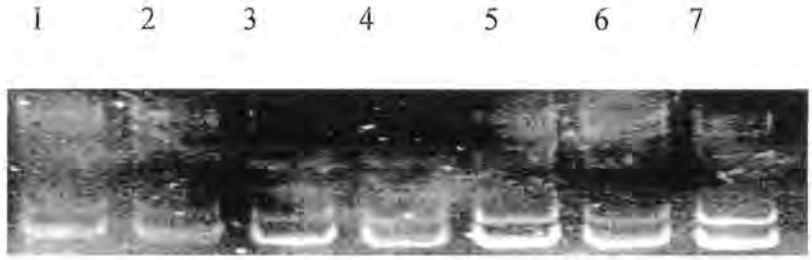
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.8: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S4538 at 9.44 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



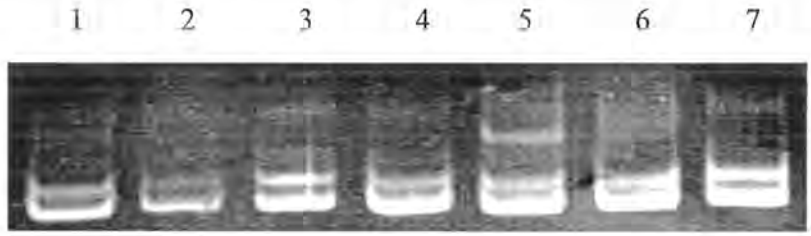
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.9: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S1560 at 12.64 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



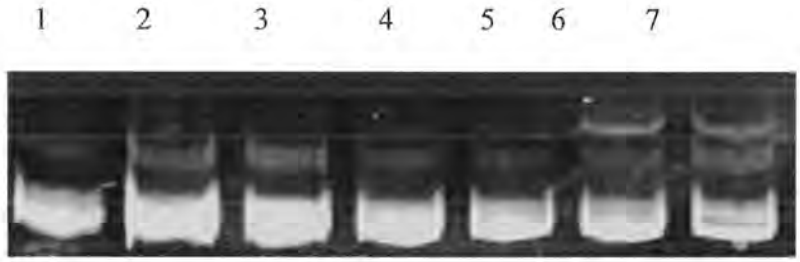
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.10: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D19S226 at 36.7 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



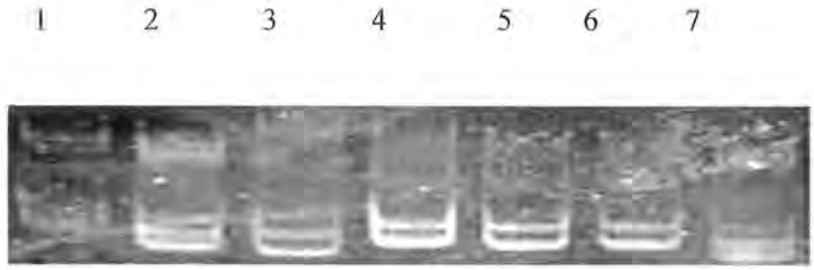
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.11: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D19S892 at 36.7 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



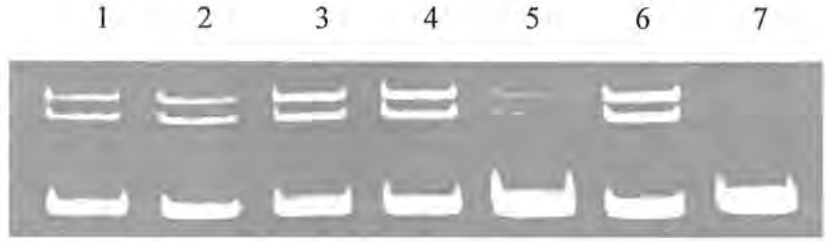
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.12: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D19S415 at 36.98 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



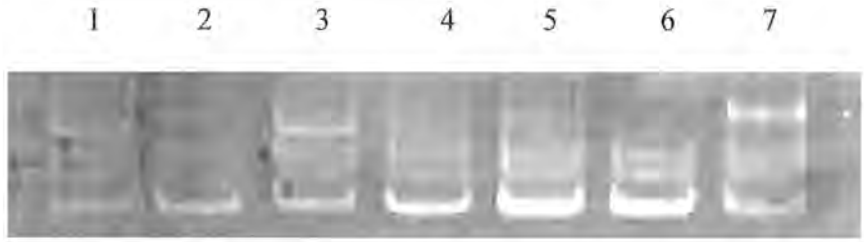
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.13: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D1S429 at 133.29 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



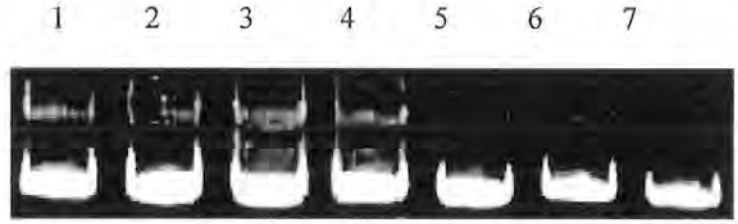
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.14: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D5S2505 at 17.59 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



