A STUDY OF PAKISTANI FAMILIES WITH LEBER'S CONGENITAL AMAUROSIS

By

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CERTIFICATE

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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.

ZAINEB AKRAM

Dedicated

To

MY PARENTS,

FOR THEIR ENDLESS LOVE,

SUPPORT &

ENCOURAGEMENT

ACKNOWLEGDEMENT

All praises to the Creator of world, The omnipotent. I would firstly like to express my gratefulness to ***Almighty Allah*** *who gave me opportunity and courage to fulfill my dream. Countless salutations upon the Holy* ***Prophet Hazrat Muhammad*** *(peace be upon him), the most perfect among all human beings, Who is forever a source of guidance and knowledge for humanity as whole.*

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Zaineb Akram

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LIST OF ABBREVIATIONS

°C	Degree centigrade
µl	Micro liter
4-HNE	4-hydroxynenal
AAV	Adeno associated virus
AD	Alzheimer disease
AF	Autofluorescence
AG	A globular
AIPL1	Aryl hydrocarbon receptor interacting protein -like 1
ALMS	Alström syndrome
ARMD	Age related macular degeneration
ATF-4	Transcription factor -4
BBS	Bardet-biedl syndrome
BCVA	Best corrected visualacuity
C.F	Counting finger
C1orf36	Chromosome 1 open reading frame 36
C6ORF152	Chromosome 6 open reading frame 152
CC	Coiled-coil
CD	Catalytic domain
CEP290	Centrosomal protein 290
CM	Centimorgan
CMV	Cytomegalovirus
COH	Cohen syndrome
CORD	Cone-rod degeneration
CRB1	Human crumbs homolog 1

CRX	Cone rod homeobox
CSNB	Congenital stationary nightblindness
ECD	Extracellular domain
EGF	Epidermal growth factor
EOG	Electrooculography
ERG	Electroretinography
FN-III	Fibronectin type iii
GC	Guanylate cyclases
GCL	Ganglion cell layer
GTP	Guanosine triphosphate
GUCY2D	Retinal guanylate cyclase
IFT	Intraflagellar transport
IgGC2	Immunoglobulin-like-c2
IMPDH	Inosine monophosphate dehydrogenase
INL	Inner nuclear layer
IQCB1	Iq motif containing b1
JBTS	Joubert syndrome
KCNJ13	Potassium inwardly-rectifying channel, subfamily j, member 13
KHD	Kinase homology domain
KID	Kinase inducible domain
L.P	Light perception
LCA	Leber's congenital amaurosis
LCA5	Leber's congenital amaurosis 5
LRAT	Lecithin Retinol Acyl transferase
M	Molar
MERTK	Mer tyrosine kinase

mg	Milligram
MKS	Meckel syndrome
ml	Milliliter
NA	Not available
NMN	Nicotinamide mononucleotide
NMNAT1	Nicotinamide mononucleotide adenylyl transferase 1
NPHP6	Nephrocystin 6
NUB1	Nedd8 ultimate buster 1
OCT	Optical coherence tomography
ONL	Outer nuclear layer
OS	Outer segment
PCM1	Pericentriolar material 1
PCR	Polymerase chain reaction
pH	Power of Hydrogen Ion
PPI	Peptidyl-prolyl isomerase
PPRPE	Preservation of Para Arteriolar Retnal Epithelium
PRC	Photoreceptor cell
RD3	Retinal degradation 3
Rdh12	Retinol dehydrogenase 12
RetGC-1	Retinal membrane guanylyl cyclase-1
RID	RPGR interacting domain
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPE65	Retinal pigment epithelium 65
RPGRIP1	Retinitis pigmentosa guanosine triphosphatase regulator

	interacting protein 1
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SLS	Senior-loken syndrome
SNP	Single nucleotide polymorphism
SPATA7	Spermatogenesis associated protein 7
TAM	Tyro3/axl/mer
TBE	Tris/borate/edta
TEMED	Tetramethylethylenediamin
TM	Tropomyosin
TPR	Tetratrico peptide repeats
TULP1	Tubby-like protein 1
UK	United kingdom
USA	United States of America

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1. INTRODUCTION

Eye is one of the five main sense organs of human. Three layers of tissue enclose the eye (Figure 1.1). Sclera and cornea compose the outer layer, whereas iris, ciliary body and choroid make the middle layer (Benedek, 1971). But innermost layer constitutes of retina which contains the photoreceptors. It acts as a light-sensitive layer in eye and covers 65% of its inner surface, measuring around 42mm in diameter. It is divided into two distinctive areas, an outer retinal pigment epithelium (RPE) and inner sensorineural retina (Koenekoop, 2004). The retina is a highly specialized tissue composed of six main cell types, namely the cone and rod photoreceptors, bipolar, horizontal, amacrine, Müller and ganglion cells (Figure 1.2).

1.1 Photoreceptors

Retina contains the real visual receptor cells of the eye i.e., the cones and rods. There are about 125 million rods and 7 million cones. The rods are long and cylindrical, and the cones are short, thick, and tapered. The rods and cones are distributed unevenly throughout the retina. The cones are concentrated more at the center of the retina, while the rods are present in the peripheral regions of the retina. Fovea is a small region in center of retina, in which very high proportion of cones is present. The fovea is rod-free and is the area of sharpest vision. Both color and spatial details are most accurately detected in the fovea (Provis *et al.*, 2013).

The rods and cones are not only structurally dissimilar, but they also play clearly different roles in vision. The rods are responsible for black and white vision, while the cones are helpful for color vision. The cones are active in bright illumination, whereas the rods are active in dim illumination (Pearring *et al.*, 2013).

The optic nerve, which carries message to the brain, leaves a small area of the retina without the receptor cells, which is referred as blind spot. The blind spot lacks rods and cone, therefore, vision is not possible in this area (Frank and Stephen, 2001).

1.2 Retinal Processes

Retinal cells communicate with one another through synaptic interactions and gap junctions. Neurotrophic factors, retinoids, growth factors and ions influence retinal cell interactions. Three important processes can be discerned in photoreceptor cells: ciliary transport, phototransduction and (a part of) the visual cycle.

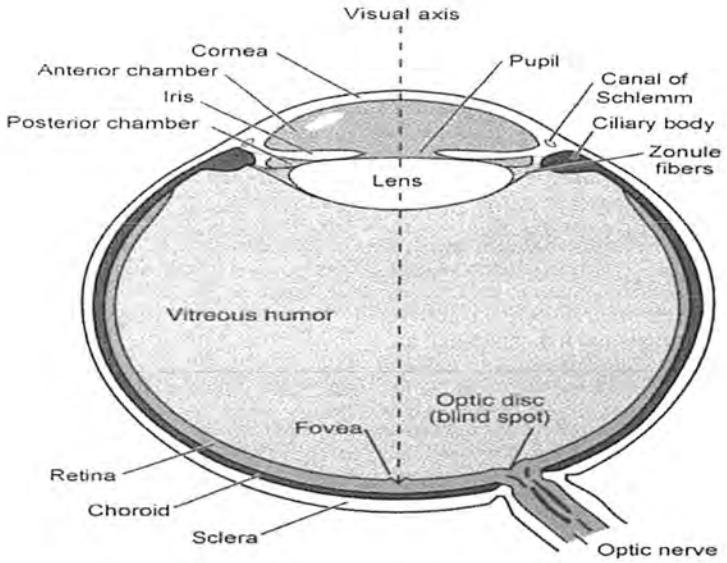


Figure 1.1: Internal structure of human eye (Adapted from Cooray *et al.*, 2007).

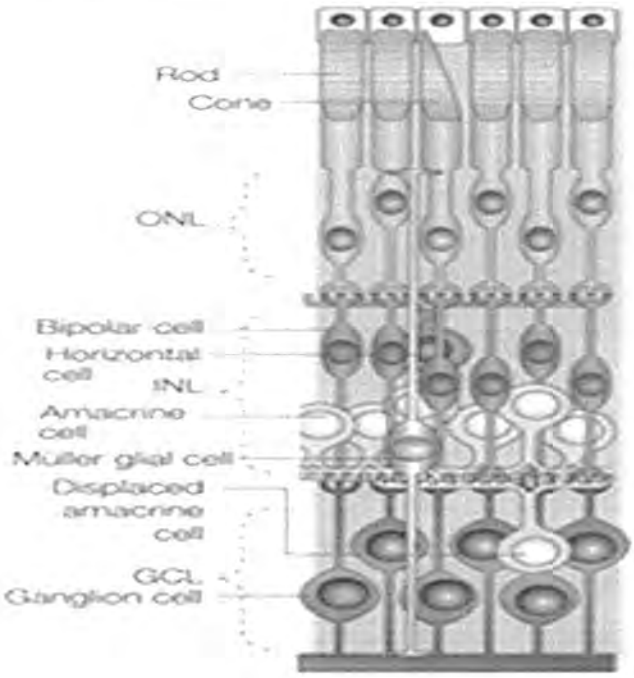


Figure 1.2: Structure of vertebrate retina (Adapted from Michael *et al.*, 2001).

Ciliary transport is carried out by the intraflagellar transport (IFT) machinery, which mediates bidirectional movement of axonemal components and proteins along the ciliaryaxoneme of photoreceptors. Second, the phototransduction cascade, a process by which the photoreceptors convert the energy of an absorbed photon into a neuronal signal. Finally, the visual cycle is a process. Its main theme is that the chromophore (all-trans retinal) that plays a role in the start of the phototransduction cascade, is recycled into newly usable chromophore (11-cis retinal) (Korenbrot, 2012).

1.3 Retinal Diseases

The retina consists of many cell types. It has the highest metabolic rate among the histological layers. So, mutation in any of the proteins involved in different functions in retina may lead to degeneration of retina. The hereditary retinal disorders are classified on basis of phenotypic differences. Disorders that affect the central retina first include macular dystrophies, like age related macular degeneration (ARMD) and cone-rod degeneration (CORD) leading to loss of central vision, while those affecting the peripheral retina early include retinitis pigmentosa (RP) and congenital stationary nightblindness (CSNB) (Musarella, 2001). Leber's congenital amaurosis (LCA) includes both peripheral and central retinal disease leading to profound loss of vision.

Most hereditary retinal disorders are bilateral and the pattern of inheritance can either be autosomal dominant or recessive. It may also be X-linked or mitochondrial in nature. In several inherited retinal disorders photoreceptor cell death occurs as a result of apoptosis (Rattner, 1999). Retinitis pigmentosa (RP), the commonest retinal dystrophy, is characterized by progressive cell death of rods and cones. Mutations involving rod cell structure and function lead to apoptosis, which have an indirect effect on the cones, causing further cone dysfunction and loss. Cone cell loss occurs when rod outer segment degenerate to more than 75% as observed by electroretinography (Cideciyan *et al.*, 1998).

Loss of rod photoreceptors causes loss of peripheral vision, motion detection, and decreased vision in scotopic conditions (nyctalopia). Loss of cone photoreceptor cells results in loss of central vision (acuity loss), trichromatic colour discrimination (dyschromatopsia), vision loss in photopic conditions (hemerelopia) and ocular discomfort under photopic conditions (photoaversion or photophobia). In many retinal degenerations apoptosis of the photoreceptor cells leads to decreased oxygen need.

This altered oxygen tension stimulates auto regulation in the retinal vasculature causing attenuation of the blood vessels as a secondary effect noted ophthalmoscopically along with optic disc pallor.

Leber's congenital amaurosis is also observed in conjunction with other systemic and syndromic disorders, like cerebellar disease (Joubert syndrome), epiphyseal disease (Saldino-Mainzer syndrome) and renal disease (Senior–Loken syndrome) (Allikmets, 2004).

1.4 Genetics of Retinal Diseases

Specifically the retinal diseases are catalogued on RetNet, a website owned by Stephen Daiger (http//www.sph.uth.tmc.edu/RetNet). Cloned genes, mapped genes and their clinical associations can be found on it. It also has protein product, primary references and links to other sites (Ropers and Hamel, 2005). Hereditary retinal diseases account for 5% of blindness all over the world and LCA is the cause of 5% of all hereditary retinal diseases. The inheritance pattern for retinal diseases may be chromosomal, monogenic or complex (Kaplan *et al.*, 1990).

1.4.1 Autosomal Dominant Inheritance

In such patients only one chromosome of the pair carries mutant allele. Generally, phenotypes which are expressed in both homozygotes and heterozygotes are dominant. In this condition the child of affected parent has 50% chances of developing the disease. In such retinal diseases difference of expression occurs due to variable expression and pleiotropy. The clinical expression may vary depending upon the genetic pattern and environment (Ayuso *et al.*, 1996). These diseases may arise due to several pathogenic mechanisms.

1.4.2 Autosomal Recessive Inheritance

When both chromosomes carry the mutant allele, the pattern of inheritance is called as autosomal recessive pattern, e.g. in congenital stationary night-blindness (CSNB), retinitis pigmentosa (RP) and LCA. The parents are usually asymptomatic. The risk of siblings to express disease is 1 in 4. Consanguinity in parental generation is strong factor for such inheritance pattern. The disorder is called recessive if in heterozygous form, 50% of gene product sufficiently performs the normal function (Tucker *et al.*, 2004).

1.4.3 X-linked Inheritance

Males are hemizygous having single X chromosome. In females most genes are expressed only on X chromosome due to random X inactivation. An example of such pattern is RP (Breuer *et al.*, 2002).

1.4.4 Digenic Inheritance

It represents inheritance due to disease causing mutations in two different genes. An example is digenic RP. In this mutation is on two genes, *ROM1* and *peripherin/rds*, located on chromosomes 11 and 6 respectively (Dryja *et al.*, 1997). Bardet-Biedl syndrome is another example showing this inheritance pattern (Fauser *et al.*, 2003).

1.4.5 Uniparental disomy (UPD)

In this case the two chromosomes should be inherited from only one parent. The identical chromosome or its segments may occur in duplicate. An example is retinal pigment epithelium 65 (*RPE65*) and MER tyrosine kinase (*MERTK*)-associated retinal dystrophy of chromosome 1 and 2 (Thompson *et al.*, 2002).

1.5 Diagnosis of Retinal Diseases

The diagnosis of heritable retinal disease is currently based on the clinical features. With the advent of molecular techniques confirmation of diseases and the effect of the mutant genes is being established. Correlation of the retinal morphology with the electrophysiology is the key to diagnosis if molecular diagnosis is unavailable. In the presence of molecular information however, a more accurate diagnosis is possible.

1.5.1 Clinical Examination

Several diagnostic methods may be applied to detect the occurrence of disease from clinical point of view, like assessment of visual activity and optical assessment, slit-lamp bio-microscopy and dilated fundoscopy. Other relevant investigations include electrophysiological (ERG, dark adaptation curves and EOG) and psychophysical testing (static or kinetic visual field tests), imaging and molecular studies. Imaging includes examination of fundus, optical coherence tomography (OCT), ophthalmoscopy, fundus fluorescein angiography and retinal autofluorescence. It is also important to perform a thorough medical and genetic examination in an effort to identify other clinical features suggestive of a syndromic form of retinal degeneration.

1.5.1.1 Electroretinography (ERG)

It is the most commonly employed electrophysiological test, where electrical response to photic stimulation of retinal cells is recorded. It has two components, photopic (cone) and scotopic (rod) (Hollander *et al.*, 2009). It records the electric potential of retinal cells after stimulation in dark and light state. The response is generated when light strikes retina and this energy is converted to electrochemical energy (Brown and Murakami, 1964) (Cone, 1964). The response is due to sodium and potassium movement in retina due to light stimulation. The reduced amount of visual pigment, rhodopsin and decreased level of cGMP in retinal diseases, closes the sodium channels, therefore reducing the response on photostimulation (Goldstein and Berson, 1969 (Berson and Goldstein, 1970). One of the basic diagnostic tools for LCA patients is that their ERG is non-detectable (Hollander *et al.*, 2008).

1.5.1.2 Electrooculography (EOG)

It records the membrane potential across RPE. Its readings become abnormal in cone, cone-rod and rod-cone retinal dystrophies (Krill, 1973). In this eye can be taken as a dipole, with cornea as positive and retina as negative pole. The electrical signals thus measured are called electrooculogram (EOG) (Bulling *et al.*, 2011).

1.5.1.3 Imaging Techniques

The imaging techniques like high resolution photography provide good information about the anterior segment and the fundus. Progression of these disorders can be detected by continuous photography. Fluorescein angiography shows the area of pigmentation. It helps in understanding pathophysiologic process of disease (Bellmann *et al.*, 2003; Trieschmann *et al.*, 2003). It can be used to differentiate different types of retinal diseases. These images vary according to the gene mutated in LCA patients. For example, they are minimal or absent in mutations of RPE65 and normal in *GUCY2D* mutations (Lorenz *et al.*, 2004).

1.5.1.4 Other Techniques

Optical coherence tomography (OCT) can give the measurement of thickness of retina. It shows the viable photoreceptors that remain till late in disease. It is a non-contact and non-invasive imaging technique (Hollander *et al.*, 2008). Live images of cellular organization and thickness of retina are shown by this (Chung and Traboulsi,

2009). Laser interferometry can assess the structural and pathological changes which may cause difference in retinal thickness (Hamada *et al.*, 2000). On the other hand by laser opthalmoscopy photoreceptor layer can be visualized (Fitzke, 2000). Dark adapted visual thresholds can measure the retinal sensitivity. Perimetric testing is used to determine the abnormalities in rod and cones threshold (Hollander *et al.*, 2008). It measures angle of peripheral vision (Chung and Traoulsi, 2009).

There are a number of hereditary retinal diseases like glaucoma, macular degeneration, optic atrophy, retinitis pigmentosa etc. They may be isolated or a part of syndrome. Leber's congenital amaurosis is also one of it, which is the most severe retinal disease.

1.6 Leber's Congenital Amaurosis

Leber's congenital amaurosis (MIM# 204000) was firstly described by Theoder Leber in 1869 (Leber, 1869), but confirmation of the diagnosis was made easier by Adolphe Franceschetti, who noted the attenuated electro-retinographic responses in patients with LCA in 1954 (Franceschetti and Dieterle, 1954). It appears as autosomal recessive trait in most cases but autosomal dominant LCA patients have also been observed. This disease causes incurable visual impairment at birth or in early childhood (Xiao *et al.*, 2011), so it is also regarded as juvenile retinal dystrophy (Koenekoop, 2004). It causes slow degeneration of the structure of retina at cellular level (Simonelli *et al.*, 2010). Patients with LCA are good models for ophthalmologists and researchers to understand normal and defective human eye development. In real, LCA does not refer to a single disorder rather it is a collection of disorders (Koenekoop, 2004). It is a heterogeneous disorder of clinical as well as genetic basis (Seong *et al.*, 2008). LCA affects approximately 1/81,000 individuals (Stone, 2007). LCA is the cause of 20% of children among all attending the blind school worldwide (Schappert *et al.*, 1959).

1.7 Clinical Symptoms of LCA

Clinically LCA is characterized to have following major features:

1.7.1 Visual Loss

LCA patients mostly experience notable visual impairment since birth. One-third of the patients show no perception of light (Hollander *et al.*, 2008). The loss of vision is usually stable or slowly progressive (Robert *et al.*, 2012). Usually the loss of vision is

due to keratoconus and cataract. It may also be caused due to macular lesions. Sometimes slight visual improvement can be seen in childhood too. This is attributed to central vision pathways that develop. Retinal maturation is not the reason behind (Hollander *et al.*, 2008).

