Application of Genomic Techniques in Identifying Genes in Families Segregating Hereditary Retinal Dystrophies

By

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Application of Genomic Techniques in Identifying Genes in Families Segregating Hereditary Retinal Dystrophies

A thesis

Submitted in partial fulfilment of the requirements for the degree of

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BIOCHEMISTRY/MOLECULAR BIOLOGY

By

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2024

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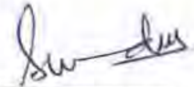
Student/Author Signature: 

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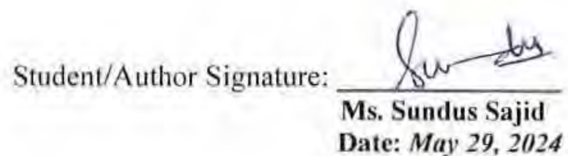

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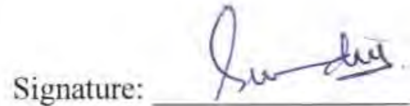
Ms. Sundus Sajid Signature: 

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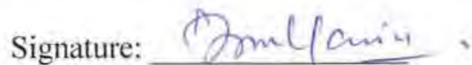
2. **External Examiner:** Signature: 
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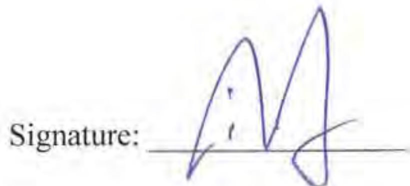

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Dedicated to my parents for their love, support and encouragement; to my husband for being a constant source of strength and to my children Ali, Ahad and Abu Bakar

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List of Abbreviations

µL	Microlitre
BBS	Bardet-Biedl syndrome
BEC	Bio-Ethics Committee
CD	Cone dystrophy
CME	Cystoid macular edema
CRD	Cone-rod dystrophy
CSNB	Congenital stationary night blindness
EOSRD	Early onset severe retinal dystrophy
ERG	Electroretinogram
GAT	Genome Analysis Toolkit
GCL	Ganglion cell layer
HM	Homozygosity Mapper
INL	Inner nuclear layer
IPL	Inner plexiform layer
JS	Joubert syndrome
LCA	Leber congenital amaurosis
MAF	Minor allele frequency
NGS	Next-generation sequencing
OCT	Optical coherence tomography
ONL	Outer nuclear layer
OS	Outer segment
PBD	Peroxisome biogenesis disorders
PCR	Polymerase chain reaction
RD	Retinal dystrophy
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
SNP	Single nucleotide polymorphism
SRD	Syndromic Retinal dystrophy
USH	Usher syndrome
VCF	Variant Call Format
WES	Whole exome sequencing

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ABSTRACT

Vision is an important sensory process that enables us to see things. This process is based on the price structure and function of retinal tissues and mutations in any gene playing role in these tissues can cause retinal dystrophies (RDs). RDs are further divided into different types based on the clinical presentation of the patients but major types include Leber Congenital Amaurosis (LCA), Retinitis Pigmentosa (RP), Cone-Rod Dystrophy (CRD and Macular Degeneration (MD). The onset, progression and severity of every RD phenotype depends on the nature of gene involved and type of mutation. Despite the great progress on the identification of RD genes, the underlying genetic defect are still unknown in large number of cases/families which require additional genetic studies. This study was initiated to recruit fifteen families with different types of RD and perform genetic analysis to identify the underlying mutations.

Analysis of the clinical information of fifteen families, recruited in this study, showed distribution in three groups. Majority of the families (8 families) belonged to LCA, whereas four families were placed in RP group while the remaining three families were placed in other RD group. Genetic analysis of these families with genome wide genotyping, homozygosity mapping, exome sequencing and Sanger sequencing resulted in the identification of underlying genetic defects in 13 out of 15 families, indicating a diagnostic yield of 86.6%. Novel pathogenic variants were identified in four families including a large deletion present on chromosome 14. The remaining nine mutations were either reported in literature or public databases. Novel mutations were identified in families A, B,C and M and in genes *SPATA7, CRB1, LCA5* and *ABCA4,* respectively.