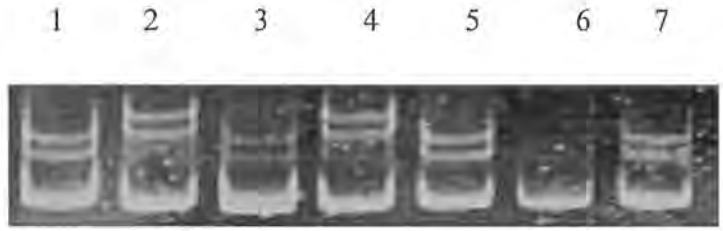
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.15: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D5S208 at 23.66 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.16: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D8S1125 at 55.67 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

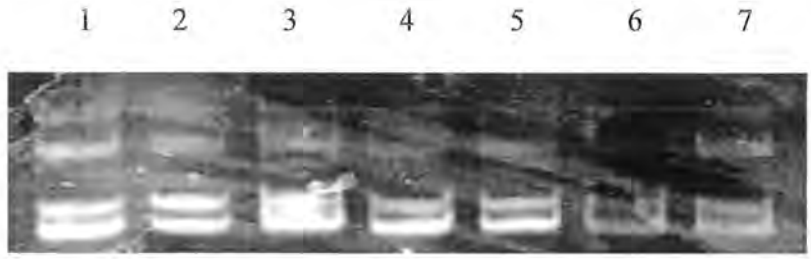


Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.17: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D8S379 at 56.67 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

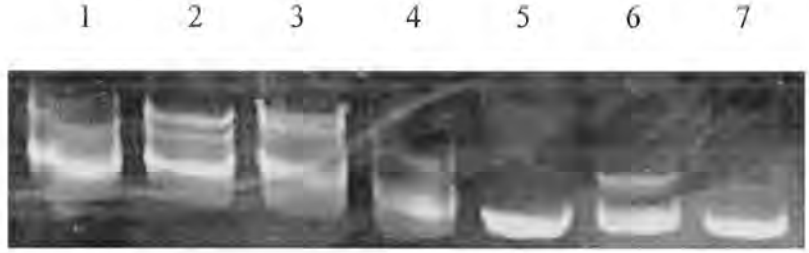
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.18: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D6S962 at 109.76 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



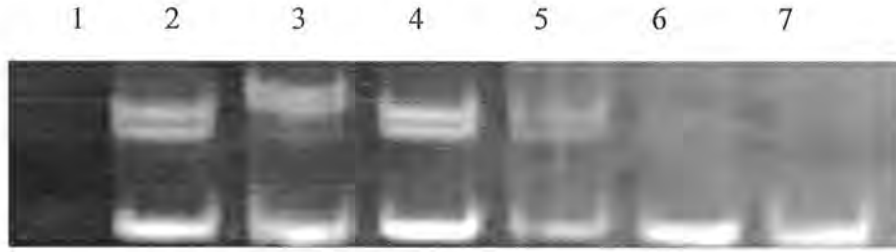
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.19: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D14S80 at 20.53 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



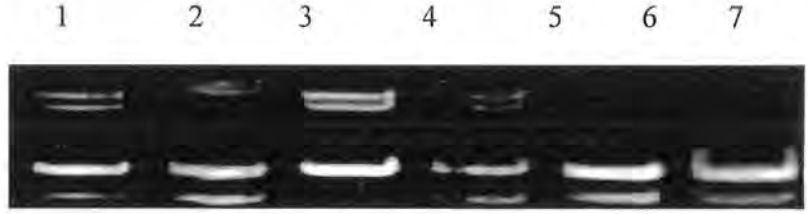
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.20: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D14S54 at 24.91 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.21: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D16S540 at 59.66 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.22: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D16S685 at 58.44 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.23: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D16S409 at 59.27 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Affected
Lane 5 IV-6 Affected
Lane 6 IV-4 Normal
Lane 7 IV-7 Normal

Figure 3.24: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D19S198 at 67.72 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