1.7.2 Nystagmus Eye Movement

Nystagmus eye movement (absence of fixation) can be observed at very early stages of LCA (Hollander *et al.*, 2008). The amplitude, also the direction and frequency of these eye vibrations depends on various factors. Some factors are visual experience in early childhood and attention given to the child. Mood of the patient and remaining vision area in retina, binocular and uniocular viewing and the direction of the gaze. These movements are involuntary (Lotery *et al.*, 2000).

1.7.3 Amaurotic Pupils

The pupils in LCA become amaurotic, i.e. it doesn't show any reaction to light.

1.7.4 Electric Signals on Electroretinogram

In LCA patients rod and cone ERG signals become minimal or absent. Franceschetti and Dieteve described it as the hallmark of LCA patients. It is a major differentiating reason between LCA and other retinal dystrophies (Robert *et al.*, 2012).

1.7.5 Retinal Appearance

Heterogenecity is observed in retinal appearance of LCA patients. Retina may appear normal at initial stages, other than that a variety of abnormal phenotypes of retina may be seen, as retinal vessel attenuation, pseudopapilledema of optic disc. In other cases maculopathy or one spicule pigmentation can be observed. And white retinal spots or marbleized retinal appearance may be observed (Hollander *et al.*, 2008). The degeneration of retina is mainly marked on its outer layers and periphery (Robert *et al.*, 2012).

1.7.6 Refractive Error

The range of refractive errors in LCA patients is high from hyperopis to myopis. Mostly patients are hyperopes. Some studies suggest that LCA phenotypes can be marked by the degree of hyperopia (Hollander *et al.*, 2008).

1.7.7 Oculodigital Sign of Franceschetti

It's an extraocular sign in LCA patients and has three main components poking of the eye, rubbing and eye pressing. The exact reason of this behavior is not known yet. It can cause degeneration of orbital fat so may be dangerous. A prominent visible symptom for LCA patients are deep eyes (enophthalmos) (Hollander *et al.*, 2008).

1.7.8 Photoaversion

Photophobia and nyctalopis (night blindness) can be regarded as prominent features of LCA (Hollander *et al.*, 2008).

1.7.9 Keratoconus

It is basically the structural change in cornea (Hollander *et al.*, 2008). It is basically non inflammatory thinning of the cornea. In 96% cases it is bilateral (Timothy *et al.*, 2009). The shape of cornea changes from normal curvature to cone like. It is the dissolution if Bowmann's membrane present between corneal stroma and epithelium of cornea (Hollander *et al.*, 2008). The prevalence rate of keratoconusis between 50 and 230 per 100,000 (Timothy *et al.*, 2009).

1.7.10 Minor Symptoms

Some minor symptoms are also shown in LCA phenotype, like cataractthat involves the clouding of eye which causes decreased vision. Strabismus in which the eyes are not properly aligned with each other. Macular atrophy in the macula is central area of the retina that provides most detailed vision. In LCA patients as retina is damaged so it leads to macular atrophy which results in vision loss.

1.8 Distinguishing Features of LCA

1.8.1 Inheritance

For LCA pattern of inheritance is autosomal recessive. According to this the expression of disease occurs only when both the chromosomes have mutant allele. However, rarely autosomal dominant pattern is also observed (Grieshaber and Niemeyer, 1998).

1.8.2 Clinical Associations

The presence of LCA has been documented with nephronophthisis in Senior-Løken syndrome; cerebellar ataxia in Saldino-Mainzer syndrome; vermishypoplasia,

oculomotor anomalies and neonatal respiratory apnea in Joubert syndrome (Ellis *et al.*, 1984). The clinical heterogeneity of Senior-Løken is indicated by the variable age of onset of the retinal and renal abnormalities (Godel *et al.*, 1979). The conorenal syndrome includes renal histopathologic changes consistent with a primarily glomerular disorder and short distal phalanges with cone shaped epiphysis (Mendley *et al.*, 1995). ERG changes have been noted in heterozygotes of renal–retinal dysplasia (Hogewind *et al.*, 1977).

LCA has also been reported with mental retardation (Tipton and Hussels, 1971). LCA has been associated with demyelination, autism, psychomotor delay and other neurodevelopmental anomalies (Curless *et al.*, 1991). LCA has also been observed in association with osteopetrosis (Keith, 1968). Joubert syndrome has been associated with LCA and multicystic kidneys (Ivarsson *et al.*, 1993). Alström syndrome presents with LCA, cardiomyopathy, short stature, deafness and diabetes mellitus. Cardiomyopathy is noted at infancy, although diabetes mellitus occurs in the second or third decade (Russell-Eggitt *et al.*, 1998). The Arima syndrome, cerebello-occulo-hepato-renal syndrome, is a rare though well defined entity that includes hepatic involvement (Matsuzaka *et al.*, 1986).

1.9 Genes Causing LCA

LCA represents genetic heterogeneity as well as variable expression, i.e. the presence of mutations in one gene leading to several ophthalmologic disorders. Approximately 130 genes are known to be involved in hereditary retinal diseases (Soeng *et al.*, 2008) and 40 loci are known for human hereditary retinal degenerations involving the photoreceptors and retinal pigment epithelium (Morimura *et al.*, 1998). Mutations in these 19 genes may lead to LCA. These are *GUCY2D* (LCA1), *RPE65* (LCA2), *SPATA7* (LCA3), *AIPL1* (LCA4), *LCA5* (LCA5), *RPGRIP1* (LCA6), *CRX* (LCA7), *CRB1* (LCA8), *NMNAT1* (LCA9), *CEP290* (LCA10), *IMPDH1* (LCA11), *RD3* (LCA12), *RDH12* (LCA13), *LRAT* (LCA14), *TULP1* (LCA15), *KCNJ13* (LCA16), *IQCB1*and *MERTK* (Perrault *et al.*, 2013). Some of these genes are also involved in other hereditary retinal diseases like retinitis pigmentosa (RP) and cone-rod dystrophy (CORD), so they can be termed as genetically related diseases (Soeng *et al.*, 2008). Different proteins important for retina development along with physiological pathways are encoded by these genes. Some examples are like, *CRB1* and *CRX* are

involved in photoreceptor morphogenesis, *AIPL1*, *GUCY2D* in phototransduction, *LRAT*, *RDH12*, *RPE65* in vitamin A cycling and *IMPDH1* in guanine synthesis. Recently, *CEP290*, *LCA5*, *RPGRIP1*, *TULP1* are shown likely to affect transport mechanisms inside photoreceptor.These genes alongwith their phenotypes and mutation frequency are given in Table 1.1.

1.9.1 Aryl Hydrocarbon Receptor Interacting Protein -Like 1 (*AIPL1*)

Aryl hydrocarbon receptor interacting protein -like 1 (*AIPL1*; MIM#604392) is located on chromosome 17p13.1 and has 6 exons. It is present in retina and pineal gland (Bett *et al.*, 2012). It is estimated to cause 5-10 % of all LCA cases (Pennesi *et al.*, 2011). At early stages it is expressed in peripheral and central retina, which becomes important for rod and cone photoreceptor development. In adult retina its presence is restricted to rod photoreceptors (Hollander *et al.*, 2008). It is expressed in adult rod, cone photoreceptors (Tan *et al.*, 2012). It is a protein of 384 amino acids (44 kD) (Hollander *et al.*, 2008). It has one peptidyl-prolylisomerase (PPI) domain. Three tetratrico peptide repeats (TPR) are also its part (Liu *et al.*, 2004). The TPR domains function as mediator for protein interactions (Hollander *et al.*, 2008). It is required for assembly of phototransduction protein, phosphodiesterase in rods and cones (Tan *et al.*, 2012).

It is included in FK-506 binding protein family (Bett *et al.*, 2012). They are known as immunophilin superfamily of proteins. Its members mostly act as molecular chaperones. *AIPL1* may show molecular chaperone function in retinal protein folding (Liu *et al.*, 2004). It forms chaperone heterocomplex with the members of Hsp70 and Hsp90 family. It also acts as chaperone for cGMP phosphodiesterase PDE6 (Bett *et al.*, 2012).

AIPL1 mutations are mainly of three types. Class 1 are the missense mutations. They are found mostly at N-terminus. Class 2 are missense mutations. Stop mutations are also included in class 2 in TPR motifs. Class 3 includes in-frame deletions. Class 1 and class 2 are linked to LCA and class 3 is usually associated with cone-rod dystrophy (CORD). It is also linked to juvenile retinitis pigmentosa (Sohocki *et al.*, 2000).

AIPL1 is identified to interact with NEDD8 ultimate buster 1(NUB1) that is a cell cycle regulator. It is required for degradation of proteins like NEDD8/NEDD8

associated proteins, FAT10/FAT10 mediated proteins. This degradation is delayed by interaction of *AIPL1* with FAT10 and NEDD8 proteins. This interaction of *AIPL1* with FAT10 protein show its involvement in regulation of FAT10 functions in retina. So, mutations in *AIPL1* that may prevent its interaction with NUB1, therefore contribute to LCA. There may be possibility that *AIPL1* may bind to newly formed FAT10 substrate before it gets associated with NUB1, thus delaying NUB1 mediated FAT10 substrate degradation.

AIPL1 has also been observed to be involved in biogenesis of rod PDE6 (Bett *et al.*, 2012). In normal cases PDE hydrolyse cGMP and balances its synthesis by gunylyl cyclase cGMP. Accumulation of cGMP causes opening of cGMP-gated channels and Ca^{+2} level increase intracellularly (Liu *et al.*, 2004). Thus it is inferred that it affects the expression, stability and biosynthesis of rod-specific cGMP phosphodiesterase by its chaperone function. *AIPL1* helps in the processing of farnesylated proteins as it is essential for the maintenance of photoreceptor structure (Ramamurthy *et al.*, 2003).

1.9.2 Retinal Guanylate Cyclase (GUCY2D)

The first gene identified to cause LCA is retinal guanylate cyclase (Tucker *et al.*, 2004). *GUCY2D* (MIM# 600179) is located on chromosome 17p13.1. Mutations in *GUCY2D* mutations lead to 6-12% of LCA cases. It encodes photoreceptor membrane specific retinal membrane guanylyl cyclase-1 (RetGC-1) (Samuel *et al.*, 2013), i.e. a protein involved in phototransduction. This protein is more expressed in outer parts of the photoreceptors. It comprises of an N-terminal extracellular domain (ECD), a membrane- spanning domain, an intracellular kinase homology domain (KHD) and another catalytic domain (CD). RetGC-1 and RetGC-2 replenish cGMP in retina after being depleted by light activated phosphodiesterase. Photoisomerization of retina starts a series of events that ultimately causes the depletion of cGMP and closes the cGMP-gated and Na^{+}/Ca^{+2}-gated channels in plasma membrane. Due to this the concentration of Na^{+}/Ca^{+2} decreases in the cell. Cell becomes hyperpolarized and the neurotransmitter release is also decreased. This decrease in Ca^{+2} levels starts the restoration of cGMP. At lower level of Ca^{+2}, RetGC proteins restore the level of cGMP in photoreceptors by the help of calcium binding proteins which stimulate RetGC-1. cGMP-gated channels open, depolarization of cells occur and signaling is restored. It was hypothesized that *GUCY2D* mutations lead to LCA due to severely reduced levels of cGMP (Tucker *et al.*, 2004).

GUCY2D mutations are also associated with several distinct phenotypes, namely Alzheimer disease (AD), cone-rod dystrophy (CORD), early onset severe retinitis pigmentosa (RP) (Perrault *et al.*, 2005).

1.9.3 Retinal Pigment Epithelium 65 (*RPE65*)

Retinal pigment epithelium (*RPE65*; MIM 180069) is another gene considered to cause 6% of all LCA cases. It is located on chromosome 1p31. Mutations in it may lead to LCA and other childhood-onset severe retinal dystrophies (Perrault *et al.*, 1999). It is 63 kDa (533 amino acids) protein showing isomerase activity (Hollander *et al.*, 2008). Loss of vision may occur by degeneration or dysfunction of the photoreceptor cells (Artur, 2010). It acts as an isomerase and thus activates the transition of all *trans*-retinyl-ester to 11-*cis*-retinol in the retinal pigment epithelium (RPE). It is essential in formation of vitamin A, which is the precursor of rhodopsin (Perrault *et al.*, 1999). Thus this conversion activates a phototrasduction cascade that signals vertebrate vision. It is also suggested that 11-*cis*- retinal is required by a sub group of cones for the transportation of opsins and other cone related phototransduction polypeptides. In case of mutated *RPE65*, may lead to mislocalization of opsins or instability of cone which in turn increases cone degeneration, thus affects vision. Measurements show that 60% of LCA patients had decreased photoreceptor functionality, in others only cone vision was observed (Artur, 2010). In 2001 successful gene therapy was used for *RPE65*- LCA patients (Boye *et al.*, 2013). Mutations in *RPE65* have been linked to severe RP (Sitorus *et al.*, 2003).

1.9.4 Spermatogenesis Associated Protein 7 (*SPATA7*)

Spermatogenesis associated protein 7 has been shown to be mutated in LCA disease, specifically type 2 (Perrault *et al.*, 2010). *SPATA7* (MIM# 609868) was first discovered in 2003 from rat. It consists of 12 exons (52.8 kb region). It is located at 14q31.3. The gene encodes a protein of 599 amino acids. It has a trans membrane domain but no functional domain has been observed till now (Mackey *et al.*, 2011). Due to the alternative splicing of exon 3, two transcripts of *SPATA7* are present, i.e. 1 and 2. It is a highly conserved vertebrate protein. It is expressed in spermatocytes as well as retina. Highest levels are present in retina, testis and brain. It has also been observed that *SPATA7* transcript version 2 is highly expressed in testis. The transcript 1 is more expressed in brain and retina (Perrault *et al.*, 2010). In retina it is expressed

in retinal layers like ganglion cell and inner nuclear layers and in the inner parts of photoreceptors. Studies suggest that its expression is important for normal retinal functions. Its significant role in development is still not known (Mackey *et al.*, 2011). The exact function of *SPATA7* is unknown. Some studies suggest its role in vesicular transport, visual perception and response to stimuli (Perrault *et al.*, 2010).

1.9.5 Retinitis Pigmentosa Guanosine Triphosphatase (GTPase) Regulator Interacting Protein 1 (*RPGRIP1*)

Retinitis pigmentosa guanosine triphosphatase (GTPase) regulator interacting protein 1 (*RPGRIP1;* MIM # 605446) gene is localized on chromosome 14 q11.2 and consists of 1287 amino acids, having 24 exons (Koenekoop, 2005). It consists of a RPGR interacting domain (RID) and another coiled-coil (CC) domain. It interacts with *RPGR*, which is an X-linked RP gene (Li *et al.*, 2009).

A unique feature of this protein is its expressions in amacrine cells which are a part of inner retina. These cells are important for photoreceptors. They also have role in development of retina and cytoplasmic trafficking. It also functions in photoreceptor disc morphogenesis (Koenekoop, 2005).

1.9.6 Centrosomal Protein 290 (*CEP290*)

Centrosomal protein 290 (*CEP290;* MIM#610142), is also called nephrocystin 6 (*NPHP6*). It is a centrosomal protein having 2479 amino acids (290 kD). It is located on chromosome 12q21.32. It spans 54 exons. Structurally it has 13 coiled coil (CC) domains, 3 tropomyosin (TM) homology domains, a hook domain, 6 RepA/Rep$^+$ protein kinase inducible domain (KID) motifs, a myosin-tail (Moradi *et al.*, 2011). The phenotypic variation spectrum of this gene is very vast. It was initially identified as candidate gene for Joubert syndrome and Senior-Loken syndrome (SLS). Soon Leber congenital amaurosis (LCA), Meckel syndrome (MKS) and Bardet-Biedl syndrome (BBS) became a part of list (Coppieters *et al.*, 2010). *CEP290* mutations are the most common cause of LCA. Its mutations are responsible for up to 6% to 22% of LCA patients (Perrault *et al.*, 2007).

CEP290 is important in cilia and centrosome functions. For example it recruits Rab8a, a small GTPase required for ciliary membrane elongation. It also has role in ciliary associated transport. It also shows interaction with pericentriolar material 1 (PCM1), and thus is involved in ciliogenesis (Moradi *et al.*, 2011). *CEP290* activates activation

transcription factor -4 (ATF-4), which along with other functions has role in skeletal and lens development. It is also seen to have role in G protein trafficking during olfactory perception. In case of *CEP290* mutation, thick inner retinal layers are observed in rod-rich regions (Perrault *et al.*, 2007).

1.9.7 Cone rod homeobox (*CRX*)

Cone rod homeobox (*CRX;* MIM# 602225) is included in Otd/Otx gene family. It maps to chromosome 19q13.3 and encodes a protein of 32.3 kDa (299 amino acids), which is basically a transcription factor important for embryonic photoreceptor development. *CRX* is mainly expressed in retina and traces are also observed in RPE (Chen *et al.*, 1997) and in nuclear layers. Mutations in the *CRX* gene are inherited as an autosomal-dominant LCA cases (Drack, 2009).

CRX has main role in differentiation of rod and cones photoreceptors and also maintains normal cone and rod function. Basically it controls expression of various genes involved in photoreception (Livesey *et al.*, 2000). These genes have *CRX* binding site on upstream region, like rhodopsin and aresstin, β-phosphodiesterase and interphotoreceptor matrix protein. It is shown that *Nrl* and *CRX* can act synergistically with each other, and show greater level of transactivation as compared to their individual work (Chen *et al.*, 1997).

Mutations in *CRX* are also linked to, autosomal dominant cone rod dystrophy and autosomal dominant retinitis pigmentosa (Drack, 2009).

1.9.8 Human Crumbs Homolog 1 (*CRB1*)

Human crumbs homolog 1 (*CRB1;* MIM # 604210) is another gene in which mutations may result in LCA. The *CRB1* gene is located at 1q31 and has 12 exons. Its alternative splicing results in the formation of two isoforms with 1376 and 1406 amino acids (Corton *et al.*, 2013). They are transmembrane proteins (Yzer *et al.*, 2006) and consist of signal peptide sequence, 19 epidermal growth factor (EGF)-like domains, 3 laminin A globular (AG)-like domains and the longer isoform also contains additional transmembrane and cytoplasmic domains (Corton *et al.*, 2013). It is expressed in retina, iris (Timothy *et al.*, 2009) and brain (Corton *et al.*, 2013). *CRB1* is important for morphogenesis of photoreceptors and also maintains organization of photoreceptors during light stimulation (Booij *et al.*, 2005).

CRB1 mutations lead to severe retinal abnormalities like focal photoreceptor degeneration. Some studies also showed asymmetric results for the two eyes of carrier. One appeared abnormal while the other was normal (Timothy *et al.*, 2009). The exact reason in unknown but it is postulated that different genetic and environmental factors may lead to haplotype of *CRB1* carrier. Mutations in *CRB1* are also linked to RP and Coats-like vasculopathy (Yzer *et al.*, 2006).