In family A, an 81.85Kb deletion was detected on chromosome 14 (hg38; chr14:88470782-88388933) which spans all the coding exons of *SPATA7* gene. The deletion breakpoints were identified by primer walking based on the information from genotype data and BAM file of individuals used for exome sequencing. The loss of *SPATA7* gene was confirmed through the amplification of exon 5 of from cDNA samples of two affected individuals. In family B a mutation (c.2424T>A; p.Tyr808Ter) was identified in *CRB1* gene, that could possibly lead to loss of function due to nonsense-mediated decay. Third novel homozygous mutation (c.1550_1551delGA) was found in the family C in *LCA5* gene. The dinucleotide deletion results in a frameshift and therefore causes an early termination of the specific protein

(p.Arg517Ile_fs*3_). Fourth novel mutation was identified in family M which was clinically diagnosed as Stargardt disease. In this case, a novel splice-site variant (c.3328+1G>C) was identified in *ABCA4* gene through whole exome sequencing. In silico data analysis showed that variant to be likely pathogenic and may result in intron retention. In the remaining nine families known mutations were identified in *AIPL1* (Family D & E), *NMNAT1* (Family F), *CRB1* (Family G), *CCDC66* (Family I), *TTC8* (Family J), *TENM1* (Family K), *ABCA4* (Family N) and *BEST1* (Family O). But in one LCA family (Family H) and one RP family (Family L) pathogenic mutations could not be identified and may require further studies.

This study provides insight to the genetic diversity of inherited retinal disorders in the Pakistani population and reports the identification of four novel mutations in families segregating heterogeneous RDs. Genetic screening of such families that belong to remote areas with less resources and health facilities will help in accurate diagnosis and family counselling for further disease management.

The work presented in this study has been partly published in the following publications;

1. Ravesh Z, El Asrag ME, Weisschuh N, McKibbin M, Reuter P, Watson CM, Baumann B, Poulter JA, **Sajid S**, Panagiotou ES, O'Sullivan J, Abdelhamed Z, Bonin M, Soltanifar M, Black GC, Amin-ud Din M, Toomes C, Ansar M, Inglehearn CF, Wissinger B, Ali M. Novel C8orf37 mutations cause retinitis pigmentosa in consanguineous families of Pakistani origin. **Mol Vis. 2015 Mar 7;21:236-43.**
2. Saqib MA, Nikopoulos K, Ullah E, Sher Khan F, Iqbal J, Bibi R, Jarral A, **Sajid S**, Nishiguchi KM, Venturini G, Ansar M, Rivolta C. Homozygosity mapping reveals novel and known mutations in Pakistani families with inherited retinal dystrophies. **Sci Rep. 2015 May 6;5:9965**.
3. Ullah E, Nadeem Saqib MA, **Sajid S**, Shah N, Zubair M, Khan MA, Ahmed I, Ali G, Dutta AK, Danda S, Lao R, Ling-Fung Tang P, Kwok PY, Ansar M, Slavotinek A. Genetic analysis of consanguineous families presenting with congenital ocular defects. **Exp Eye Res. 2016 May;146:163-171**.
4. **Sajid S**, Rabia Basharat, Ehsan Ullah, Muhammad Arif Nadeem Saqib, Memoona Rasheed, Muhammad Ansar. Identification of Disease-Causing Mutations Using Homozygosity Mapping and Whole Exome Sequencing of

Index Cases of Inherited Retinal Dystrophy Families. Molecular Vision **(Manuscript under revision)**

INTRODUCTION

1.0 Retinal dystrophies (RDs)

RDs are a group of heterogeneous inherited disorders which exhibit clinical and genetic diversity. Currently available literature has catalogued 25 different types of visual disorders under RDs. Generally, RDs are characterized by the degeneration of the retinal photoreceptor cells and the retinal pigment epithelium (RPE) (Nentwich and Rudolph, 2013, Sahel et al., 2014) and therefore clinical presentation of the patients vary with the nature and type of cell loss. Additionally RDs also exhibit variation in terms of age of onset, rate of progression and disease severity (Zheng et al., 2015). Common clinical features observed in patients with RDs include, nyctalopia, peripheral vision loss, central vision loss, deformation of RPE and abnormal or no electroretinogram (ERG) responses (Heckenlively, 1988). But these clinical features overlap between different types of RDs and in majority of the cases ophthalmologist face difficulties in reaching at the final diagnosis. For example, in cone dystrophy (CD), only the central vision is affected while in cone-rod dystrophy (CRD) central vision impairment is followed by peripheral vision loss (Hamel, 2006). Retinitis pigmentosa (RP), another type of RD, causes severe loss of peripheral retinal layer that is accompanied by central vision loss. Congenital stationary night blindness (CSNB), mainly involves rod photoreceptors but Leber congenital amaurosis (LCA) features complete loss of vision since early childhood (Rivolta et al., 2002).