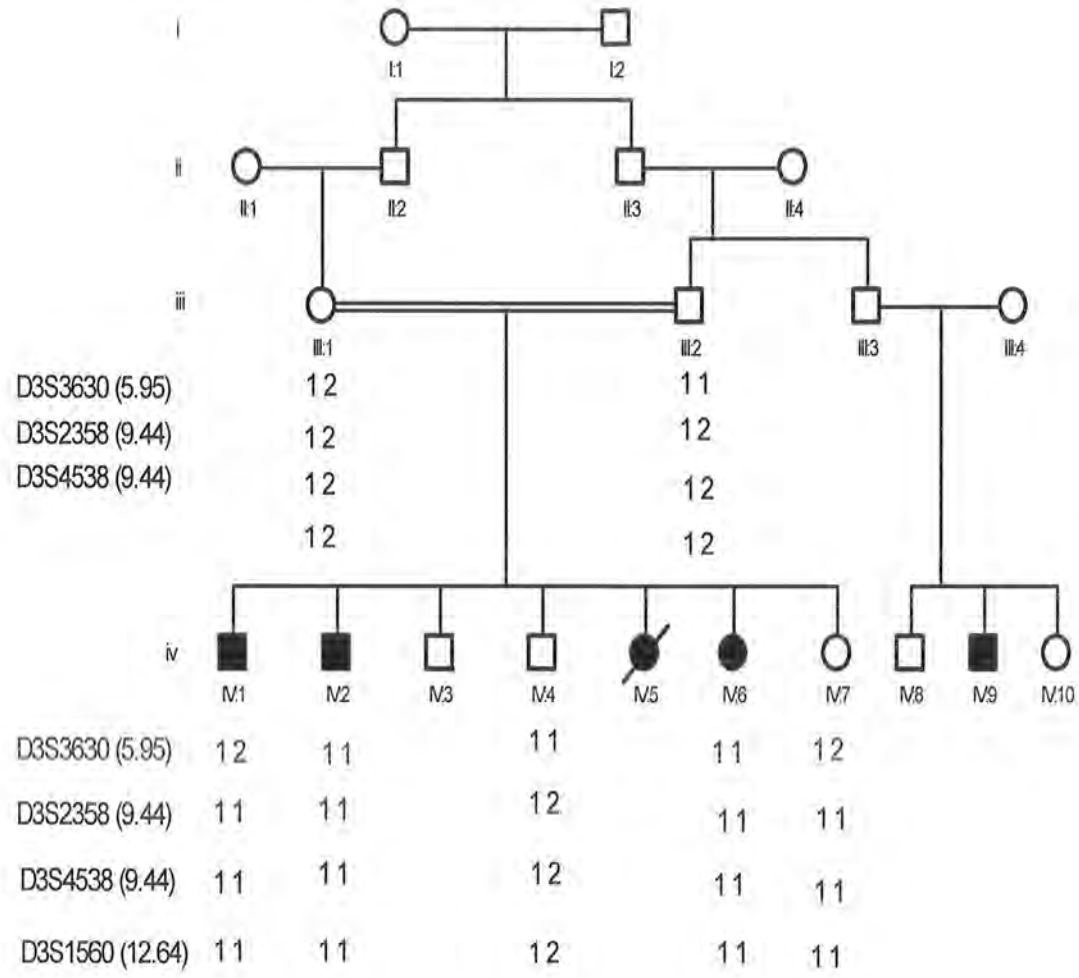


Figure 3.25: Haplotype of family A showing homozygosity stretch shared by the affected individuals at MRT2A locus on chromosome 3.

Project:	NAWAZ	Inheritance:	Recessive
Family name:	TOTALS	Common allele:	99.90 %
Used map:	Marshfield (sex averaged)	Disease allele:	0.10 %
Marker positions:	20 ok / 2 ? / 0 outside	LC1 PCOPY rate:	0.00 %
Allele frequencies:	Equal distribution of alleles	LC1 PENET wt/mt:	0.00 %
CALC interval:	Entire chromosome	LC1 PENET mt/mt:	100.00 %

Marker	CHR	cM	LOD	Theta
1.D19S415	19	42.28	1.4658	0.0000
2.D3S1560	3	18.97	0.7904	0.1000
3.D19S226	19	42.28	0.5210	0.1000
4.D16S540	16	58.47	0.4753	0.1000
5.D16S409	16	58.46	0.1046	0.1500