1.9.9 Retinal Degradation 3 (*RD3*)

RD3 (MIM#180040) is also known as *C1orf36* (chromosome 1 open reading frame 36), and is located at 1q32.1. It has three exons. Its protein is of 194 amino acids (22.7kDa). It consists of putative coiled-coil domains at amino acids and many casein kinase II and protein kinase C phosphorylation sites (Preising *et al.*, 2012). It has been identified as photoreceptor GC-binding protein. It has a main role in stable expression and membrane vesicle trafficking of GC-1 and GC-2 in photoreceptors.GC-1 and GC-2 are guanylate cyclases, present in the photoreceptor cells. They have essential role in phototransduction by increasing the production of cGMP and regulating Ca^{+2} levels. Firstly a mutation in *RD3* may cause defect in GC functioning. Their ability to synthesize cGMP in photoreceptors may be lost. It leads to closure of cGMP-gated channels and in turn drop in Ca^{+2} level. A controlled concentration of intracellular Ca^{+2} is required for proper function and survival of photoreceptors. So excessive high or low Ca^{+2} levels may kill them (Azadi *et al.*, 2010). Secondly *RD3* deficiency may lead to stress in endoplasmic reticulum due to miss-trafficking of GC in photoreceptors which may lead to photoreceptor degeneration (Preising *et al.*, 2012).

1.9.10 Inosine Monophosphate Dehydrogenase Type I (*IMPDH1*)

Inosine monophosphate dehydrogenase type I (*IMPDH1;* MIM#146690) mutations may also rarely cause LCA. This gene is present on chromosome 7q32.1. It produces enzyme *IMPDH1* (Azadi *et al.*, 2010). In most mammals including human beings, two genes *IMPDH1* and *IMPDH2* are present. The enzymes encoded by them are 84% identical at amino acid level. *IMPDH1* is 10 times more expressed in retina than *IMPDH2*. It is highly expressed in peripheral part of retina.

The mutations in *IMPDH1* that may cause retinal diseases have not shown effects on its enzyme activity. The proteins bind to single stranded nucleic acids i.e. RNA and DNA (Bowne *et al.*, 2006). Therefore it plays role in transcription, translation,

posttranslational modification, different aspects of RNA metabolism. Studies show that the mutations causing the retinal diseases change this nucleic acid binding capacity of *IMPDH*.

1.9.11 Leber's Congenital Amaurosis 5 (*LCA5*)

LCA5 (MIM# 604537) is also called *C6ORF152* (chromosome 6 open reading frame 152). It is mapped to 6q11–q16 chromosomal region (Ozgül *et al.*, 2006). The gene has 9 exons. It is expressed in cilium of photoreceptors and microtubules. Mutations in *LCA5* account for 1-2% of LCA. A protein lebercilin, of 697 amino acids is encoded by this gene. This is involved in ciliary functions in photoreceptors (Jacobson *et al.*, 2009). Mutations in *LCA5* therefore may lead to disruption of ciliary functions that in turn results in LCA.

1.9.12 IQ motif containing B1 (*IQCB1*)

IQ motif containing B1 (*IQCB1;* MIM#609237) is also known as *NPHP5* (Wang *et al.*, 2011). This gene encodes IQ domain protein that has an important role in function of cilia. This protein is located at connecting cilia on photoreceptors along with *CEP290*. Here they form a complex that is involved in transport of molecules to the outer layers of photoreceptors. *IQCB1* mutations lead to nonfunctional or absence of *IQCB1* protein (Estrada *et al.*, 2011). They are the cause of LCA and Senior- Løken syndrome (SLSN) (Wang *et al.*, 2011). SLSN is characterized by retinal defects and nephronophthisis. *IQCB1* mutations may result in LCA without other syndromic phenotypes. *IQCB1* mutations may cause LCA without nephronophthisis, but some patients may develop kidney diseases in later stages of life. So it is inferred that patients with LCA disease due to *IQCB1* mutations are at high risk of renal failure. This although may not be recognizable in early stages, due to mild symptoms, but may lead to sudden death due to imbalance of electrolytes and fluids. LCA patients are therefore recommended to have screening for *IQCB1* mutations so that onset of kidney disease may be detected. The occurrence or severity of kidney failure may be dependent on unknown modifier alleles or environmental factors (Estrada *et al.*, 2011).

1.9.13 Retinol Dehydrogenase 12 (*RDH12*)

Retinol dehydrogenase 12 (*RDH12;* MIM#608830) is present on chromosome 14q23.3. It has 7 exons (Sun et al., 2007). It is highly expressed in retina, in inner

layers of rods and cones. It is a member of dehydrogenase/reductase family and subfamily of dual specificity retinol dehydrogenases. Vitamin A analogue (11-*cis* retinal) is required in photoreceptor cells to absorb photons, for the process of phototransduction. For this purpose dietary vitamin (all-*trans* retinal) is converted to 11-*cis* retinal chromophore in retinal pigment epithelium (RPE). This is called the visual cycle. *RDH12* is very important for this cycle and acts as $NADP^+$- dependent oxidoreductase (Lee *et al.*, 2010). Mutation in *Rdh12* leads to reduced production of all *trans*-retinol from all *trans*-retinal (Sun *et al.*, 2007). Rod and cone dystrophy is the result of impairment of this visual cycle in 10.5% of the cases (Perrault *et al.*, 2004). *RDH12* also protects rods and cones from toxic excess caused by retinaldehyde. It enters into the photoreceptors from rhodopsins (Lee *et al.*, 2010), i.e. it protects against the light stimulated apoptosis of photoreceptors. For example, it is involved in detoxifying 4-hydroxynenal (4-HNE) (Mackey *et al.*, 2011), which is one of the most abundant and most toxic compound of lipid peroxidation (Merchette *et al.*, 2010). *RDH12* mutations have also been linked with one family of autosomal-dominant retinitis pigmentosa (Mackey *et al.*, 2011).

1.9.14 Lecithin Retinol Acyl transferase (*LRAT*)

Lecithin retinol acyltransferase (*LRAT;* MIM#604863) is located on chromosome 4q31. It encodes protein of 230 amino acids (25.7 kDa) and is part of NlpC/P60 thiolpeptidase protein superfamily. It is expressed in liver, RPE, small intestine, pancreas, colon and brain (Kiser *et al.*, 2012). This protein acts as an enzyme for conversion of all-*trans*-retinol into all-*trans*-retinyl esters in RPE. It acts in visual cycle in formation of 11-*cis*-retinaldehyde which is chromophore for rhodopsin. It is the storage form of all-*trans*-retinol (vitamin A) (Ruiz *et al.*, 2001). Mutations in *LRAT* gene may result in deficiency of *LRAT* enzyme, which will result in decreased concentration of 11-*cis*-retinal chromophore. Thus it will decrease the level of functional visual pigment and lead to death of photoreceptors. *LRAT* absence effects three steps of retinoid metabolism. Firstly, absorption of vitamin A from small intestine. Secondly, storage of vitamin A in liver cells, thirdly,uptake of vitamin A by tissues (Kiser *et al.*, 2012). Degeneration of cone occurs more rapidly than rod (Dev Borman *et al.*, 2012). Therefore it leads to severe retinal dystrophy like LCA (Ruiz *et al.*, 2001).

1.9.15 MER Tyrosine Kinase (*MERTK*)

MER tyrosine kinase (*MERTK;* MIM#604705) is the cause of LCA in 0.6% of patients. It is included in TAM (Tyro3/Axl/Mer) receptor tyrosine kinase family (Mackay *et al.*, 2010). *MERTK* is found on chromosome 2q14.1. It consists of 19 exons encoding protein of 999 amino acids (110 kDa) (Marchette *et al.*,2010). It is expressed in monocytes/macrophages, testis and epithelial cells. It has extracellular domains, namely two immunoglobulin-like-C2 (IgGC2) and two fibronectin type III (FN-III) domain and an intracellular tyrosine kinase domain (Strick and Vollrath, 2010). It is involved in cell proliferation and survival, down-modulation of pro inflammatory signals.

As a receptor it is involved in signal transduction and also in phagocytic uptake of degraded membranes of photoreceptors by RPE. This is important for retina survival (Shelby *et al.*, 2013). Due to mutation if the protein is not normally functioning the apoptotic debris will acuumulate in the retinal spaces which leads to degeneration of rods and cones and vision loss. Mutations in *MERTK* are also associated with rod cone dystrophy and macular atrophy (Lipinski *et al.*, 2013).

1.9.16 Potassium Inwardly-Rectifying Channel, Subfamily J, Member 13 (*KCNJ13*)

It is located on chromosome 2q37 (Döring and Kaarschin, 2000) and has 3 exons. It encodes a protein Kir 7.1 of 360 amino acids (Sergouniotis *et al.*, 2011). *KCNJ13* (MIM#603208) is expressed in many tissues like kidney, intestine, stomach, thyroid, spinal cord, brain, and eye (Pttnaik *et al.*, 2012). In eye it is localized to RPE membranes. The encoded protein is a low-conductance inwardly rectifying potassium channel (Sergouniotis *et al.*, 2011). It is composed of two transmembrane a helices, M1 and M2, and a pore loop. It normally is in homotetramer form.

These channels have great effect on transport of ion, metabolite and fluid inside the eye, by influencing the membrane potential (Yang *et al.*, 2008). In mutated form of gene, functional homotetramer will not be produced (Sergouniotis *et al.*, 2011), which lead to premature depolarization, causing leaky channels. Due to mutation the Kir channels formed may not localize properly to the RPE membrane, to mediate potassium influx (Pttnaik *et al.*, 2012). So mutated form of gene effects the channels and inturn the transport and concentration of molecules in eye leading to LCA.

1.9.17 Nicotinamide Mononucleotide Adenylyl Transferase 1 (*NMNAT1*)

Nicotinamide mononucleotide adenylyl transferase 1 (*NMNAT1;* MIM#608700) has 4 exons and is located at 1p36.22. Its protein is of 279 amino acids that plays crucial role in NAD^+ synthesis. The protein acts as an enzyme that catalyzes the formation of NAD^+ from ATP and nicotinamide mononucleotide (NMN) (Chiang *et al.*, 2012). This NAD^+ homeostasis is essential for DNA metabolism and cell signalling (Koenekoop *et al.*, 2012). In addition to this enzymatic role, *NMNAT1* acts as chaperon for the protection of retina against neurodegeneration. The mutations in *NMNAT1* may lead to defective level of NAD^+, which may not be enough to prevent the degeneration of photoreceptors (Chiang*et al.*, 2012).

1.9.18 Tubby-Like Protein 1 (*TULP1*)

Tubby-like protein (*TULP1;* MIM#602280) is located on chromosome 6p21.3, has 15 exons and is specifically expressed in retina. Its mutations are cause of LCA in 1.7% patients (Mataftsi *et al.*, 2007). It belongs to TULP family of proteins (TUB, TULP1, TULP2, TULP3), which play important role in vertebrate development at embryonic level and functioning of central nervous system. *TULP1* is mainly expressed in rod, cone photoreceptors (Kannabiran *et al.*, 2012) and is involved in traficking of proteins, like rhodopsin inside the eye. It also acts as transcription factor, for the downstream genes of photoreceptors. Results show that mutations in tubby genes cause degeneration of cochlea and photoreceptors (Ajmal *et al.*, 2012).

1.10 Gene Replacement Therapy

This procedure has come up as a solution to LCA disease. It is in its pre-clinical stages yet and efforts are being put to make it more efficeint procedure. Animal models of LCA have been used to test this process, that had human like mutations.

Adeno associated virus (AAV) is used as gene delivery system, as it can easily transduce through rod, cones and RPE. It has non-toxic nature and its effect is non-immunogenic and long lasting (Weber *et al.*, 2003).

Table 1.1: Candidate genes for LCA, along with their function, phenotypes and mutation frequency (Adapted and modified from Fazzi *et al.*, 2003).

Gene	Locus	Function	Expression	Phenotype Associated With LCA	Mutation Frequency
GUCY 2D	17p13. 1	Phototransduct ion (cGMP synthesis)	PRCs	Poor vision, mild-to-severe hyperopia, normal fundus appearance, photophobia	2.7-21
RPE65	1p31.3 - 1p31.2	Visual Cycle (retinyl esters to 11-*cis*-retinol)	RPE	Night blindness, photo attraction, nystagmus, mild-to-moderate hyperopia, relatively good vision early in life and later deterioration, bull's eye maculopathy, atrophic lesions	1.7-9
SPATA 7	14q31. 3		Retina	Poor vision, retinal atrophy, attenuated vessels	1.7-2.9
AIPL1	17p13. 2	Maintenance of rod photoreceptor, chaperone and protein farnesylation	In rods of central and peripheral retina, pineal glands	Poor vision, night blindness, late onset, pigmentary retinal changes, maculopathy, keratoconus or cataracts, decreased macular thickness	1.1-7.8
LCA5	6q14.1	Protein transport	Ubiquitous expression in early embryonic stages, connecting cilium, centriole and microtubules of PRCs in adults	Low vision (CF-20/100), severe hyperopia, macular colobama, peripheral pigment mottling	1.7-6
RPGRI P1	14q11. 2	Protein transport	Outer segment of rod photoreceptor cells, kidney, brain, heart, liver and spleen	Occasional night blindness, initially normal retinal appearance, progressive pigmentary retinopathy, loss of vision (20/20-LP) , intact ONL	4-28
CRX	19q13. 33	Transcription factor	PRCs, retinal inner nuclear layer and pineal gland	Severe vision loss (20/300-LP), infantile nystagmus, cataract, pigmentary retinopathy	0.6-3.4
CRB1	1q31.3	Cell-cell interaction	PRCs, retinal inner nuclear layer and iris	PPRPE, Coats-like response, keratoconus, thickened and disorganized retina	9-16
LCA9	1p36				

Gene	Locus	Function	Expression	Phenotype Associated With LCA	Mutation Frequency
IMPDH1	7q32.1	Regulation of cell growth	Ubiquitous	Rare, diffuse RPE mottling, moderate hyperopia, low vision	8.3
RD3	1q32.3	Transport of retinal guanylate cyclase	Subnuclear localization	Poor vision, atrophic and pigmentary maculopathy, RPE atrophy, optic nerve pallor	0.1
RDH12	14q24.1	Vitamin A cycle (converts all-tran s/ 11-cis-retinal to all-trans/11-cis-retinol)	RPE	RPE atrophy, pronounced attenuation of retinal arterioles, maculopathy, visual function in early life followed by a progressive decline	1-2
LRAT	4q32.1	Visual Cycle (converts all-trans-retinol to retinyl esters)	RPE	Night blindness, retinal atrophy, peripheral RPE atrophy, maculopathy, moderate optic nerve pallor	< 1.0
TULP1	2q37.1	Protein transport and synaptic development	PRCs	Reading vision in early stages, severe pigmentary retinopathy, early bull's eye maculopathy, moderate-to-severe myopia, color vision anomalies	<1-13
KCNJ13	2q13	Regulation of K transport	RPE, neural retina, CNS, epithelial cells of kidney, lung, small intestine, testes	NA	0.9
MERTK	3q13.33	OS phagocytosis	Macrophages, dendritic cell, RPE	NA	0.9-2.3
IQCB1		Protein transport across cilia	Photoreceptor and primary cilia of renal epithelial cells	NA	8

(C.F=Counting Finger, L.P= Light Perception, NA=Not Available, ONL= Outer Nuclear Layer, OS=Outer Segment, PPRPE= Preservation of Para Arteriolar Retinal epithelium, PRC=Photoreceptor cells, RPE= Retinal Pigment Epithelium).

1.10.1 Clinical Trials

The animal model that has been most extensively studied in respect of gene therapy is Briard dog. This breed of dog is affected by LCA caused by *RPE65* mutations. Visual recovery was seen when recombinant adeno-associated virus (AAV) serotype 2 having a chicken h-actin promoter/cytomegalovirus (CMV)-enhancer, wild type canine *RPE65* cDNA was administered to three briard dogs (Acland *et al.*, 2001). Further studies on models undergoing gene therapy showed removal of some symptoms of LCA, like nystagmus movement was lost, ERG responses had increased and lipid droplets in RPE were depleted (Narfstrom *et al.*, 2003). 3 years after the therapy, rod and cones functions were stabely restored in these models (Acland *et al.*, 2005).

Gene replacement therapies for other genes have shown promising results like restoration of photoreceptor functions and visual restoration in mouse model of Gucy2D or GC1 deficiency (guanylate cyclase 1 knockout) (Pasadhika *et al.*, 2010).

Stable restoration of structural and functional defects in various LCA models have been shown for other genes like *MERTK*, *LRAT*, *AIPL1*, *RPGRIP1* (Shukla *et al.*, 2006). *CEP290* is also being explored as candidate for gene therapy, as it is the most frequently mutated gene for LCA (Cideciyan *et al.*, 2007).

1.10.2 Human Clinical Trials

In 2007, clinical trials of AAV-*RPE65* gene replacement were initiated at three places (Bainbridge *et al.*, 2008; Maguire *et al.*, 2008). These trials demonstrated the, feasibility, efficacy and safety of gene therapy for LCA patients.

Bainbridge and coworkers gave AAV 2/2-hRPE65 sub-retinal injections with human *RPE65* promoter to 3 patients (Bainbridge *et al.*, 2008). Prior to treatment, structural integrity of the retina was analyzed using fundus photography, fundus autofluorescence and OCT. Additionally, visual acuity, contrast sensitivity, color vision, and cone flicker sensitivities were performed and perimetric procedures enabled the assessment of visual fields, before and after treatment was conducted. Ultimately, patients were submitted to obstacle course testing, at different illumination conditions, to evaluate their real-world visual performance.Results showed that only one of the patients showed visual improvement after gene therapy was performed. He probably had less advanced retinal disease at time of intervention,

which may explain the lack of visual improvement in the other patients. In this study, no serious adverse effects were reported (Bainbridge *et al.*, 2008).

AAV2-h*RPE65*v2 with chicken b-actin promoter was injected in Philadelphian study by Maguire and co workers in 3 patients (Maguire *et al.*, 2008). Two weeks post-surgery, all patients self-reported vision improvement in dimly lit environments . Clinically significant improvements in visual acuities were also observed. measures of nystagmus frequency and pupillary constriction were much improved after treatment, which reflects enhanced retinal sensitivity, and better transmission of visual input signals to the brainstem. Of note, clinical benefits remained stable during the 6 months of follow-up. Importantly, no serious systemic adverse events were reported in any of the patients. However, a full-thickness macular hole developed in one of the patients. This can possibly be explained by the proximity between the site of the retinotomy and the foveal region (Hauswirth *et al.*, 2008).

Improved visual acuities in both studies are likely due to photoreceptor rescue but, since no detectable improvements in ERG responses were reported in either study, it is impossible to confirm this assumption (Hauswirth *et al.*, 2008; Koenekoop 2008). Differences in visual outcome between the two trials may be due to several factors, including differences in patient genotypes, or differences in the promoters that were use to enhance *RPE65* expression. Furthermore, better visual acuities at baseline in the Bainbridge study might be another contributing factor. Common to both studies were the facts that low doses of the viral vector were used, and patients were at an age considered old, in which photoreceptor loss is likely to already have commenced (Koenekoop 2008). The results showed that functional improvements might be better in younger patients (Bainbridge et al. 2008, Hauswirth *et al.*, 2008, Maguire et al. 2008) and with higher doses of the vectors (Hauswirth *et al.*, 2008).