To date 281 gene are reported to cause inherited RDs worldwide. Some inherited RDs are observed in patients as a sole clinical feature, which primarily affect vision, and are categorised as nonsyndromic RDs. Alternatively, patients may present additional systemic manifestations at the time of RD diagnosis by ophthalmologist, which are categorised as syndromic RDs (SRDs). SRDs are also heterogeneous and are divided into different groups depending on the type and nature of additional clinical features. SRDs approximately represent 20-30% of inherited retinal dystrophies but major types include Joubert syndrome (JS), Usher syndrome (USH) and Bardet-Biedl syndrome (BBS) (Werdich et al., 2014, Ran et al., 2014). Some SRDs are rare and are only reported in a limited number of patients including a Golgi apparatus-related disorders (Cohen syndrome), endoplasmic reticulum-associated disorders (Wolfram syndrome), lysosomal storage disorders (Platt et al., 2018) and peroxisome biogenesis disorders (PBD) (Argyriou et al. 2016; (Perea-Romero et al., 2021). Currently 98 genes have been identified for SRDs, but underlying genes are yet unknown for 8 loci mapped on

different human chromosomes (The Retinal Information Network, RetNet: https://sph.uth.edu/retnet/; last accessed January 2023).

1.1 Retina: Major tissue

Retina is a specialised tissue which is composed of different retinal layers. Six types of neurons are organized in three layers; outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). The ONL and INL layers are separated by outer plexiform layer (OPL), whereas inner plexiform layer (IPL) separates INL and GCL layers (Fig. 1.1). Human eye has a narrow field of view but can achieve higher spatial resolution and acuity due to the presence of millions of sensory (Rod and cone) photoreceptors within the retinal pigment epithelium (RPE). Rods are specialized for low light vision whereas cone photoreceptors mediate day light and colour vision. Human retina consists of ~105 million and 6 million rod and cone photoreceptors, respectively. Both photoreceptors have four distinct regions: the outer segment (OS), the inner segment (IS), the cell body containing nucleus and the synaptic region (Lamb, 2013).

The OS of both photoreceptors is a sensory cilium containing hundreds of stacked membranous discs which are filled with the opsin pigments. Opsins are classified into three types based on their spectral sensitivity. Three types of cone photoreceptors i.e. L (564nm), M (533nm) and S (437nm) are present in the human retina to allow response to variable wavelength. Whereas, vertebrate retina only has rod photoreceptors containing rhodopsin (Zelinger and Swaroop, 2018).

The integrity of photoreceptors and their functioning (Phototransduction pathway) is critical for normal vision and any mutation in the retina specific genes that can alter their structure and function, may result in the dysfunctional phototransduction cascade causing partial or complete loss of vision (Wright et al., 2010).

1.2 Phototransduction cascade

The process of phototransduction takes place in the OS, where the visual pigment captures photons of light and convert them into electrical signals. OS has all the components and proteins which are required to convert light photons into electrical responses. Both rod and cone photoreceptors have OS (though each have unique morphology) but their response to photons vary due to differences in the photoreceptor

pigments and the nature and expression levels of molecular machinery. However, a generalized phototransduction pathway is briefly summarized below;

As the light falls on the back of the retina, the photoisomerization of 11-*cis* retinal into all-*trans* retinal produces active rhodopsin, which is capable of activating Transducin, a photoreceptor-specific G protein. The activated transducin then stimulates the activity of phosphodiesterase (PDE) that specifically hydrolyzes cGMP (Fig. 1.2). The cGMP levels are increased in the darkness and this cGMP maintains the transport of certain ions through specific channels on the plasma membrane. In the presence of light, cGMP levels undergo graded decrease and this ultimately closes the cGMP-gated channels ensuing hyperpolarization of the cell (Arshavsky et al., 2002).