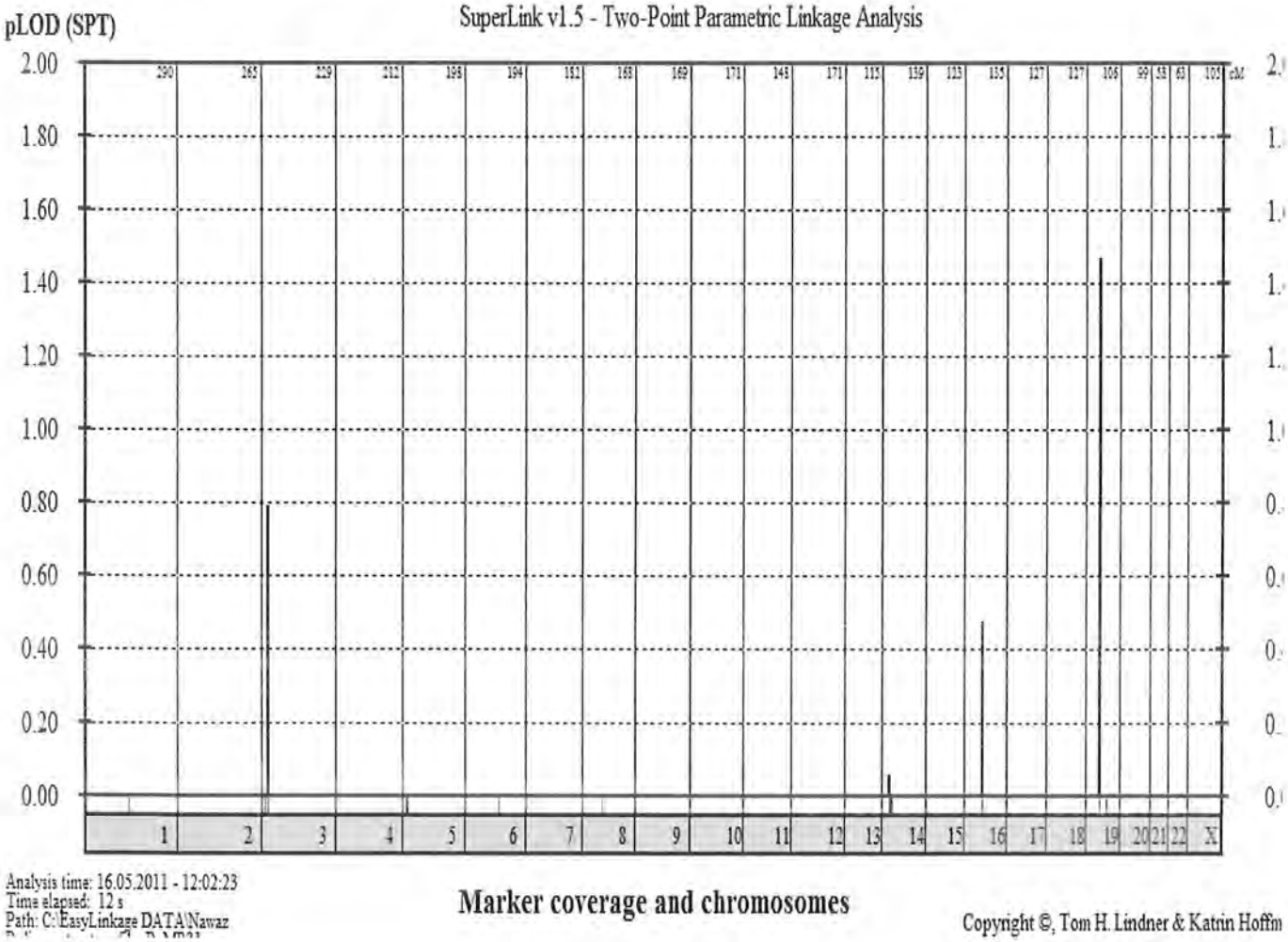


Figure 3.26: Graphical representation of LOD scores calculation using easyLINKAGE plus v5.02, with polymorphic microsatellite markers genotyped in family A. Highest LOD score of 1.46 was obtained with marker D19S415 on chromosome 19 in family A.

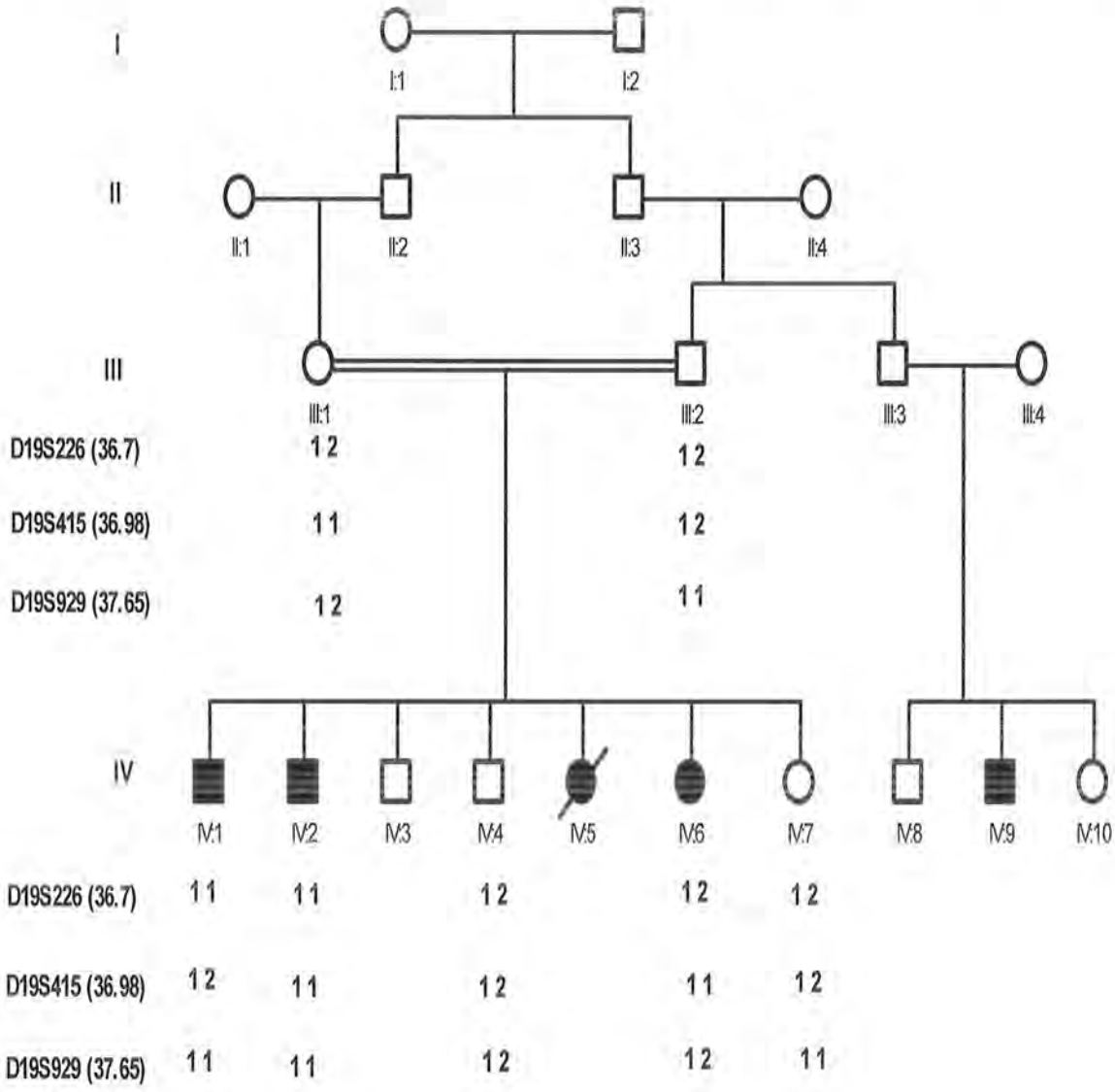
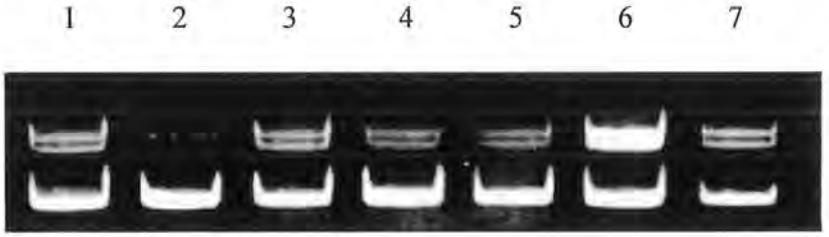


Figure 3.27: Haplptype of family A showing homozygosity on chromosome 19.