In a parallel study, Cideciyan and colleagues performed gene therapy to three human LCA patients, ranging from 21-24 years old, restoring normal RPE65 function through AAV2-mediated gene transfer (Cideciyan *et al.*, 2008). After treatment, rod-dependent night vision increased in sensitivity up to 63000 fold in the three patients, an improvement observed exclusively in the retinal areas exposed to therapy. Moreover, the magnitude of improvement differed among patients, and those with a better preserved photoreceptor layer in the treated area were found to have greater rod rescue. Cone-mediated vision was also found to have robustly improved. Of note,

none of the patients showed a decrease in nystagmus frequency. It was suggested that the increase in rod sensitivity probably relates to an increased 11-cis-retinal synthesis mediated by the *RPE65* transgene. Surprisingly, Cideciyan *et al.*, (2008) found that although therapy appeared to successfully restore normal retinoid cycle activity, it did so but with abnormally slow rod kinetics. Indeed, after a desensitizing light flash, rod recovery in two of the patients progressed slowly, lasting at least 8 hours. The investigators suggest that the prolonged rod recovery could relate to slowed delivery of 11-cis-retinal from the RPE to the rod photoreceptors either due to a reduced synthesis rate, possibly relating to limited transgene expression, or to an increased obstruction to its inter or intracellular transport, eventually related to the underlying retinal degeneration (Cideciyan *et al.*, 2008). The finding of slow post-treatment rod kinetics has practical implications since it will surely influence the design of future clinical trials. Cideciyan et al highlight this issue alerting for the fact that maximum increase in vision after gene therapy can only be judged after patients have undergone a long period of dark adaptation, in order to provide enough time for full rod photoreceptor recovery. Likewise, comparisons of visual function between patients within or between trials cannot be made unless rigorous attention is given to previous light exposure and length of dark adaptation (Cideciyan *et al.*, 2008).

AAV2-h*RPE65* was used in 3 patients by Hauswirth et al (Jacobson *et al.*, 2006). The initial results of these trials are very promising and one has to wait to see results of next stages of clinical trials of *RPE65* gene therapy. Various other gene delivery procedures have also been developed. Photoreceptor replacement using cell based therapies can also prove to be a good option. Rod and cones transplantation in retina in animal models has also been proved feasible (Shukla *et al.*, 2007). Altogether, these favorable results will certainly allow for the development of future human clinical trials, in which new, previously unexplored premises will surely be addressed, bringing further insights into the emerging field of ocular gene therapy, particularly that related to LCA. Additionally, gene therapy's success in human LCA patients has nurtured hope regarding the possibility of its applicability to other visually debilitating diseases, such as Stargardt disease.

1.11 Scope of Study

The basic reason for this study is to add information to the genetic causes of LCA. After so much of research going on this area, still in 30% of LCA cases underlying

genetic information is not known (Shukla *et al.*, 2007). The study is based on families with eye diseases showing autosomal recessive inheritance. Candidate genes which are known to be involved in disease are first checked. If none of the candidate gene proves to be the cause than the study is elaborated further. Different techniques are then used to find the causative gene. All this practice helps make a database of genes, mutations in which lead to disease. This can be further used to develop the therapy techniques.

2. MATERIALS and METHODS

2.1 Families

The research work was carried out on two families (Family A and Family B) suffering from autosomal recessive Leber's congenital amaurosis (LCA). At the time of blood collection, pedigree was drawn according to the illustration of Bennett *et al* (1995). Other related information regarding onset of disease and its symptoms were noted during the visit of respective areas.

2.2 Blood Collection

Blood was drawn in 10ml syringes from each individual, was stored in standard potassium ethylene diamine tetra acetate vacutainer tubes (K3 EDTA tubes) (BD biosciences, Franklin Lakes USA).These tubes were brought to and stored in laboratory at the Department of Biochemistry, Quaid-i-Azam University, Islamabad.

2.3 Genomic DNA Extraction

To extract DNA from the blood we used Organic method, i.e., Phenol-Chloroform extraction method. For this purpose 750µl blood of each individual was taken in separate micro centrifuge tubes (1.5ml). Afterwards equal volume, i-e. 750 µl of Solution A was added in the tubes, and were kept at room temperature for 15-20 minutes with occasional mixing. Centrifugation was done at 13,000 rpm (revolutions per minute) for 60 seconds. The supernatant was then discarded and 400 µl of Solution A was added in the pellet. Again centrifugation was done at 13,000 rpm for 1 minute and supernatant was again discarded. Later 500 µl of solution B, 12 µl of Sodium Dodecyl Sulphate (SDS 20%) and 5µl Proteinase K (20 mg/ml) were added in the pellet and tubes were kept at 37° C overnight in incubator.

Next day, 500 µl of freshly prepared Solution C and Solution D was added in the tubes. The tubes were inverted several times and centrifugation was done for 10 minutes at 13,000 rpm. The pellet was discarded and the upper aqueous layer was collected in new tubes. Afterwards 500 µl of Solution D was added and centrifugation was repeated for 10 minutes at 13,000 rpm. Again the aqueous phase was collected in new tubes and 55 µl of sodium acetate (3M, pH 6) along with 500 µl of isopropanol was added to DNA. The tubes were inverted several times and centrifuged at 13,000 rpm for 10 minutes. The DNA was precipitated and washed with 200 µl of chilled

ethanol (70%) and was centrifuged for the last time for 7 minutes at 13,000 rpm. The pellet was dried by keeping at room temperature for 30 minutes. Finally, it was dissolved in 100-200 µl of Tris-EDTA (TE Buffer).

Horizontal gel (1%) electrophoresis was performed to analyze the extracted DNA and gels were documented on Gel Doc system (SYNGENE, UK).

2.4 Polymerase Chain Reaction (PCR)

PCR was performed for the amplification of the genomic DNA. The reaction mixture is of 25 µl and is performed in 0.2 ml PCR tubes (Axygen, USA).

The reaction mixture contained

- 1-1.5 µl DNA of individual
- 2.5 µl 10X buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3)
- 1.5-1.8 $MgCl_2$ (25 mM)
- 0.5 µl dNTPs (10Mm, Fermentas UK)
- 0.3 µl of each forward and reverse marker (0.1 M)
- 0.5 Taq DNA Polymerase (Fermentas, UK)
- 17.6-18.4 µl PCR Water

The tubes were vortexed and were given short spin. T1 thermal cyclers (Biometra, Germany) were used for PCR, by applying following conditions:

- Initial whole genome denaturation : 95°C for 5 minutes
- Denaturation : 95 °C for 1 minute
- Primer annealing : 54-65 °C for 45 seconds
- Extension : 72 °C for 1 minute
- Final extension : 72 °C for 10 minutes

→ 40 Cycles

The PCR products were separated on 8% polyacrylamide gel. Its contents are:

- 30% Acrylamide (29:1 of acrylamide and N, N' Methylene bisacrylamide)
- 10 X Tris/Borate/EDTA (TBE) (Tris 0.89 M, Borate 0.89 M, EDTA 0.02 M)
- 10% Ammonium persulphate
- Tetramethylethylenediamin (TEMED)
- Distilled Water

The electrophoresis was carried out by using vertical gel tank model V16-2 (Life Technologies, USA). The PCR samples were mixed with 5 µl Bromophenol dye (0.25% bromophenol, 40% sucrose) and were loaded into the wells of the gel. The gels were then visualized under ultraviolet light and images were acquired by using Gel Doc system (SYNGENE, UK).

2.5 SPATA7 Sequencing

2.5.1 Primer Designing

Ensemble genome browser was used to download the sequence of SPATA7 (ENSG00000042317) gene. Primer 3.0 software was used to design primers. Blast like alignment tool (BLAT) was used to check the specificity. Forward and reverse primers for each exon with single hit were selected and purchased from Gene link (USA). The primer list is given in Table 2.2.

2.5.2 First PCR

Firstly for each exon amplification of 25 µL reaction mixture was done by the same procedure of PCR as described earlier. DNA of normal and affected individual of each family was taken.

2.5.3 PCR Product Purification

For PCR product purification PCR Cleanup kit was used. By this kit PCR product is purified and unincorporated primers, nucleotides and enzymes are removed. Following steps are involved in this process:

1. 100 µl of PCR-A buffer (DNA binding buffer) was initially added to 20 µl of PCR product and tubes were vortexed for mixing.
2. PCR column was placed in 2 ml microfuge tube. Mixture of PCR product and PCR-A was added to PCR column and centrifugation was done for 1 minute at 12, 000 rpm.
3. Filtrate was discarded from the tube and 700 µl of Buffer W2 (Desalting buffer) was added into column followed by centrifugation at 12, 000 rpm for 1 minute.
4. Filtrate was again discarded and 400 µl of W2 buffer was added.
5. PCR column was transferred to a new microfuge tube (1.5 ml) and 20-25 µl pre-warmed eluent (2.5 mM Tris-HCl, pH 8.5) was added to membrane and

left at room temperature for 1 minute. Centrifugation was done at 12, 000 rpm for 1 minute to elute purified PCR product.

Finally the eluent was run on 2% agarose gel to check integrity of PCR product and the specific products were selected for sequencing.

2.5.4 Sequencing PCR

CEQ 8800 (Beckman coulter, Germany) sequencer was used for sequencing. The reagents for sequencing reaction include:

- Purified PCR product (4-5 µL)
- Reverse or forward primer (2 µL)
- DTCS sequencing kit (2.5-3 µL)
- PCR water (as required)

The reagents mentioned above were utilized to make 10 µL reaction mixtures and amplified in T1 thermocyclers (Biometra, Germany) by using following conditions:

- Initial whole genomic denaturation: 96 °C for 10 minutes
- Denaturation: 96 °C for 20 seconds
- Primer annealing: 54-65 °C for 20 seconds
- Extension: 60 °C for 4 minutes

30 cycles

- Final extension: 65-72 °C for 10 minutes

At the end of PCR, reactions were stopped by using 3 M sodium acetate (pH 5.2), 100 mM sodium EDTA (pH 8) and glycogen (20mg/mL). The contents were mixed by vortex and purified PCR products were transferred to sample plates and a drop of mineral oil was laid on it before sequencing (ceq8800 Beckman Coulter, Germany). Products were purified and washed by using 100 % and 70 % ethanol, respectively. Both steps were followed by centrifugation at 13, 000 rpm for 20 minutes.

2.5.5 Analysis

BioEdit software version 7.1.3.0 was used for data analysis for variations. Ensemble genome browser gave us the gene sequence. These sequences was imported to BioEdit software and aligned. The reverse primer data was also aligned with reference sequence. The alignment option used was ClustW Multiple Alignment. The sequence changes that were observed were checked in all the available members of the family to observe segregation pattern.

Table 2.1: List of microsatellite markers for LCA

S.No.	Gene	Markers	Distance (cM)
1	RPGRIP1	D14S122	4.91
		D14S742	9.27
2	AIPL1	D17S1149	19.95
		D17S1298	13.23
3	GUCY2D	D17S720	23.27
		D17S1879	32.73
4	NMNAT1	D1S1597	28.78
5	TULP1	D6S1611	57.04
		D6S1051	58.46
6	RDH12	D14S1038	57.19
		D14S1004	70.12
7	CRX	D19S543	71.49
		D19S902	75.28
8	CABP4	D11S4076	72.3
		D11S4136	80.2
9	SPATA7	D14S1063	83.69
		D14S1066	87.22
10	LCA5	D6S1282	86.81
		D6S1031	93.14
11	CEP290	D12S853	100.4
		D12S1598	101.32
12	RPE65	D1S3467	96.03
		D1S2761	105.16
13	MERTK	D2S293	119.81
		D2S1891	122.2
14	IMPDH1	D7S2543	124.42
		D7S530	132.96
15	LRAT	D4S43049	156.69
		D4S413	160.2
16	CRB1	D1S533	199.85
		D1S1660	202.08
17	RD3	D1S1667	221.7
		D1S2827	225.64
18	KCNJ13	D2S2344	242.78
		D2S2973	249.8

Table 2.2: List of sequencing primers for SPATA7 gene

Primer Name	Forward Sequence	Reverse Sequence	Product size
SPATA7-Exon 5	TTGGCCAGTTTGTCATATCTTG	GGAAAAATTTGCTGACCCTAAA	570
SPATA7-Exon 6A	TCAGAAAGGAAATTGATTGCTC	GGGTGCTTTCGAAATGACTAAC	516
SPATA7-Exon 6B	GTCATCACAAATGGTCCTGAGA	TTCCAATCAAAAGGGCACTATC	466
SPATA7-Exon 8	AAGTGCTGGATGGATAGAAAAA	AACACCAAAATAGATTGGAGCA	401
SPATA7-Exon 11	CAGGAAAGGATTAGTCTTCAGC	CAGTGCTGAAAATCTCACAGGA	385
SPATA7-Exon12A	AATCCTGTGAGATTTTCAGCAC	ATAACCCCTTCTGAATGCTTTG	382
SPATA7-Exon12B	CACCAAAGGATGAGAACGAGA	GCAGCACAGAAAACCAATAGAG	427

3. RESULTS

3.1 Description of Families

3.1.1 Family A

Patients of family A were suffering from LCA. The pedigree is shown in Figure 3.1. The pedigree shows four generations. There were two affected male members of the family in fourth generation labeled IV-6 and IV-7. The pedigree analysis indicated that the disease is transmitted in autosomal recessive pattern. For linkage studies blood was taken from five members of the family including two affected (IV-6, IV-7) and three normal (III-1, III-2, IV-4) individuals.

3.1.1.1 Clinical Examination

Physical examination of the patients showed no malformation in ocular structure. The pupils responded to light and photophobia was not observed. DNA was extracted from blood of all five members available. It was stored at 4°C for further analysis.

3.1.2 Family B

Patients of family B also suffered from LCA. The pedigree is shown in Figure 3.2. Blood was collected from nine members of family including affected (III-3, IV-7, IV-8, V-1, V-2) and five normal (III-4, III-5, IV-3, IV-4, IV-6).

DNA was extracted for seven members that are III-3, III-4, III-5, IV-3, IV-6, IV-7, IV-8 and V-1, and stored at 4°C for further analysis.

3.1.2.1 Clinical Examination

Among the patients, IV-8 was not able to see, while IV-7 showed cataract. Tearing of eyes was not normal. V-1 showed cataract on left eye and V-2 was unable to see.

3.2 Linkage Analysis

The families were genotyped by using microsatellite markers for the candidate genes involved in LCA.

3.2.1 Family A

In Family A, all the five DNA samples were genotyped for the microsatellite markers specific for each candidate gene. The markers are listed in table 2.1. Analysis was done to locate regions of homozygosity shared by all affected individuals. The results showed that the affected individuals are heterozygous for parental alleles for a number of microsatellite markers (Figure 3.4-3.34).

A region of gene *RPE65* showed homozygosity for two microsatellite markers, D1S1180 and DIS2761 at 104.28 and 105.16 (Figure 3.26 and 3.27 respectively).The region was further genotyped for other markers (D1S3467, D1S1162) but the homozygosity did not continued ahead. So, the linkage to *RPE65* gene was not confirmed.

Another region of gene *SPATA7* also showed homozygosity. The markers D14S1063 and D14S1044 at 83.69 cM and 83.36 cM respectively (Figure 3.16 and 3.15), showed homozygous allele pattern for all affected individuals. All normal individuals were heterozygous. To confirm this linkage four other markers (D14S67, D14S1066, D14S256 and D14S617) were used to genotype family A members. All these markers showed the same pattern of homozygosity, (Figure 3.17 to 3.21). So the linkage was confirmed for *SPATA7*. Markers D14S61 and D6S1031 at 72.82 cM and 93.14 cM respectively were heterozygous in affected as well as normal individuals. These established the proximal and distal boundaries of minimal critical region identified in family A. The haplotype for SPATA7 gene linkage for Family A is given in Figure 3.3.

3.2.2 Family B

DNA samples of all available family members available were genotyped for microsatellite markers for all the candidate genes. The results obtained showed no region of homozygosity for any of the marker. As no linkage was observed, family B is excluded (Figure 3.35-3.60).

So, on the basis of this research it can be inferred that no known gene for LCA is the cause of disease in family B.

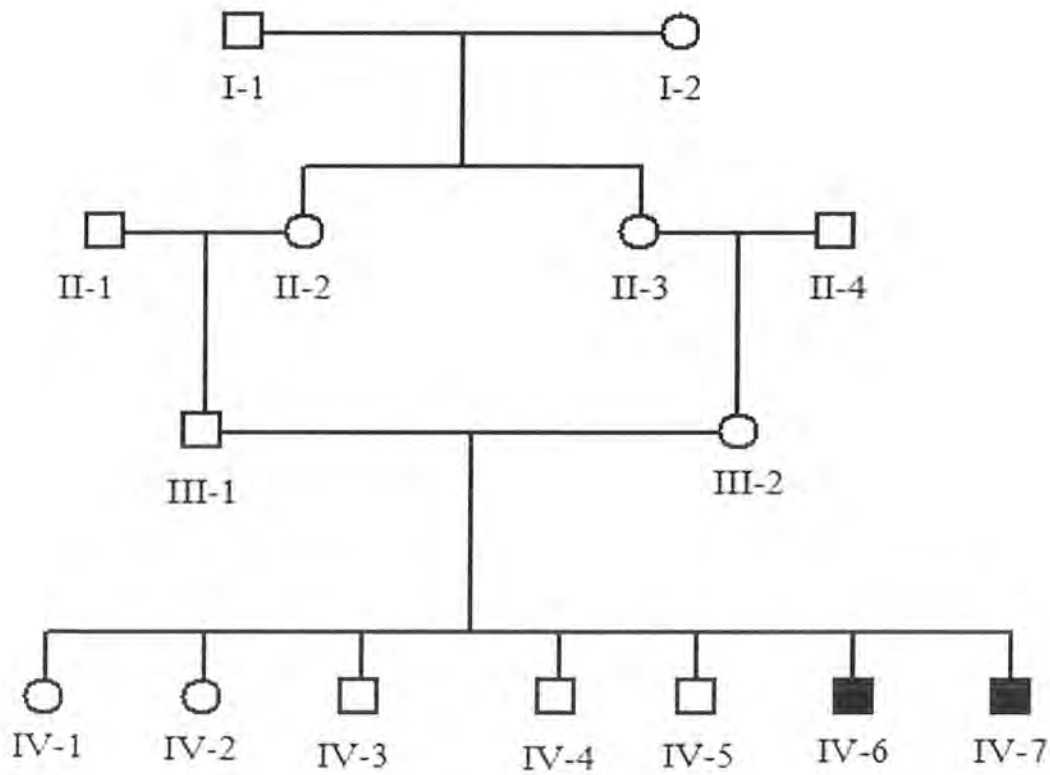


Figure 3.1: Pedigree of family A with LCA.