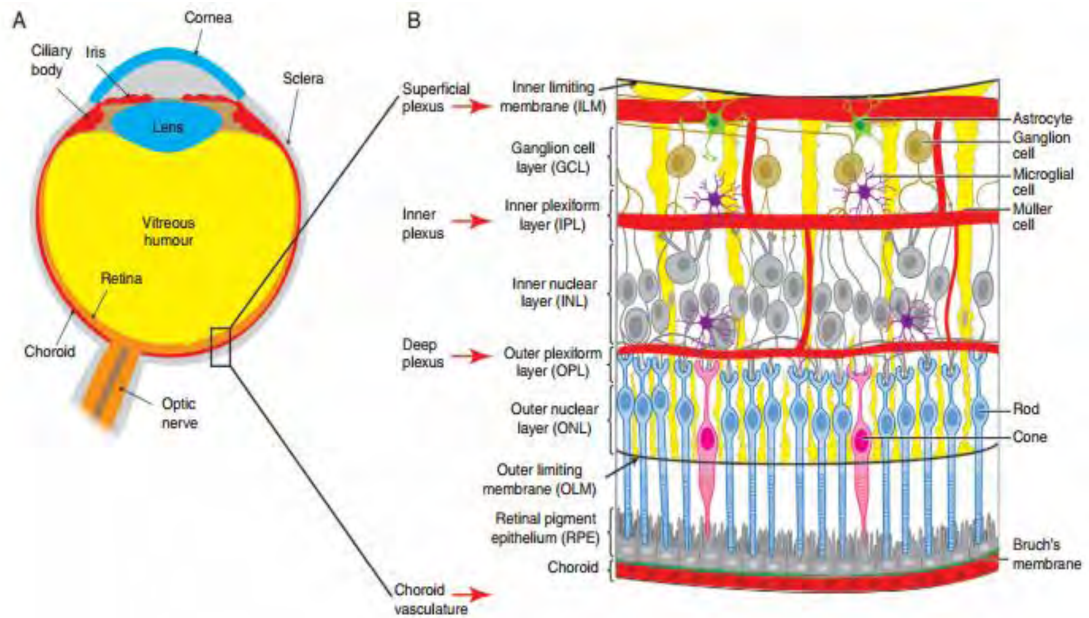


Figure 1.1: (A) Schematic representation of Human eye. (B) Organization of retinal neurons, blood vessels and glia. Adapted from (Coorey et al., 2012).

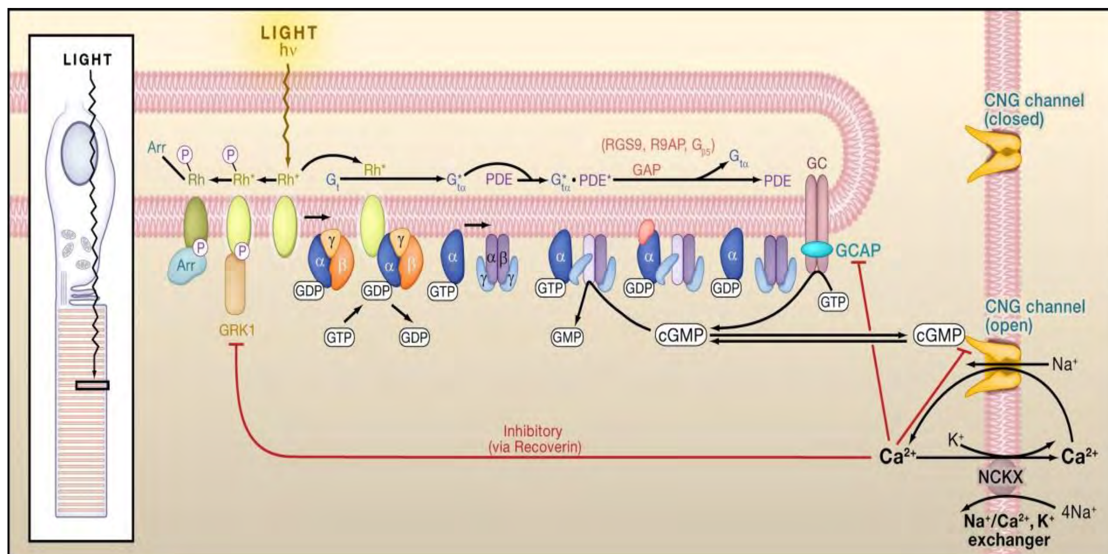


Figure 1.2: Phototransduction cascade in vertebrate rod cells. Adapted from (Yau and Hardie, 2009).