FAMILY B

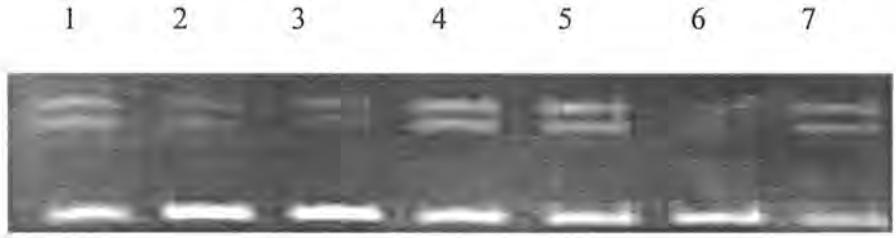
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Normal
Lane 5 IV-3 Normal
Lane 6 IV-4 Affected
Lane 7 III-5 Affected

Figure 3.28: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D4S2297 at 123.65 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



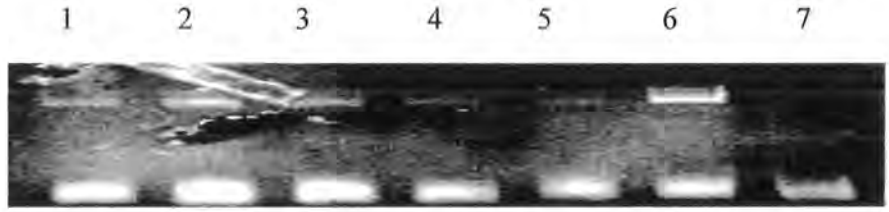
Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Normal
Lane 5 IV-3 Normal
Lane 6 IV-4 Affected
Lane 7 III-5 Affected

Figure 3.29: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D4S1522 at 125.35 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



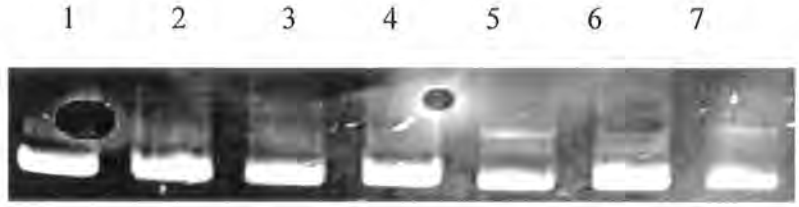
Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.30: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D4S191 at 125.89 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.31: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S2358 at 9.44 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

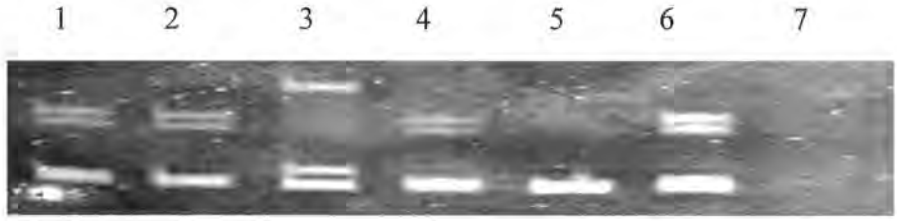


Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.32: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S1620 at 10.31 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

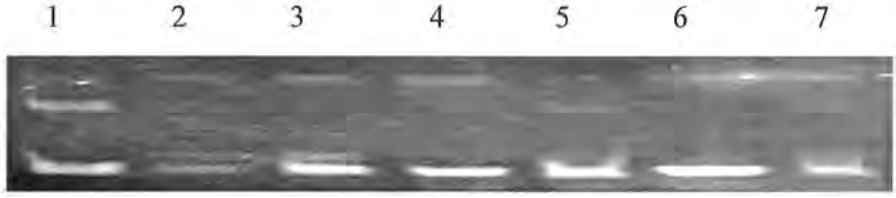
Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.32: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D3S1560 at 12.64 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



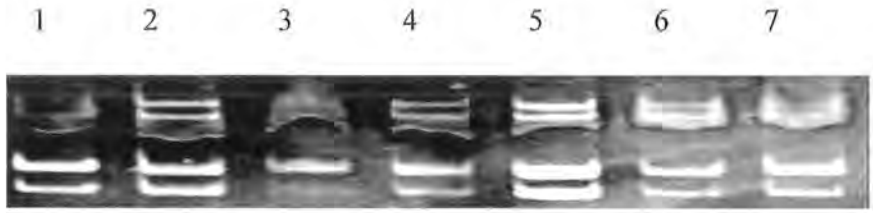
Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.33: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker GATA133A08 at 133.63 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