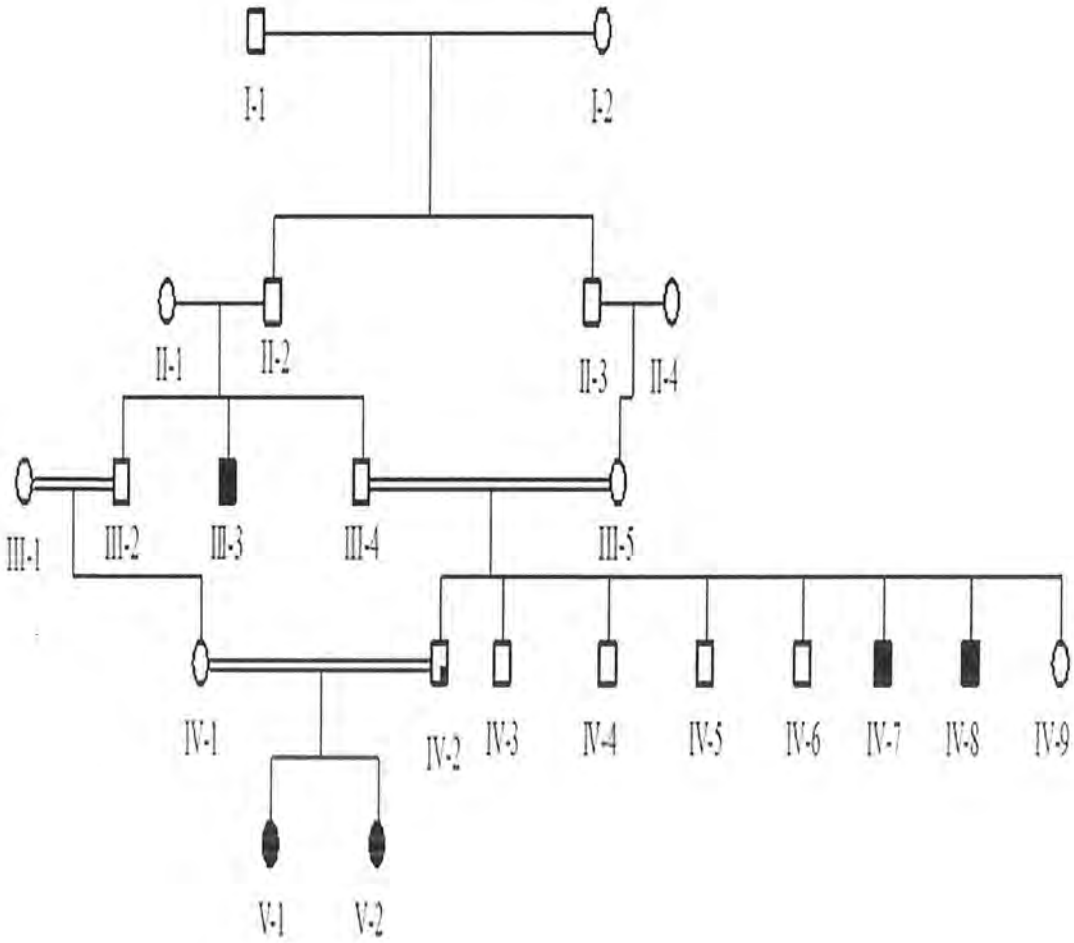
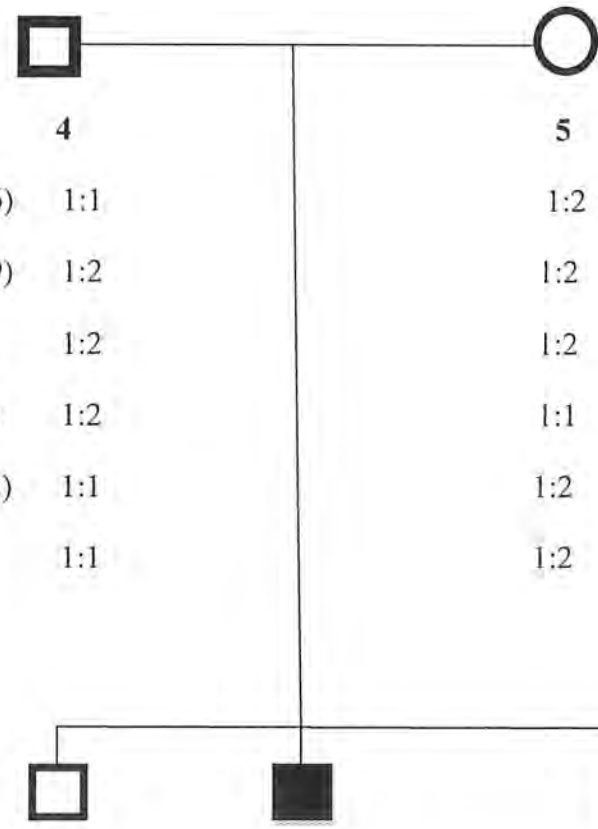


Figure 3.2: Pedigree of family B with LCA.



	4	5
D14S1044 (83.36)	1:1	1:2
D14S1063 (83.69)	1:2	1:2
D14S67 (84.85)	1:2	1:2
D14S256 (85.52)	1:2	1:1
D14S1066 (87.22)	1:1	1:2
D14S68 (87.22)	1:1	1:2

	3	1	2
D14S1044 (83.36)	1:2	1:1	1:1
D14S1063 (83.69)	1:2	1:1	1:1
D14S67 (84.85)	1:2	1:1	1:1
D14S256 (85.52)	1:2	1:1	1:1
D14S1066 (87.22)	1:2	1:1	1:1
D14S68 (87.22)	1:2	1:1	1:2

Figure 3.3: Haplotype for Family A showing homozygous region of *SPATA7* gene.

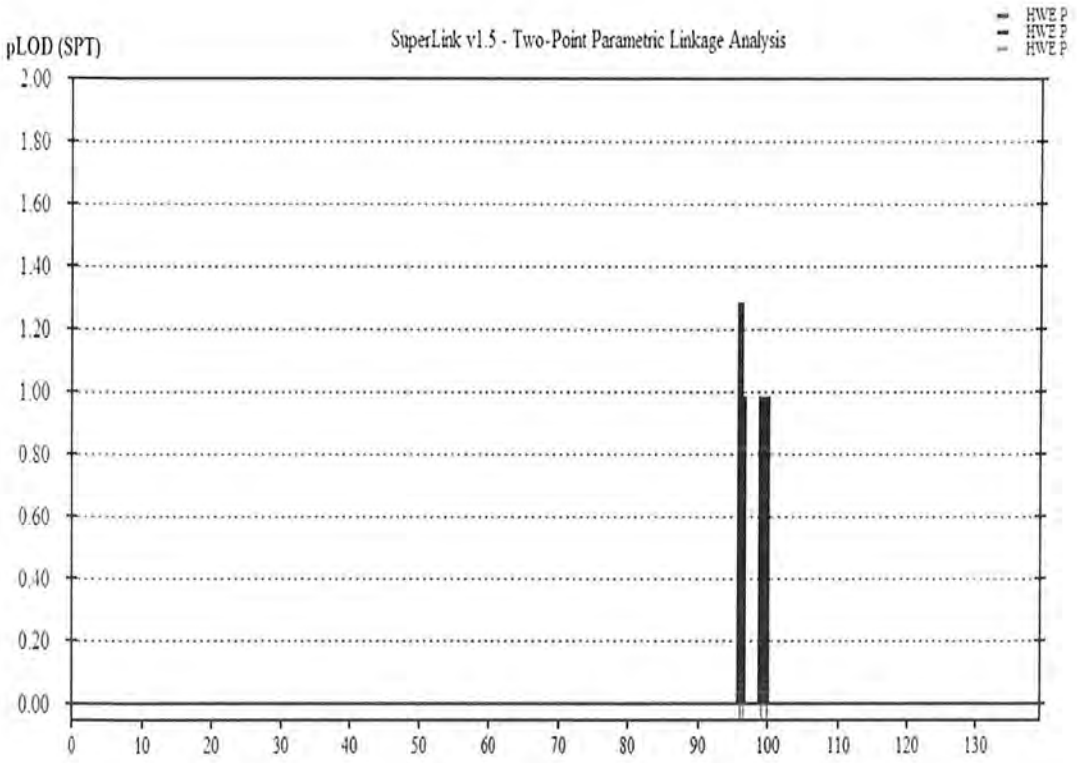


Figure 3.4: LOD score analysis of *SPATA7* linkage in family A.

3.3 Family A Results

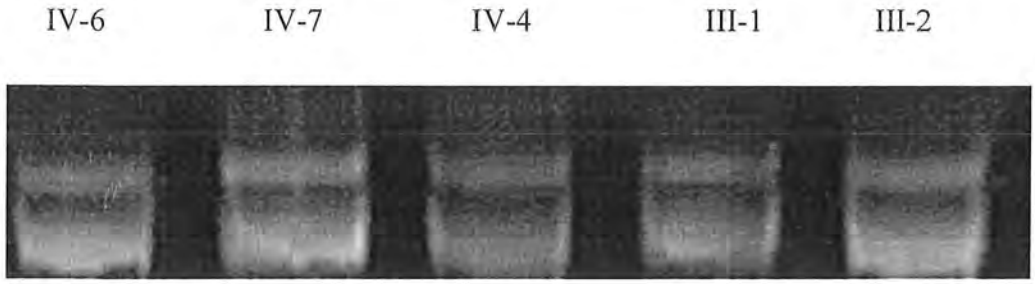


Figure 3.5: Electropherogram of 8% polyacrylamide gel of marker D14S742 at 9.2 cM.

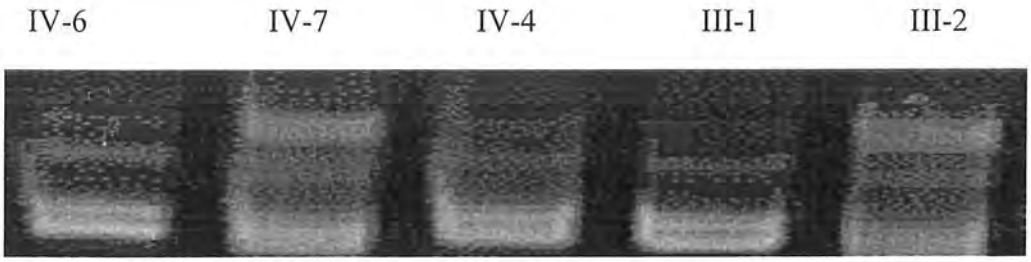


Figure 3.6: Electropherogram of 8% polyacrylamide gel of marker D14S28 at 10.32 cM.

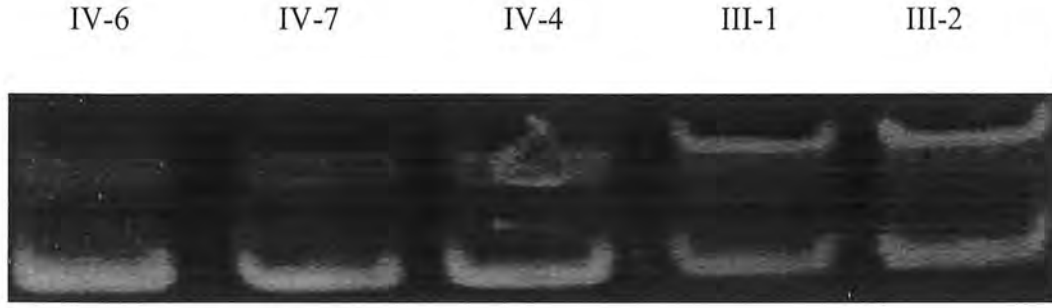


Figure 3.7: Electropherogram of 8% polyacrylamide gel of marker GATA158H04 at 19.95 cM.

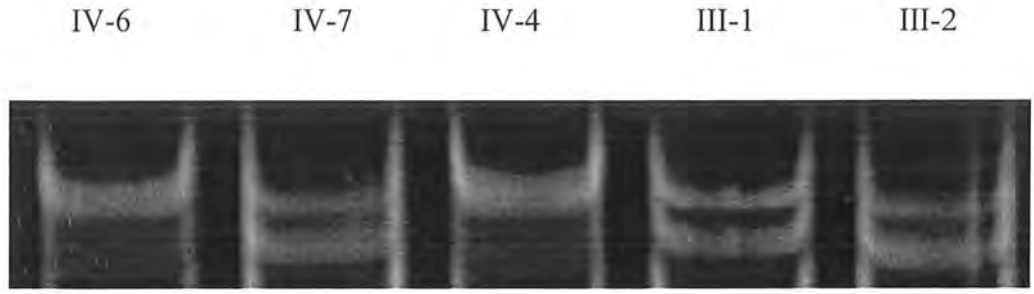


Figure 3.8: Electropherogram of 8% polyacrylamide gel of marker D17S786 at 25.58 cM.

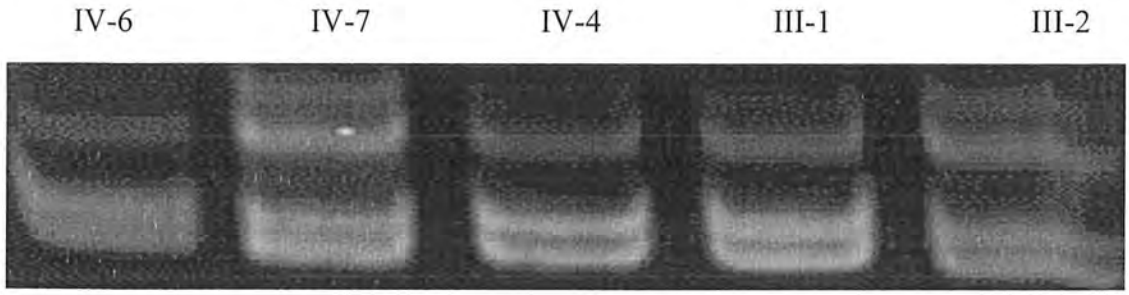


Figure 3.9: Electropherogram of 8% polyacrylamide gel of marker D17S1879 at 32.73 cM.

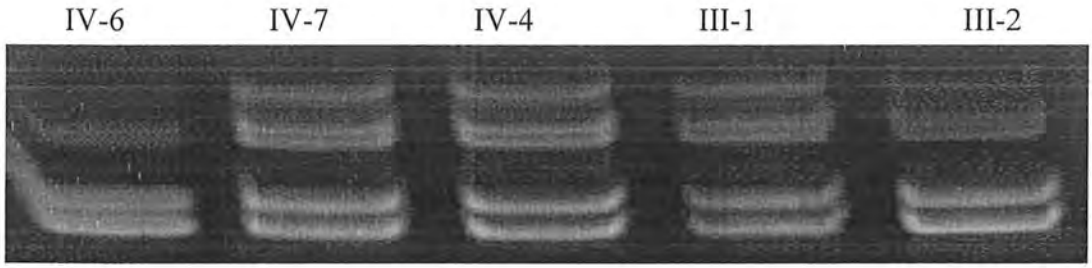


Figure 3.10: Electropherogram of 8% polyacrylamide gel of marker D6S1611 at 57.04 cM.

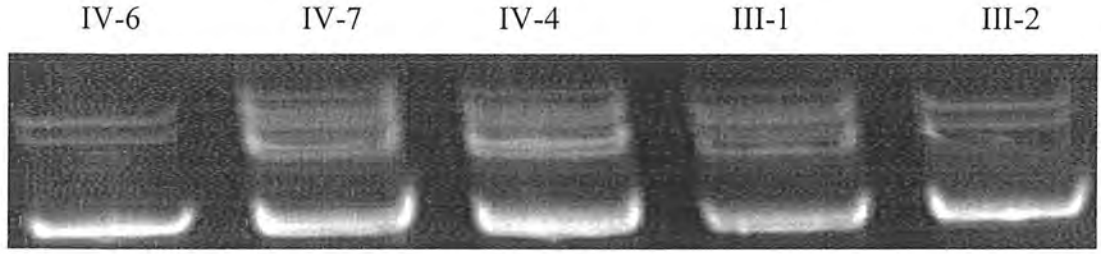


Figure 3.11: Electropherogram of 8% polyacrylamide gel of marker D6S1051 at 58.46 cM.

Figure 3.12: Electropherogram of 8% polyacrylamide gel of marker D14S1038 at 57.19 cM.

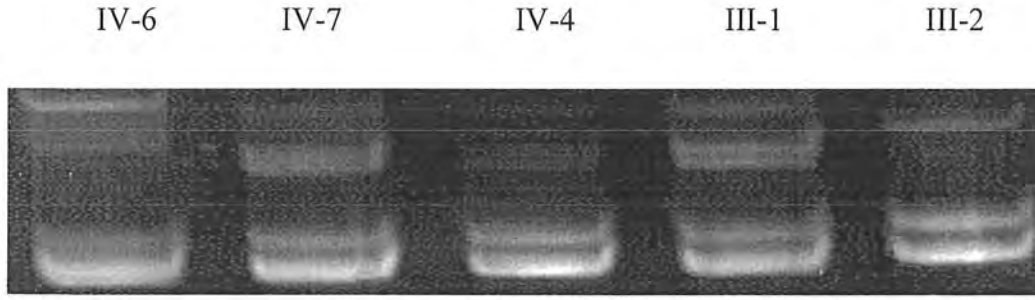


Figure 3.13: Electropherogram of 8% polyacrylamide gel of marker D14S1004 at 70.12 cM.

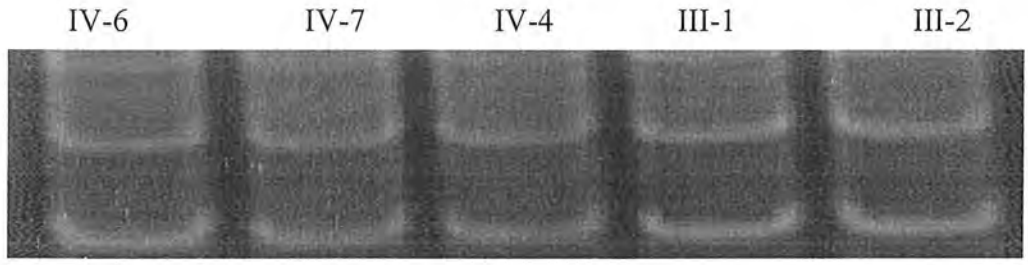


Figure 3.14: Electropherogram of 8% polyacrylamide gel of marker D19S543 at 71.49 cM.

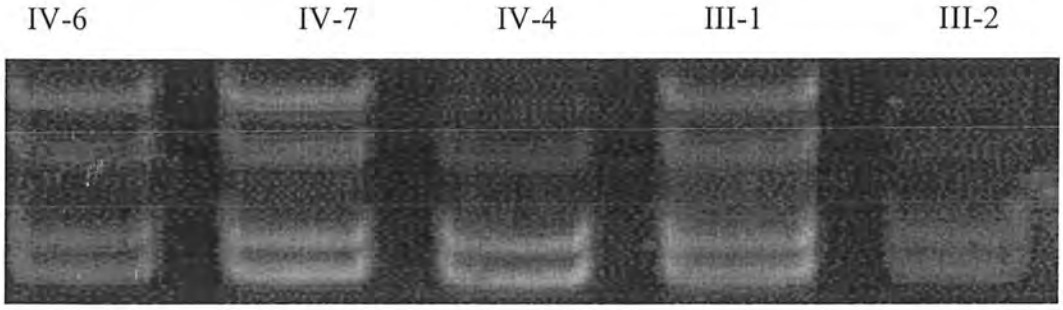


Figure 3.15: Electropherogram of 8% polyacrylamide gel of marker D19S902 at 75.2 cM.

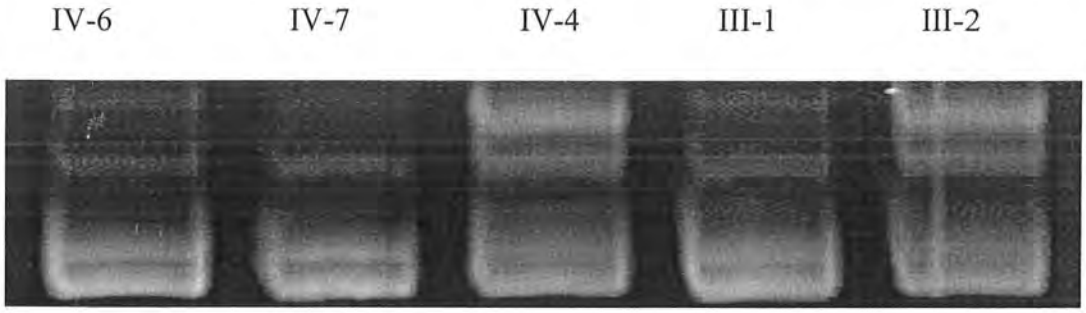


Figure 3.16: Electropherogram of 8% polyacrylamide gel of marker D14S1044 at 83.36 cM.