1.3 Types of RDs

Nonsyndromic RDs are divided into seven major groups including RP, LCA, congenital stationary night blindness (CSNB) and cone-rod dystrophy (CRD). These nonsyndromic RDs are reported in all possible modes of inheritances. The syndromic RDs are also divided into groups but major types include USH and BBS.

1.3.1 Retinitis pigmentosa (RP)

RP is the most frequently found nonsyndromic RD and it exhibits extensive genetic and clinical heterogeneity (Inglehearn, 1998). The prevalence varies among different parts of the world and it could be 1 in 1000 – 1 in 4000 (Dandona et al., 2001, Kannabiran et al., 2012). There are three modes of inheritance of RP; autosomal dominant (ad), autosomal recessive (ar) and X-linked (xl). Digenic factors as well as the mitochondrial genome involvement is possible but is rarely reported. The xlRP, adRP and arRP makes up 10-15%, 15-20% and 20-25% of all reported RP cases, respectively, but remaining 40-55% cases are usually sporadic (Bundey and Crews, 1984, Maubaret and Hamel, 2005, Li et al., 2010).

RP is characterized by initial degeneration of rod photoreceptors, which can be extended to cone degeneration at later stages of life. The early loss of rod cells causes nyctalopia or night vision loss in the patients (Tsujikawa et al., 2008). Another characteristic of RP is the progressive loss of peripheral vision resulting in "tunnel vision". In some of the RP cases, central vision gets affected by the age of 60 but mostly patients are legally blind by the age of 40 years (Hartong et al., 2006).

Syndromic features are present in 20-30% of RP patients. One of the most frequent syndromic form of RP is Usher syndrome (USH) which has an associated hearing impairment and is present in approximately 18% of RP cases (Boughman et al., 1983). USH is divided into three forms depending upon the (i) onset of the visual and hearing impairment, (ii) the complexity of disease and (iii) the occurrence of vestibular areflexia. In addition to three established USH subtypes, patients from many families have been reported which show symptoms different from currently known subtypes and are therefore, categorized as, atypical USH (Liu et al., 1998, Bashir et al., 2010, Khateb et al., 2014). To date, 18 loci have been mapped for USH, but underlying genes have been identified at 15 loci (RetNet).

Another common syndromic form of RP is Bardet-Biedl syndrome (BBS). The other characteristics of BBS include; polydactyly, type-II diabetes, obesity, cognitive dysfunction, hypogonadism and renal abnormalities are also present in some cases (Forsythe and Beales, 2013). It accounts for 5-6% cases of RP (Haim, 2002). According to RetNet, 18 genes have been identified so far.

Joubert syndrome (JS) is also one of the syndromic forms of RP and it has features like; hypotonia, developmental delay, molar tooth sign, breathing problems and defects in renal system. JS is associated with different types of RDs (Romani et al., 2013). The number of genes that have been known for JS are more than 40 (Vilboux et al., 2017), but causative genes are still unknown in approximately 6% cases (Gana et al., 2022)

1.3.2 Leber Congenital Amaurosis (LCA)

Theodore Leber initially described LCA as a group of severely impaired, autosomal recessive RDs (Perrault et al., 1999). LCA is characterized by severe vision loss from birth or the first few months of life, nystagmus, poor pupillary light responses, oculodigital sign, and undetectable or severely abnormal full field electroretinogram (ERG). Prevalence of LCA varies in different countries of the world and ranges from 1 in 33,000 to 1 in 80,000. The common mode of inheritance of LCA is autosomal recessive but there is a single gene known for autosomal dominant LCA (Koenekoop, 2004, Stone, 2007). Currently single 13 genes are known for autosomal recessive LCA.

LCA exhibit close similarity with another RD i.e. early onset severe retinal dystrophy (EOSRD). Among the currently identified genes mutations in few can cause both types of disorders. For example, mutations in *GUCY2D*, *NMNAT1*, *CEP290* and *AIPL1* genes cause LCA, whereas mutations in *RPE65*, *LRAT* and *RDH12* are common in EOSRD phenotype. Similarly, LCA must be separated from some syndromic forms like; Zellweger syndrome, JS, Senior-Loken syndrome (SLS) especially during early infancy where ocular phenotypes are indistinguishable (Lambert et al., 1989, Casteels et al., 1996).