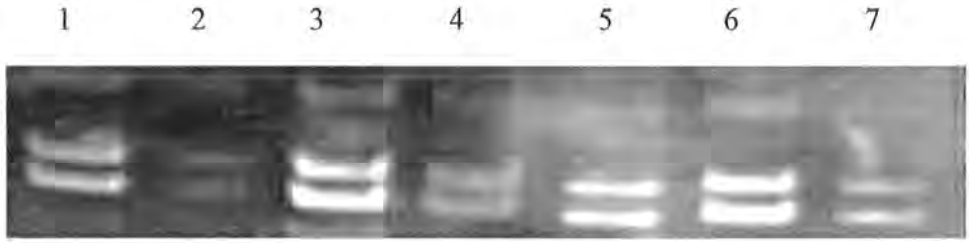
Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.34: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D1S3723 at 134.79 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



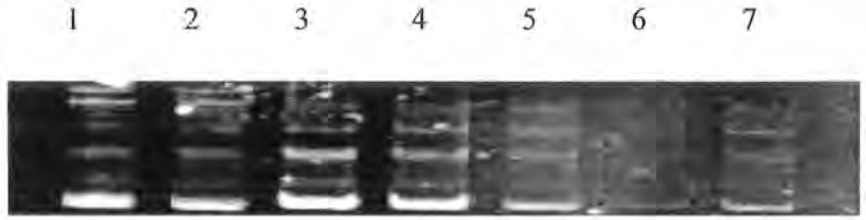
Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.35: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D5S2505 at 17.59 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



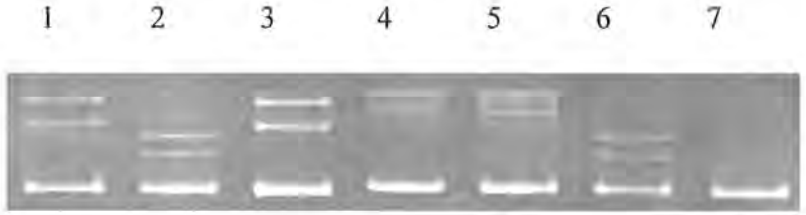
Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.36: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D5S208 at 23.66 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Normal
Lane 5 IV-3 Normal
Lane 6 IV-4 Affected
Lane 7 III-5 Affected

Figure 3.37: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D8S1125 at 55.67 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.



Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Normal
Lane 5 IV-3 Normal
Lane 6 IV-4 Affected
Lane 7 III-5 Affected

Figure 3.38: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D8S379 at 56.67 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Normal
Lane 5 IV-3 Normal
Lane 6 IV-4 Affected
Lane 7 III-5 Affected

Figure 3.39: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D14S615 at 21.3 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7

Lane 1 III-1 Normal
Lane 2 III-2 Normal
Lane 3 IV-1 Affected
Lane 4 IV-2 Normal
Lane 5 IV-3 Normal
Lane 6 IV-4 Affected
Lane 7 III-5 Affected

Figure 3.40: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D16S409 at 59.27 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

Lane 1	III-1	Normal	Lane 5	IV-3	Normal
Lane 2	III-2	Normal	Lane 6	IV-4	Affected
Lane 3	IV-1	Affected	Lane 7	III-5	Affected
Lane 4	IV-2	Normal			

Figure 3.41: Electropherogram of the ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D16S540 at 59.66 cM. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numerals indicate their positions within generation.