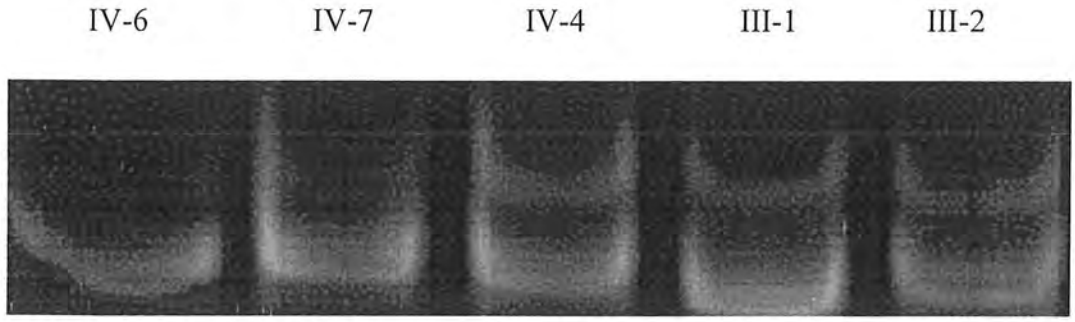


Figure 3.17: Electropherogram of 8% polyacrylamide gel of marker D14S1063 at 83.69 cM.

Figure 3.18: Electropherogram of 8% polyacrylamide gel of marker D14S67 at 84.85 cM..

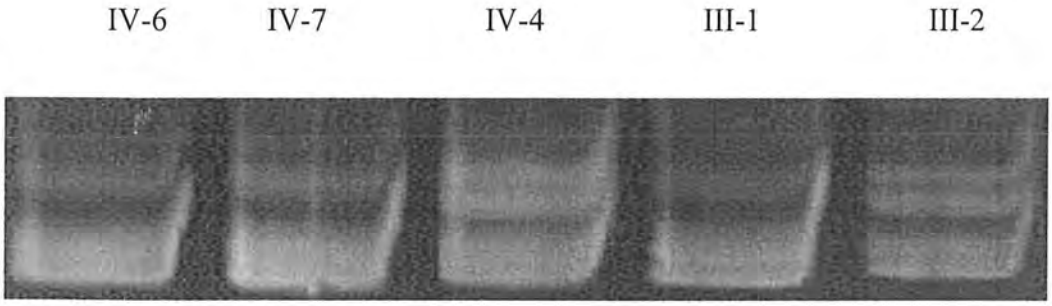


Figure 3.19: Electropherogram of 8% polyacrylamide gel of marker D14S1066 at 87.22 cM.

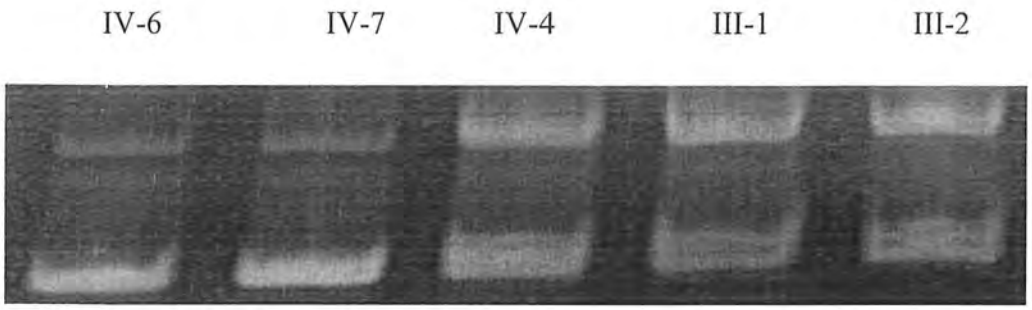


Figure 3.20: Electropherogram of 8% polyacrylamide gel of marker D14S617 at 92.74 cM.

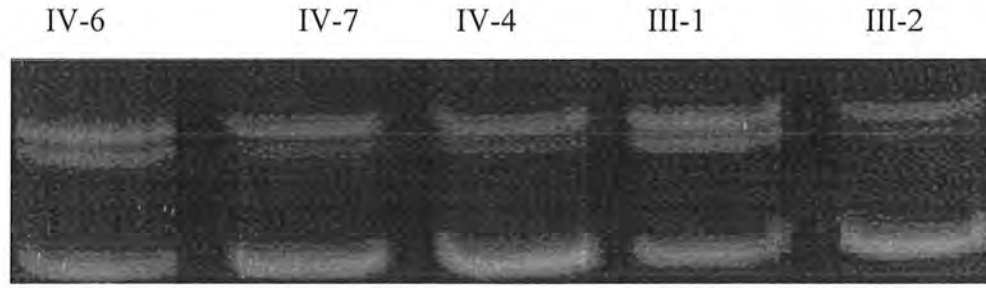


Figure 3.21: Electropherogram of 8% polyacrylamide gel of marker D6S1282 at 86.81 cM.

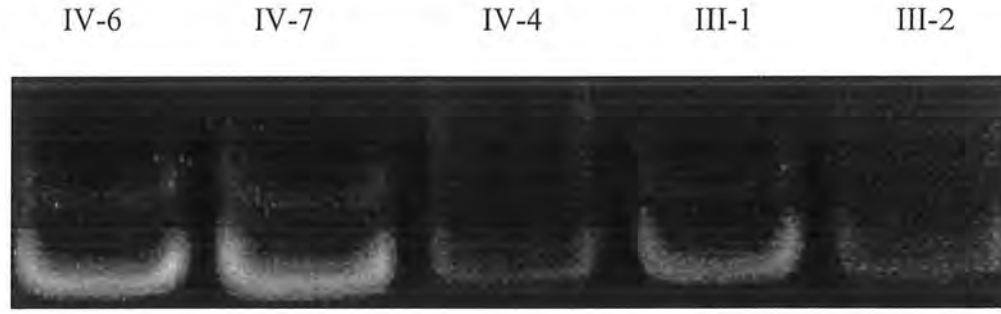


Figure 3.22: Electropherogram of 8% polyacrylamide gel of marker D6S1031 at 93.14 cM.

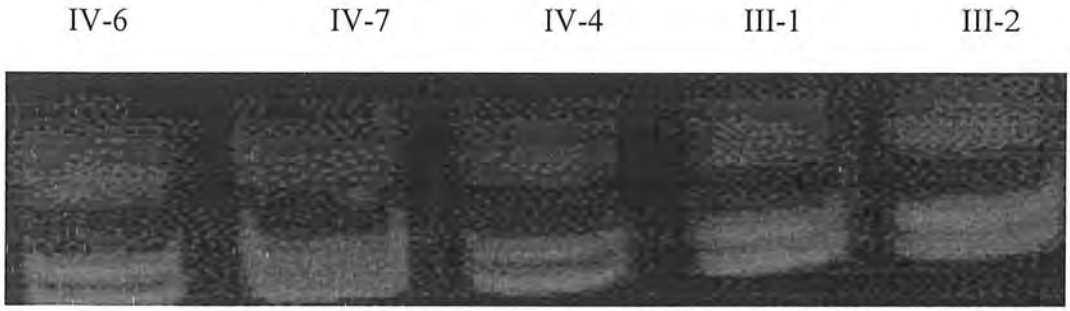


Figure 3.23: Electropherogram of 8% polyacrylamide gel of marker D12S853 at 100.4 cM

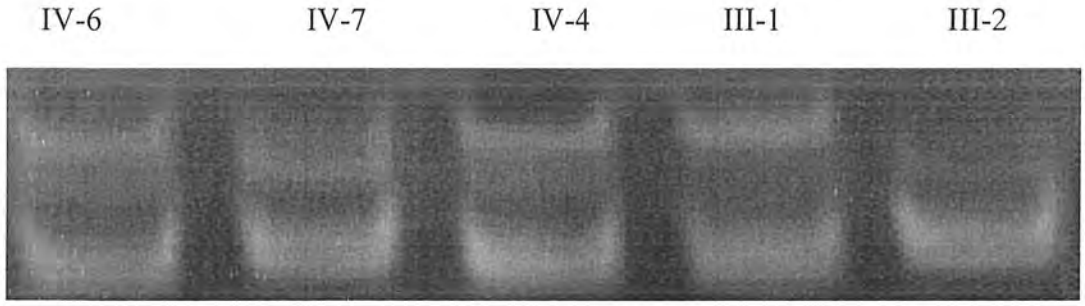


Figure 3.24: Electropherogram of 8% polyacrylamide gel of marker D12S1678 at 102.18 cM.

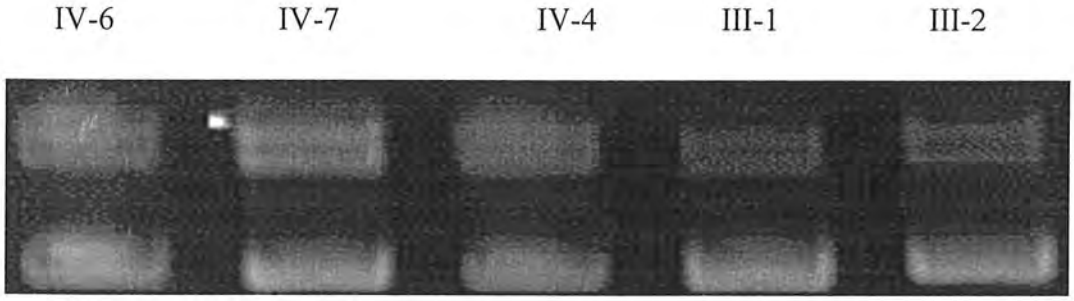


Figure 3.25: Electropherogram of 8% polyacrylamide gel of marker D1S1162 at 102.18 cM.

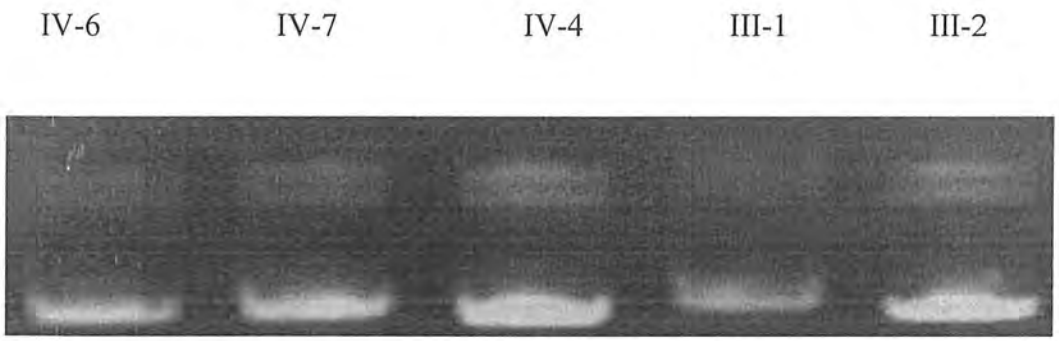


Figure 3.26: Electropherogram of 8% polyacrylamide gel of marker D1S1180 at 104.28 cM.

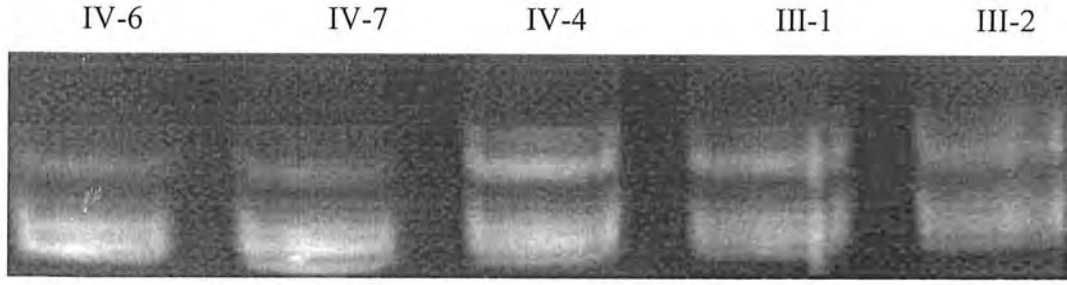


Figure 3.27: Electropherogram of 8% polyacrylamide gel of marker D1S2761 at 105.16 cM.

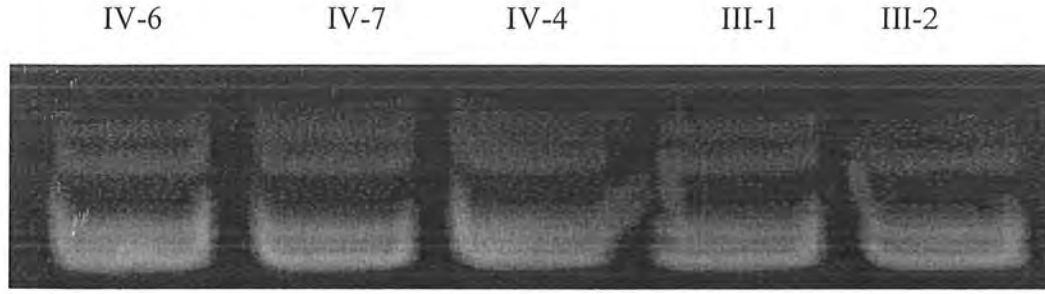


Figure 3.28: Electropherogram of 8% polyacrylamide gel of marker D7S2543 at 124.42 cM.

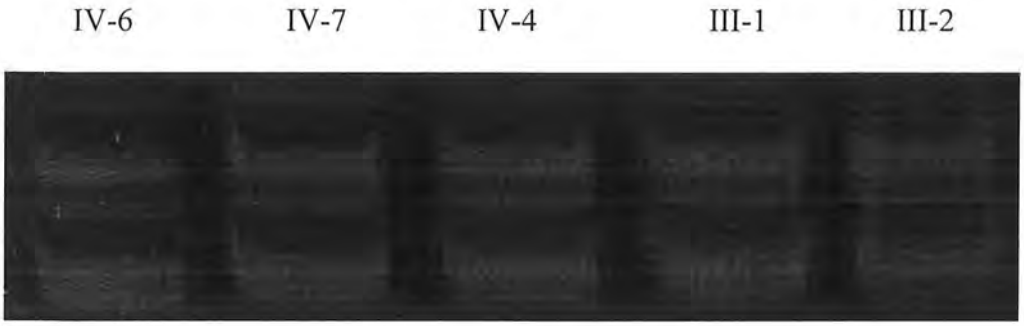


Figure 3.29: Electropherogram of 8% polyacrylamide gel of marker D7S530 at 132.9 cM.

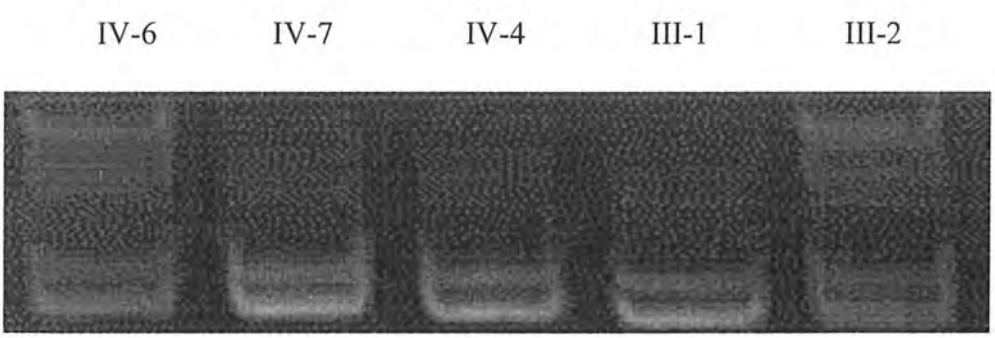


Figure 3.30: Electropherogram of 8% polyacrylamide gel of marker D4S3016 at 158.47cM .

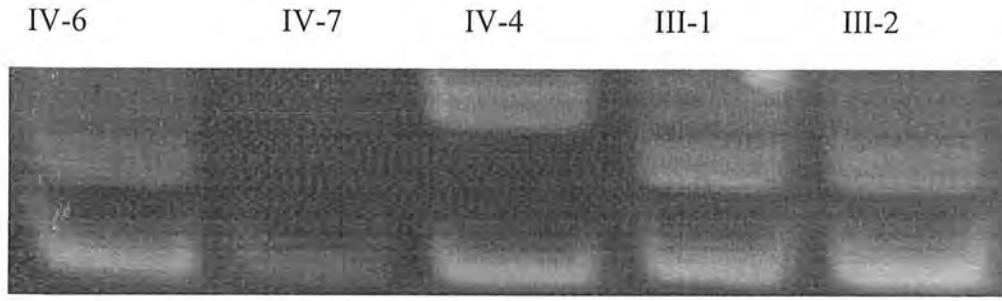


Figure 3.31: Electropherogram of 8% polyacrylamide gel of marker D4S413 at 160.2 cM

.

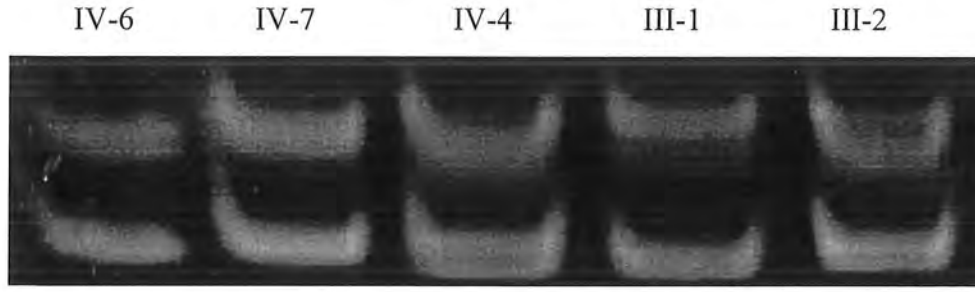


Figure 3.32: Electropherogram of 8% polyacrylamide gel of marker D1S533 at 199.85 cM.

IV-6 IV-7 IV-4 III-1 III-2

Figure 3.33: Electropherogram of 8% polyacrylamide gel of marker D1S2840 at 201.88 cM.

IV-6 IV-7 IV-4 III-1 III-2

Figure 3.34: Electropherogram of 8% polyacrylamide gel of marker D1S1667 at 221.7 cM.

IV-6 IV-7 IV-4 III-1 III-2

Figure 3.35: Electropherogram of 8% polyacrylamide gel of marker D1S2827 at 225.64 cM .

3.4 FAMILY- B RESULTS

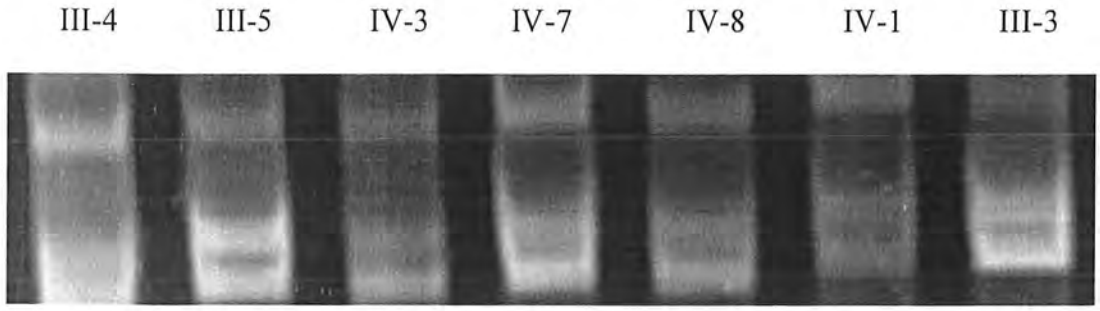


Figure 3.36: Electropherogram of 8% polyacrylamide gel of marker D14S1043 at 4.91 cM.

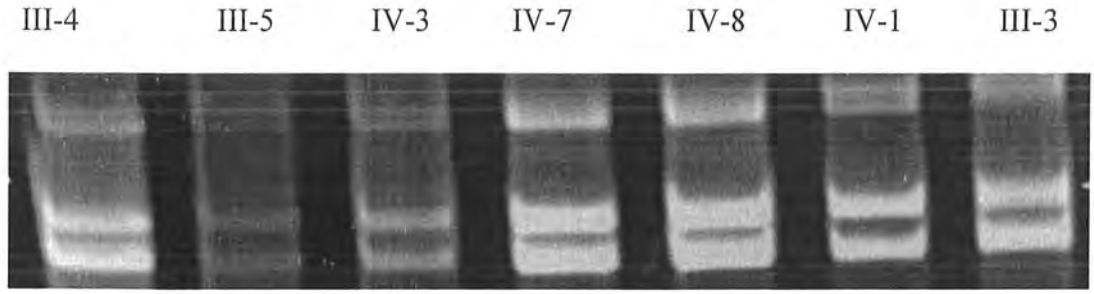


Figure 3.37: Electropherogram of 8% polyacrylamide gel of marker D14S1070 at 5.27 cM.

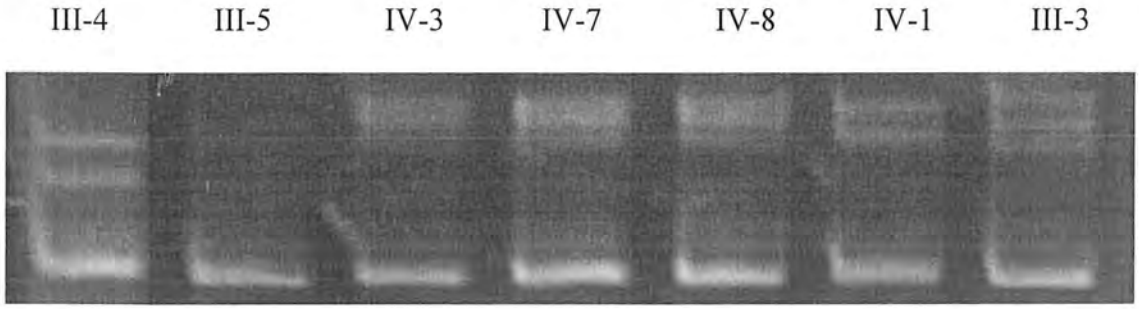


Figure 3.38: Electropherogram of 8% polyacrylamide gel of marker D17S678 at 18.96 cM.

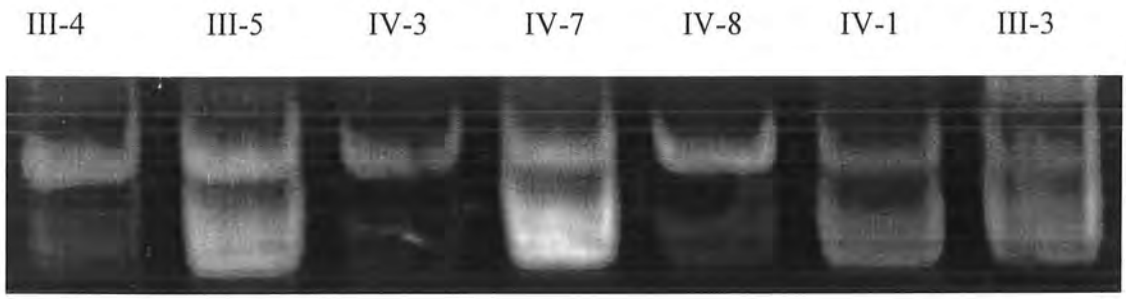


Figure 3.39: Electropherogram of 8% polyacrylamide gel of marker D17S938 at 19.66 cM.

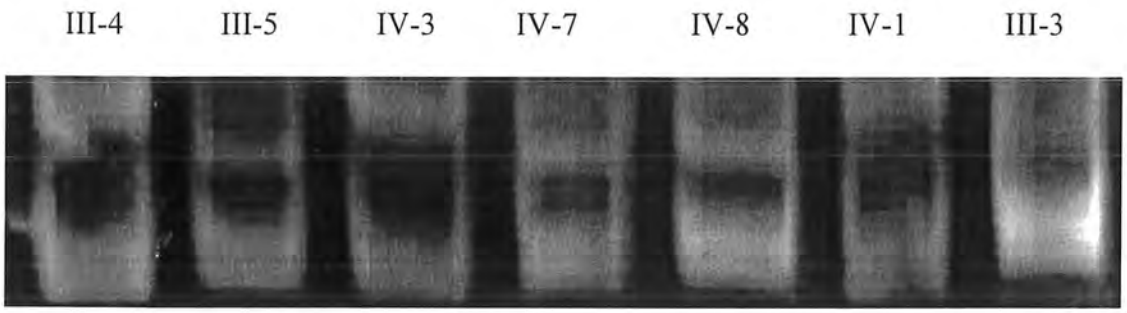


Figure 3.40: Electropherogram of 8% polyacrylamide gel of marker D17S1879 at 32.73 cM.

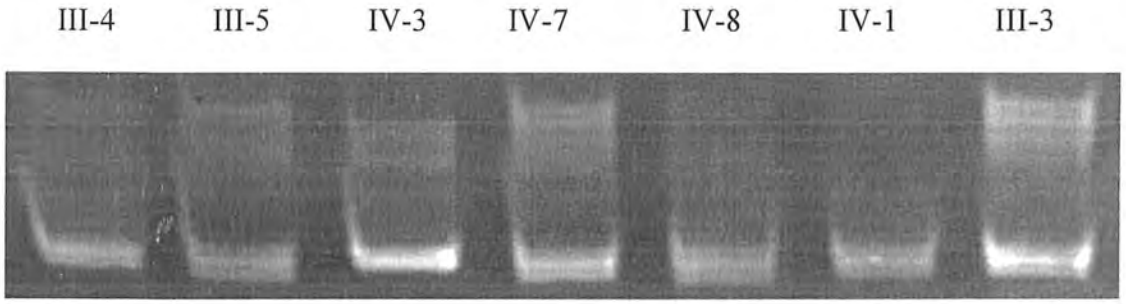


Figure 3.41: Electropherogram of 8% polyacrylamide gel of marker D1S1597 at 28.78 cM.

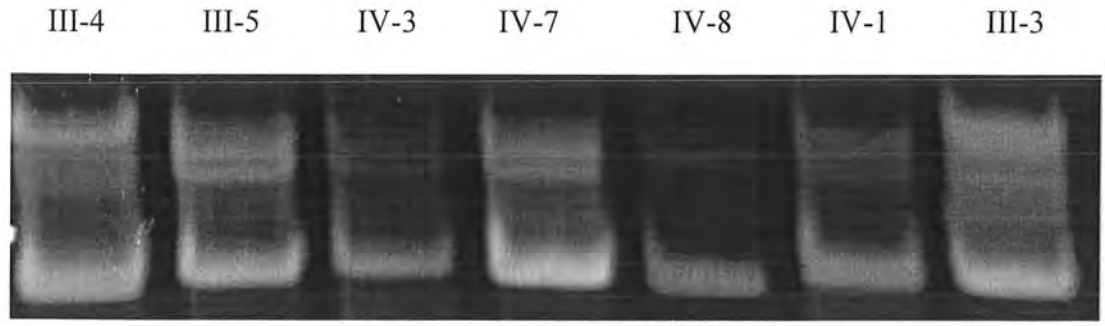


Figure 3.42: Electropherogram of 8% polyacrylamide gel of marker D6S1051 at 58.46 cM.

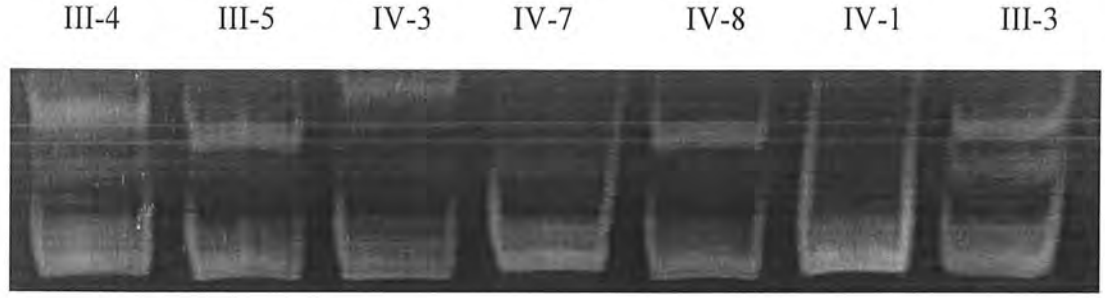


Figure 3.43: Electropherogram of 8% polyacrylamide of marker D14S1038 at 57.19 cM.

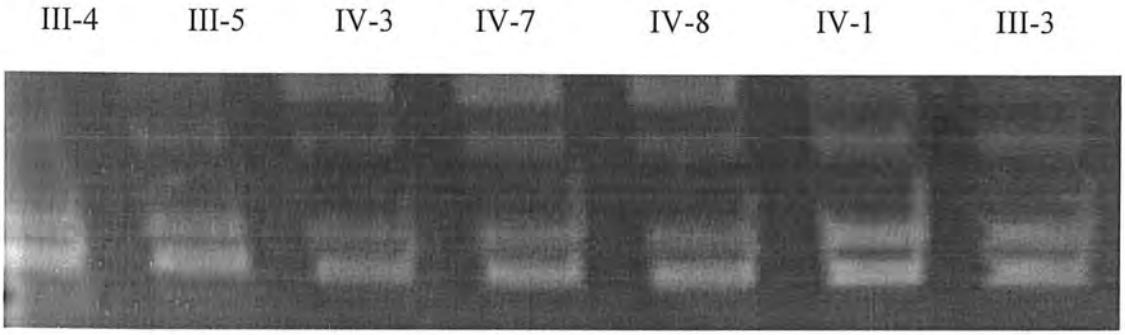


Figure 3.44: Electropherogram of 8% polyacrylamide gel of marker D19S902 at 75.28 cM.

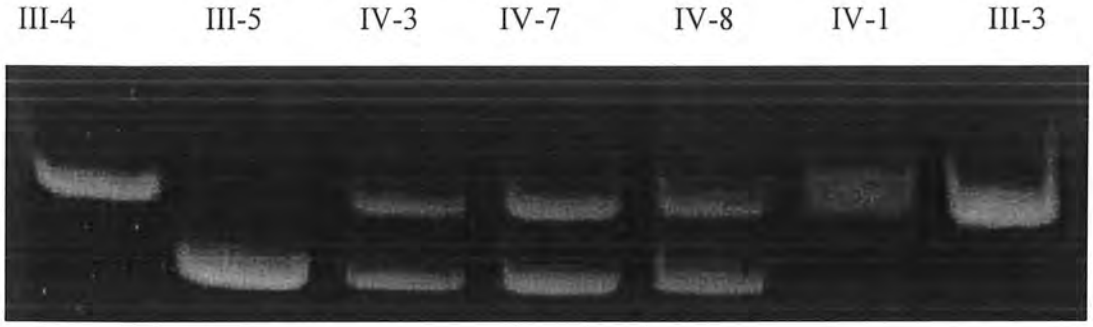


Figure 3.45: Electropherogram of 8% polyacrylamide gel of marker D19S246 at 82.48 cM.

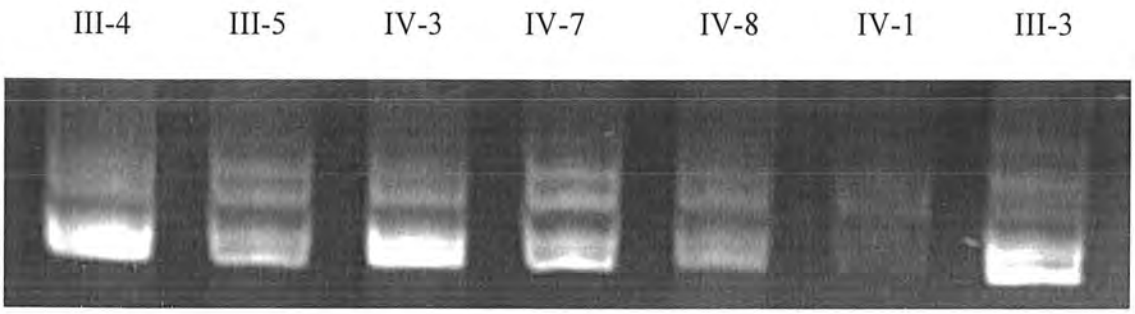


Figure 3.46: Electropherogram of 8% polyacrylamide of marker D14S1066 at 87.22 cM.

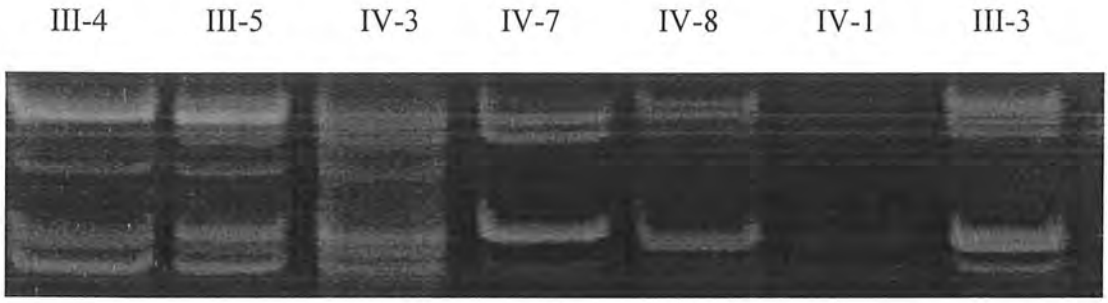


Figure 3.47: Electropherogram of 8% polyacrylamide gel of marker D6S1282 at 86.81 cM.

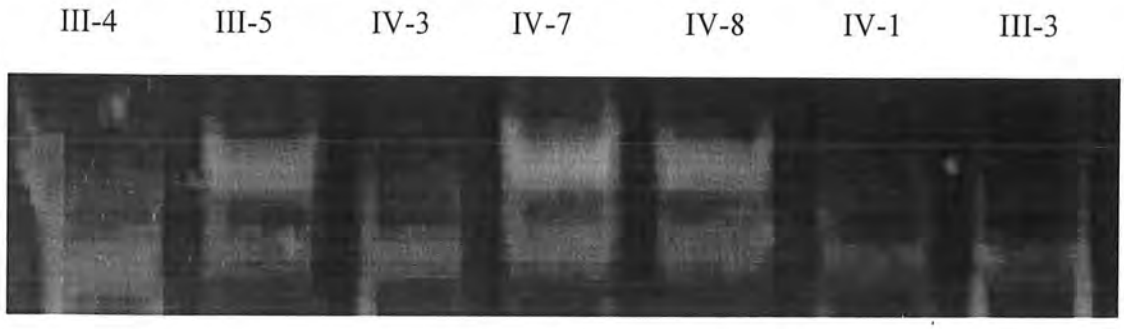


Figure 3.48: Electropherogram of 8% polyacrylamide gel of marker D12S1678 at 102.18 cM.

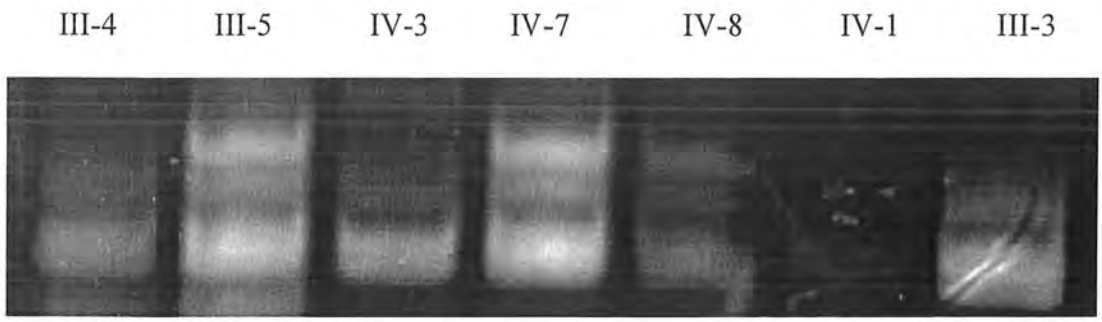


Figure 3.49: Electropherogram of 8% polyacrylamide gel of marker D12S1678 at 102.18 cM.

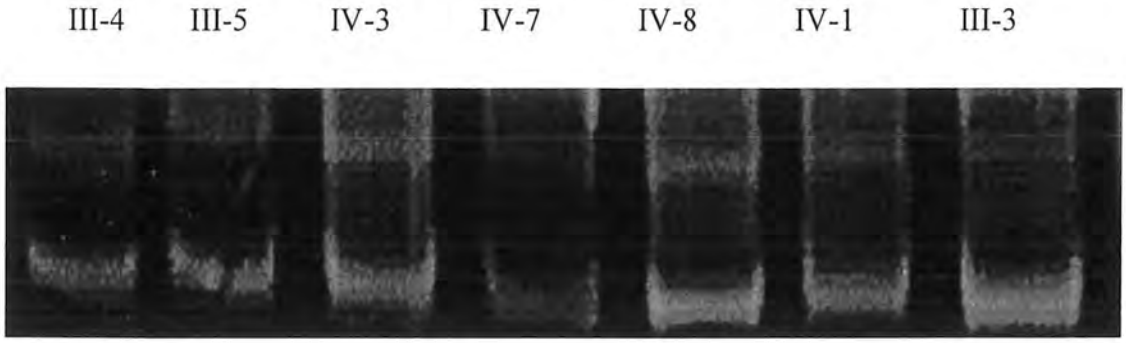


Figure 3.50: Electropherogram of 8% polyacrylamide gel of marker D1S3467 at 96.03 cM.

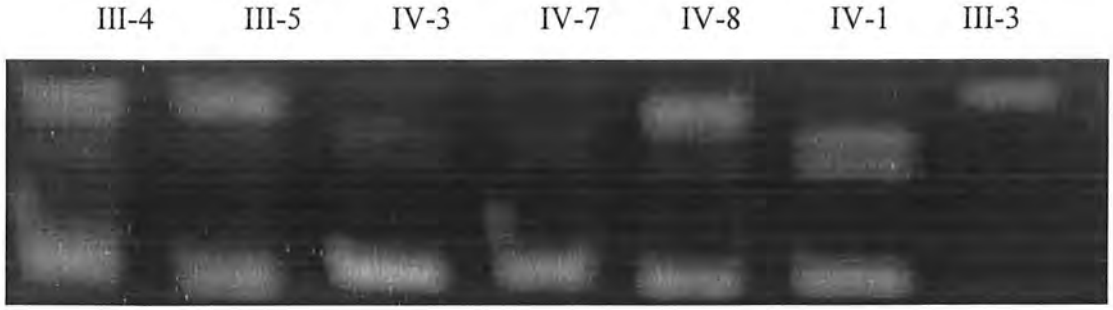


Figure 3.51: Electropherogram of 8% polyacrylamide gel of marker D1S1162 at 102.18 cM.