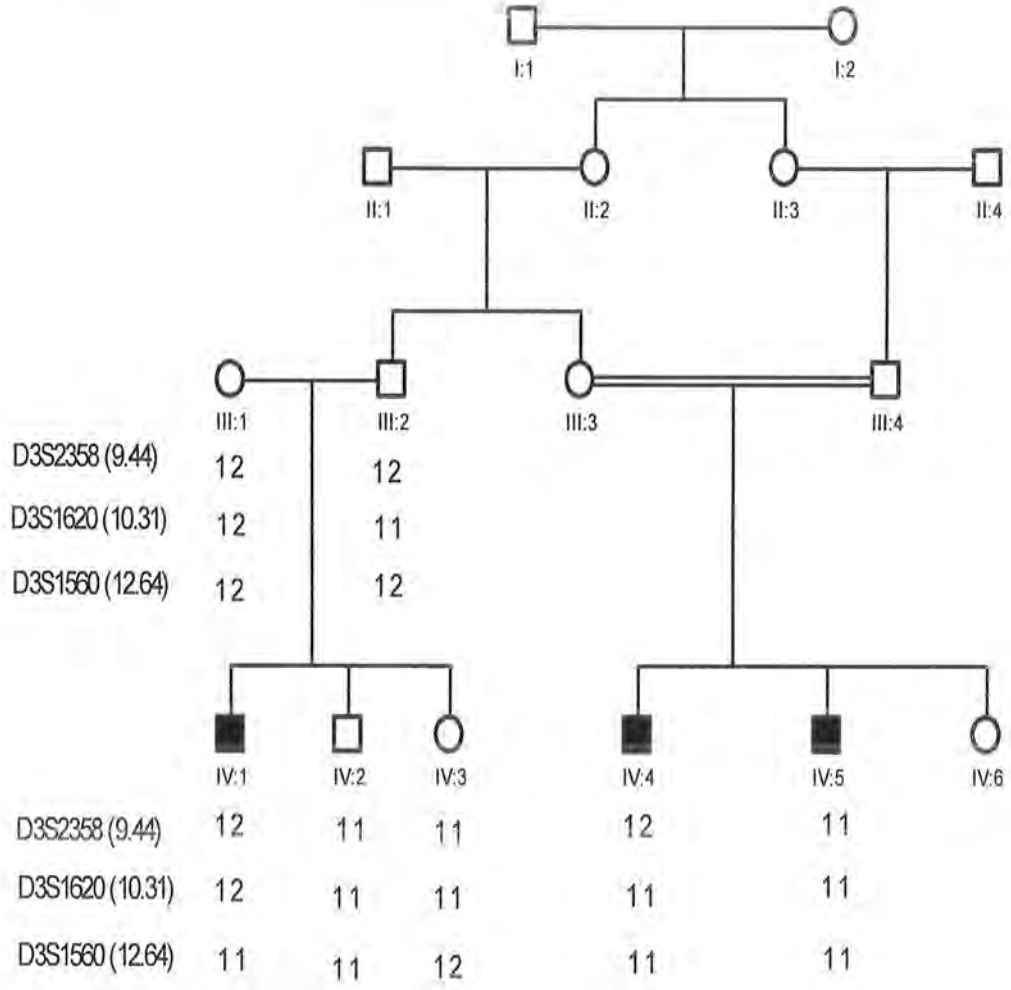


Figure 3.42: Haplotype of family B showing homozygosity on chromosome 3.

Project:	NEW FOLDER	Inheritance:	Recessive
Family name:	TOTALS	Common allele:	99.90 %
Used map:	Marshfield (sex averaged)	Disease allele:	0.10 %
Marker positions:	16 ok / 1 ? / 0 outside	LC1 PCOPY rate:	0.00 %
Allele frequencies:	Equal distribution of alleles	LC1 PENET wt/mt:	0.00 %
CALC interval:	Entire chromosome	LC1 PENET mt/mt:	100.00 %

Marker	CHR	cM	LOD	Theta
1.D3S1560	3	18.97	1.7287	0.0000
2.D1S429	1	136.89	1.4277	0.0000
3.D16S540	16	58.47	0.9677	0.0000
4.D8S1125	8	60.88	0.6824	0.1000
5.D4S191	4	126.16	0.2663	0.1500

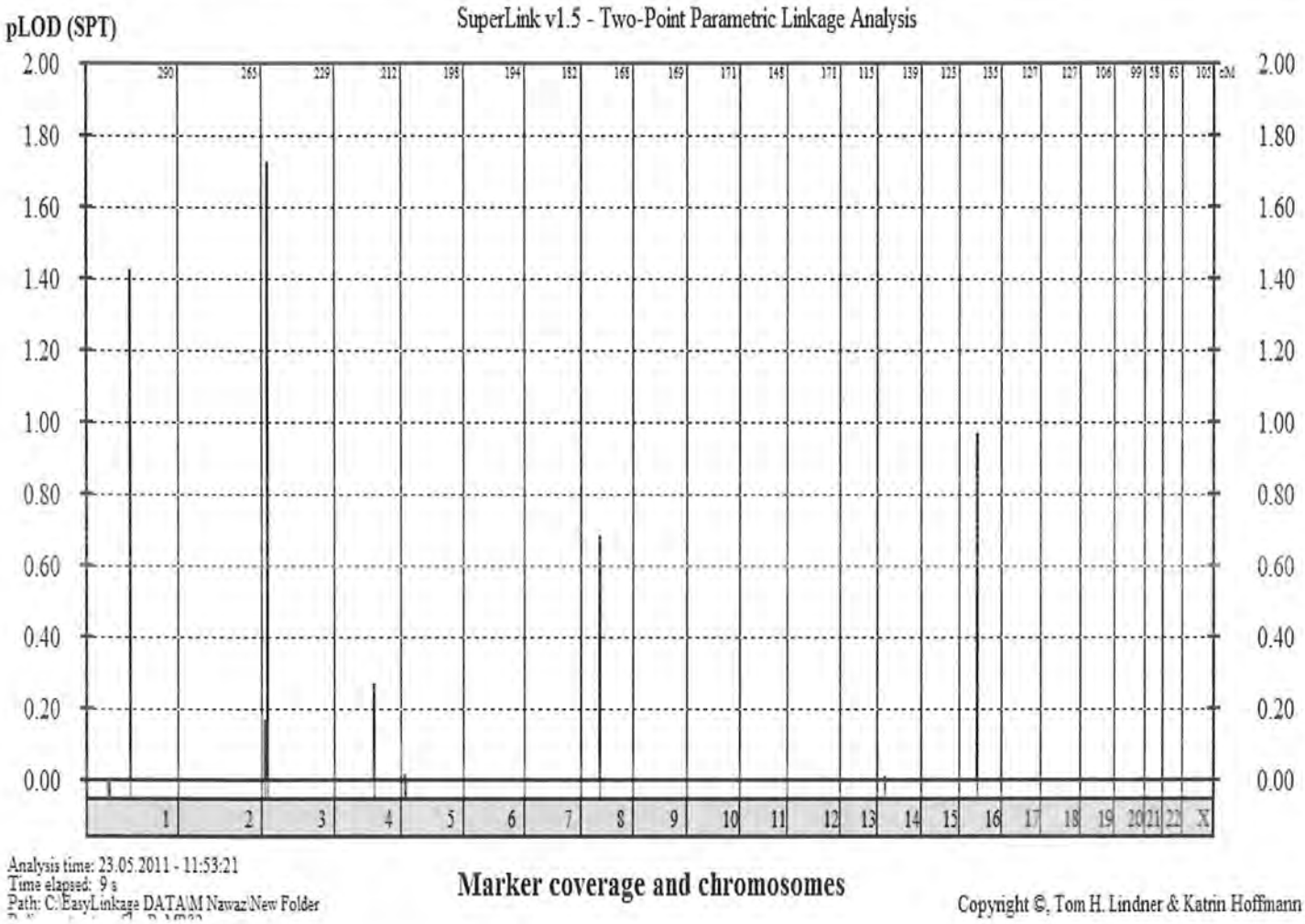


Figure 3.43: Graphical representation of LOD scores calculated by easy LINKAGE plus v5.02, with polymorphic microsatellite markers genotyped in family B. Highest LOD score of 1.72 was obtained on chromosome 3 with marker D3S1560.