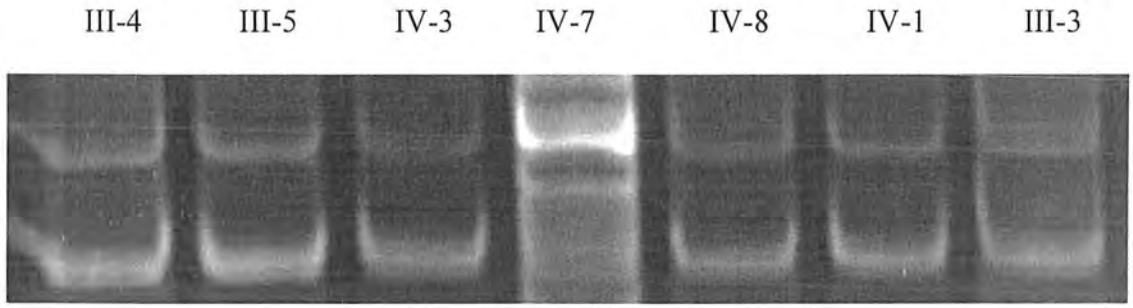


Figure 3.52: Electropherogram of 8% polyacrylamide gel of marker D2S1892 at 123.19 cM.

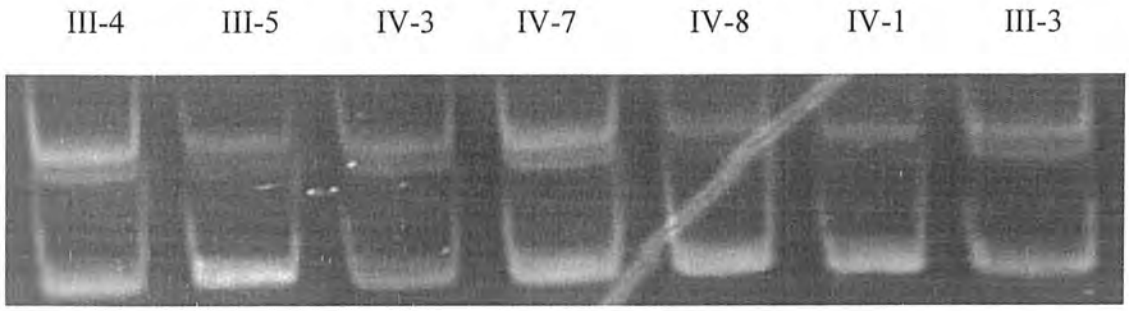


Figure 3.53: Electropherogram of 8% polyacrylamide gel of marker D2S2954 at 121.24 cM.

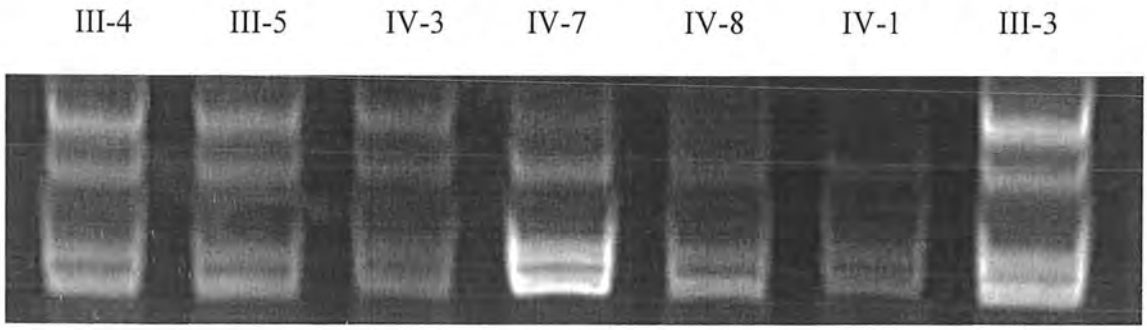


Figure 3.54: Electropherogram of 8% polyacrylamide gel of marker D7S2543 at 124.42 cM.

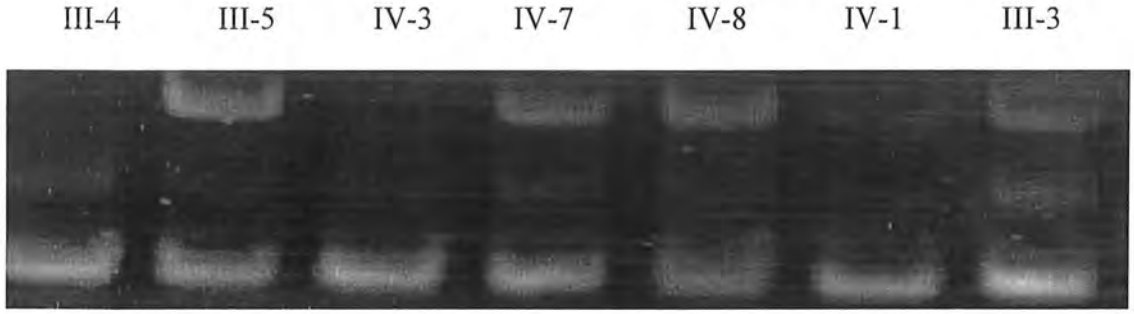


Figure 3.55: Electropherogram of 8% polyacrylamide gel of marker D7S1809 at 128.42 cM.

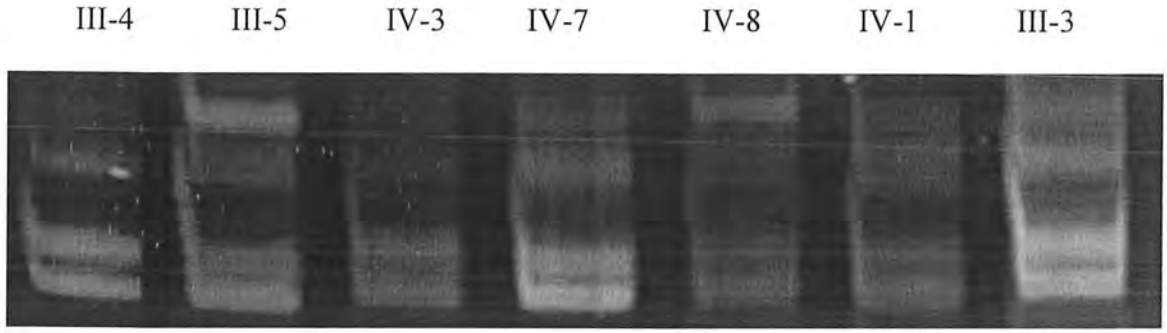


Figure 3.56: Electropherogram of 8% polyacrylamide gel of marker D4S3016 at 158.47 cM.

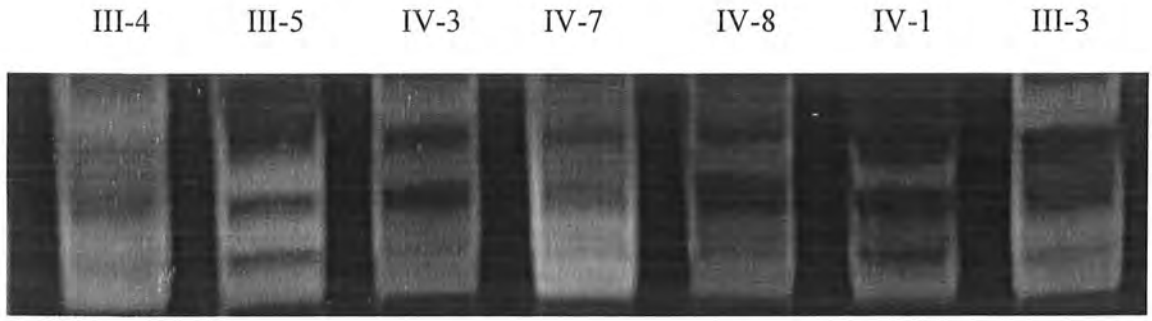


Figure 3.57: Electropherogram of 8% polyacrylamide of marker D4S3021 at 157.11 cM.

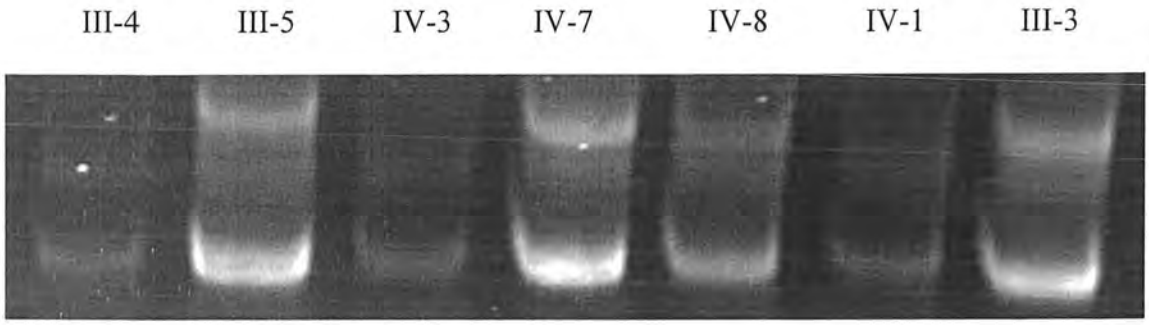


Figure 3.58: Electropherogram of 8% polyacrylamide gel of marker D1S1660 at 202.08 cM.

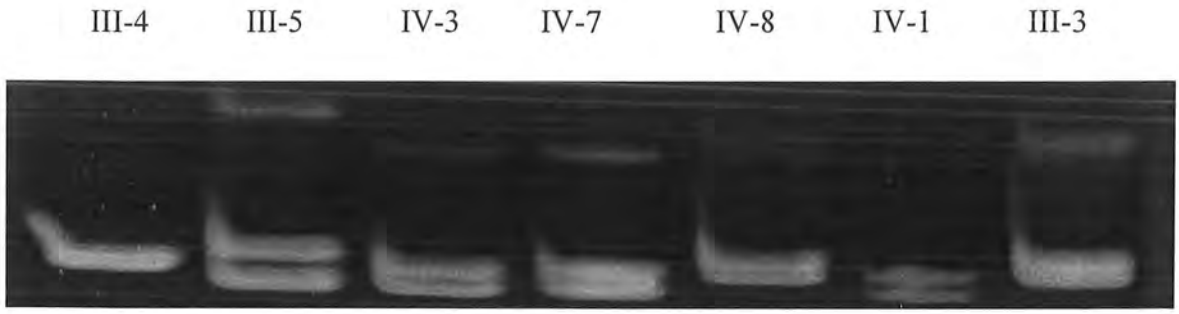


Figure 3.59: Electropherogram of 8% polyacrylamide gel of marker DIS1667 at 221.7 cM.

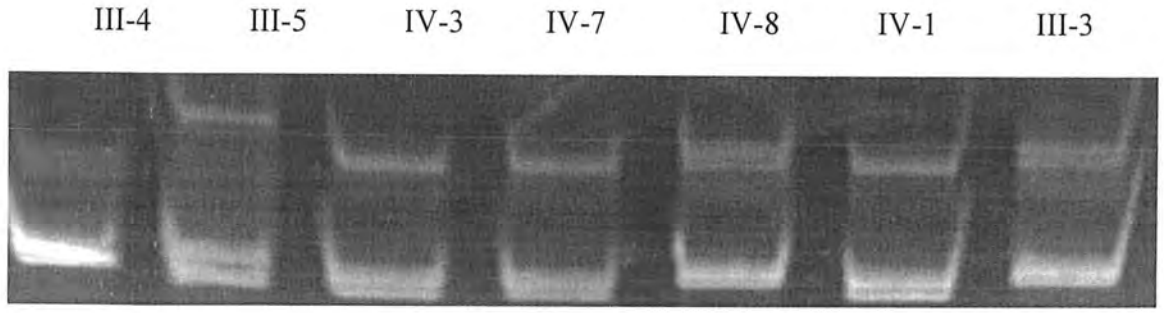


Figure 3.60: Electropherogram of 8% polyacrylamide gel of marker D1S1667 at 221.7 cM.

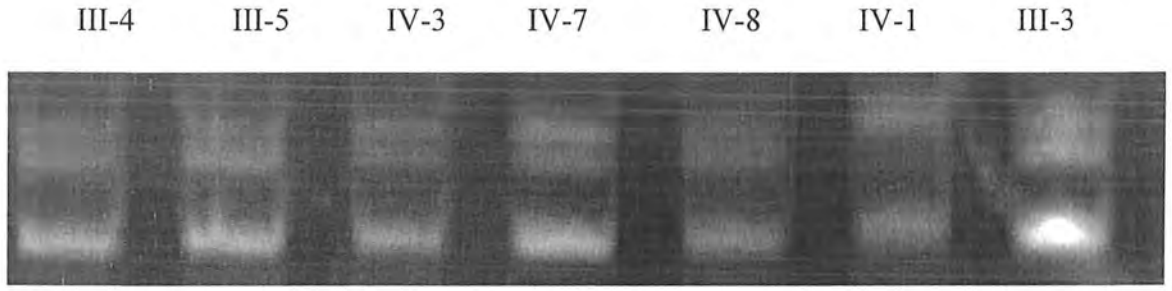


Figure 3.61: Electropherogram of 8% polyacrylamide of marker D2S2344 at 242.78 cM.

4. DISCUSSION

LCA is one of the most sever retinal dystrophy (denHollander *et al.*, 2008). It is diagnosed in earlier stages of childhood by symptoms like nystagmus eye movement, sluggish photomotor reflex, oculo-digital signs of Franceschetti, inability to follow light or objects and normal fundus. Electrophysiological examination confirms the disease by showing no retinal response.

Non syndromic form of LCA is isolated but the syndromic form can be associated with diseases like cardiomyopathy (Cohen syndrome, COH; MIM# 216550), nephronophtisis (Senior-Loken syndrome, SLSN; MIM# 266900), cone-shaped epiphyses of the hand and cerebellar ataxia (Saldino-Mainzer syndrome; MIM# 266920), deafness, obesity and diabetes mellitus (Alström syndrome, ALMS; MIM# 203800) or vermis hypoplasia, oculomotor anomalies and respiratory problems in the neonatal period (Joubert syndrome, JBTS; MIM# 610188) (Perrault *et al.*, 2007). It usually exhibits autosomal recessive hereditary pattern but rarely may also show autosomal dominant pattern of inheritance. Retinal diseases have enormous heterogeneity which is contributed by complex genetic inheritance and differential severity of mutant alleles at single locus. (Wang *et al.*, 2009)

To date, 19 genes have been identified for LCA (Perrault I *et al.*, 2013). Mutation frequency varies for specific genes (Shukla *et al.*, 2012) as shown in Table 1.1. Mutations in these genes are responsible for almost 70 % of LCA cases. It shows that some genes are still unidentified. High-throughput screening methods like mutation chips and exome sequencing are now being used, to locate the cause of disease in patients. They have increased the pace of genetic research (Perrault *et al.*, 2013).

The undergoing developments in treatment side hold promise for LCA patients. Gene therapy, photoreceptor replacement and development of electronic prosthesis are being used to cure LCA (Shukla *et al.*, 2012).

The families were collected from different areas of Pakistan. Their phenotypes and family history showed similarities to LCA. The linkage analysis of Family B did not give any region of homozygosity for the affected individuals. All candidate genes were analyzed. Thus,

Family B was excluded. So, advanced research methodologies like SNP microarray, axome sequencing have to be carried out to know the cause of disease.

Molecular screening was done for Family A and data showed linkage in a region. When observed, *SPATA7*, which is a candidate gene for LCA is located in this region of homozygosity. Homozygous pattern was shown for six markers (D14S1063, D14S1044, D14S67, D14S256, D14S1066 and D14S617). The homozygosity continued for 3.86 cM. The markers D14S61 and D6S1031 at 72.82 cM and 93.14 cM showed flanking region of this linkage.

The LCA3 locus was identified in 1998by linkage analysis in a large consanguineous family from Saudi Arabia (Perrault *et al.*, 2013). *SPATA7* gene consists of 12 exons that expand approximately 52.8 kb region. Relatively late onset of *SPATA7* expression has been speculated in retina (Wang *et al.*, 2009). Two isoforms of *SPATA7* are known to be expressed. Isoform 1 was highly expressed in neuronal tissues and isoform 2 is highly expressed in testis. Its structure shows one transmembrane domain but no known functional domains (Perrault *et al.*, 2013). Two overlapping but distinct retinal diseases, i.e. RP and LCA are linked to *SPATA7* mutations.

Studies show that *SPATA7* may be involved in some novel pathway and additional studies are to be carried out to reveal its function. The function of *SPATA7* is unknown but it has been suggested that it may have a role in vesicular transport (Wang *et al.*, 2009). It may also have role in visual perception and response to stimuli (Perrault *et al.*, 2010).

Wang *et al.* (2009) studied LCA within Saudi Arabian origin. These patients exhibit symptoms like poor visual fixation and function from birth, nystagmus, astigmatism and undetectable electroretinogram. These mutations may result in early protein truncations. This non sense mediated decay may lead to severe loss of function decay and destruction of message (Wang *et al.*, 2009).

LCA was also studied by Perrault *et al.*, (2010). These patients experienced nystagmus movement of retina since childhood. Fundus was normal in beginning but with time it showed salt and pepper appearance (Perrault *et al.*, 2010).

In 2011, Mackay *et al.,* work was related to LCA and severe RP. Different tests were performed to confirm the disease like, monocular best corrected visualacuity (BCVA), perimetry, slit lamp biomicroscopy, retinal autofluorescence (AF) imaging and electroretinography. The fundi of all patients showed severe RPE atrophy and minimal interretinal pigmentation (Wang *et al.,* 2009).

In Chinese population SPATA7 was found to be the cause of 4.6% of LCA cases in the study by Li *et al.*, (2011). In their study they found 3 variants in SPATA7. Out of these mutations 2 were pathogenic but 1 was neutral. To confirm these mutations as causative agents of LCA needs additional information. (Lin *et al.,* 2011). List of found mutations in SPATA7 till date are given in Table 4.1. According to the table mutations is SPATA7 have been extensively identified in exon 5, exon 6, exon 8, exon 11 and exon 12 till now in different studies. So, sequencing was first targeted to these exons.

Table 4.1: List of known mutations of *SPATA7* in literature.

Type of Mutation	Exon/intron	Family Origin	Reference
nonsense mutation (c.322C/T)	5	Saudi Arabian	Wang *et al.*, (2009)
frameshift mutation (c.961dupA)	8	Middle Eastern	
nonsense mutation (c.1183C→T)	11	Portuguese	
frameshift mutation (c.1546delA)	12	French Canadian	
frameshift mutation (253C→T)	5	Pakistani	Mackay *et al.*, (2011)
frameshift mutation (253C→T)	5	Pakistani	
frameshift mutation (253C→T)	8	Bangladeshi	
Duplication of A in both strands (c.961dupA)	8	Pakistani	
• 3 bases deletion frame shift (p.L89KfsX3). • 4 bases deletion (c1227_1229delCAC)	• 5 • 12	British Caucasian	
• Non sense mutation (c.763c>t) • (c.1161-1g>c)	• 6 • Intron 11	French	Perrault *et al.*, (2010)
Homozygous mutation (c.845+1g>a)	Intron 6	Greek	
• nonsense mutations (c.322c>t) • nonsense mutation (c.1983c>t)	• 5 • 11	Unrelated parents	
c.3G>T		French	

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