DISCUSSION

DISCUSSION

Mental retardation (MR) or intellectual disability (ID) is a neurodevelopment disorder caused by genetic and environmental factors (Raymond, 2006). MR is characterized by suboptimal functioning of the central nervous system (CNS) resulting in, significant limitations both in intellectual functioning and adaptive behavior as expressed in conceptual, social and adaptive skills, originating before 18 years of age (Luckasson *et al.* 2002). MR shows a worldwide prevalence of around 2% and is a frequent cause of severe disability. Significant excess of MR and its functional reflection in the children of consanguineous matings suggest that autosomal recessive forms of MR are relatively common (Kuss *et al.*, 2011). MR occurs as isolated disorder or accompanied with other maladies affecting social and intellectual abilities of patients (Frints *et al.*, 2002). A study survey among Pakistani population shows 8.4 percent children with serious retardation and 48.2 percent children with mild retardation (Durkin *et al.*, 1998), which is due to consanguinity as dominant marriage pattern and low socioeconomic conditions (Bashir *et al.*, 2007).

Marriage between close biological kins is widely regarded as genetically disadvantageous in Western societies, but consanguineous unions remain preferential in North Africa, the Middle East and large parts of Asia particularly in Pakistan, India, Iran and Saudi Arabia (Zlotogora *et al.* 2007), due to which a positive association has been repeatedly shown between consanguinity and inherited disorders. Examples include deafness, retinal dystrophies, intellectual and developmental disability, complex congenital heart disease, thalassemia and primary microcephaly (Saggar and Bittles, 2008).

In this study two families (A and B) suffering from moderate to severe mental retardation were selected from remote areas of Khyber Pakhtunkhwa (KPK) Province of Pakistan. Clinical evaluation of affected members of both families (A,B) revealed moderate to severe mental retardation, low IQ level with no fitz history, speech disability, normal hearing, vision and no facial dysmorphism. None of the patients showed any other additional congenital malformations or facial dysmorphisms and neurological problems. Head circumferences, body heights and weights were normal in the effected individuals of both families, indicating segregation of non syndromic MR.

The homozygosity mapping was used to identify genes underlying MR in these families. Because of heterogeneity of disease, all reported loci with similar clinical presentation were checked for linkage in both families A and B.

In family A, markers D3S2358 (9.44 cM), D3S4538 (9.44 cM) and D3S1560 (12.64cM) flanking the *CRBN* gene on chromosome 3, showed homozygosity in all affected individuals and one normal individual. Then genotyping of additional polymorphic microsatellite markers ruled out the chances of linkage of this family to MRT2A locus, carrying *CRBN* gene. The results of all genotyped markers for this locus were analyzed for two-point LOD score using easy-LINKAGE plus V 5.02, which yielded an insignificant LOD score of 0.79. The data for all other genotyped loci was also analyzed using easy-LINKAGE plus V 5.02, which yielded a maximum two-point LOD score of 1.46 at marker D19S415 which again, is insignificant. Absence of homozygosity and insignificant LOD score ruled out the chances of linkage to all known loci in family A.

Similarly in family B homozygosity mapping was used to screen out known ARNSMR loci and genotyping results with polymorphic microsatellite markers were observed that showed heterozygous allele patterns in these effected individuls for all loci. The data for genotyped loci was analyzed using easy-LINKAGE plus V 5.02, which yielded a maximum two-point LOD score of 1.72 at marker D31560 and 1.42 at marker D1S429. However, genotyping of additional flanking markers ruled out the presence of disease associated homozygosity in the effected individuals of this family. Hence the molecular analysis showed the absence of homozygosity and insignificant LOD score ruled out the chances of linkage to all tested loci in family B.

Since genotyping results failed to detect linkage to the known ARNSMR loci in both families A and B, therefore the absence of linkage in both families indicates the presence of new loci that requires further investigations. It is proposed that genome wide scan might lead to identification and exploration of the disease genes in both families (A and B). Additionally microarray based SNP genotyping can also be used for genome scan in these families and the possibility of involvement of recently identified loci (MRT13-33) in pathogenicity of families (A, B) is still required to be ruled out as a follow up study. This study supports the genetic heterogeneity of MR in Pakistani population as predicted

in Iranian population by Najamabadi *et al.*, (2010), so additional studies are required to explore all MR associated genes. An additional step in searching for novel potential regions may include detection of genomic copy number alterations by high resolution whole-genome CGH array. CGH array combined with sequencing of the MR-causing genes would be effective strategy for this purpose. However, most of these tests are currently not available in Pakistan as a clinical service. Following the identification of additional genes causing non-syndromic MR, high throughput features of resequencing microarrays may enable efficient identification of molecular defects in the known genes in affected individuals using a single microarray platform.

In Pakistan mental retardation is one of the prominent congenital disorder that contributes much in population to be disabled. This study will facilitate to understand the causes and molecular basis of heterogeneity of complex forms of intellectual disability (ID) and identification of new genes causing MR.

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