1.3.3 Cone/Cone-rod dystrophy

Cone dystrophy (CD)/cone-rod dystrophy (CRD) both come under the heterogenous group of RDs, presenting pre-dominantly cone dysfunction (Roosing et al., 2014). The disease appears usually in first or second decade of life (Michaelides et al., 2004). Prevalence of both CD/CRD is approximately 1 in 40,000 individuals worldwide.

CD/CRD both diseases follow all the three patterns of inheritance with autosomal recessive as the most common. Currently 23 genes are known to cause CD and CRDs.

1.3.4 Congenital Stationary Night Blindness

Congenital stationary night blindness (CSNB), is a large group of RDs which are non-progressive (Zeitz, 2007). There are four types of CSNB: Riggs, Schubert–Bornschein, fundus albipunctatus, and Oguchi disease (Tsang and Sharma, 2018). Patients with CSNB experience night blindness, myopia, strabismus, and/or nystagmus. The most common mode of inheritance is X-linked. But CSNB also follows autosomal recessive and autosomal dominant patterns (Miyake et al., 1986). The frequency of X-linked CSNB cases is around 57%, autosomal recessive cases are 40% which also include sporadic and those which have fundus abnormalities while autosomal dominant account for only 2.1% of CSNB cases (Zeitz et al., 2015).

1.3.5 Stargardt Disease

Stargardt disease (STGD1) is a common cause of macular dystrophy and has onset during second decade of life. STGD1 patients initially present central vision loss, yellow flecks around the macula and retinal mid-periphery, and progressive atrophy of RPE (Del Pozo-Valero et al., 2020). STGD1 is inherited in autosomal recessive manner and is mainly caused by mutations in ABCA4 gene, which is also known to cause other types of RDs like RP and CRD (Riveiro-Alvarez et al., 2013).

1.4 RD Diagnosis

Clinical characteristics of RDs can be revealed by different clinical tests including; psychophysical tests, fundus examination, slit-lamp examination, optical coherence tomography (OCT) and electroretinogram (ERG) (Hartong et al., 2006).

In case of RP, fundus examination at early stage can appear normal, but initial signs are observed during the disease progression like the formation of granules on RPE. The changes in retinal pigment are early signs of the disease and sometimes a mottled effect is observed without affecting the vasculature. As the disease progresses, loss of RPE occurs in the form of patches, narrowing of arterioles, bone spicule formation and death of the photoreceptor cells. In more than 40% of cases, another feature observed is cystoid macular edema (CME) (Adackapara et al., 2008).

LCA is characterized with severe form of visual loss that usually appears at the time of birth or just after a few months. This vision loss is followed by many phenotypic features like; nyctalopia, a continuous sideways movement of pupil, poor responses towards light, the "occulodigital" sign that is, continuous poking of eye by the individual and non-detectable electroretinogram (ERG). In case of LCA fundus may appear normal at initial stages but with the passage of time it may develop abnormal appearances. The other less common features may include, hyperopia, myopia, strabismus, cataracts, keratoconus, disc edema, macular pseudo coloboma and attenuation of retinal vasculature (Heher et al., 1992, Koenekoop, 2004).

OCT also shows unaffected outer retina in majority of cases although, abnormalities in foveal cones do appear with severity level that reaches upto loss of foveal cones (Pasadhika et al., 2010, Jacobson et al., 2013).

The early symptoms of CRD include; decreased visual acuity that is usually observed during the first decade of life, intense photophobia, dyschromatopsia that varies from individual to individual and variable degree of nystagmus as well (Michaelides et al., 2004). There is continuous progression in reduction of visual acuity. Night blindness and peripheral vision loss becomes apparent with the progression of disease that is due to the dysfunctioning of rod photoreceptor cells preceding cone photoreceptors (Roosing et al., 2014).

Patients of CSNB often suffer visual disturbances at night or in dim light. They also have a delay in dark adaptation in one of the sub-groups, photophobia has also been reported. CSNB patients also show symptoms like; nystagmus, strabismus, reduced visual acuity, myopia and fundus abnormalities (Zeitz, 2007).

1.5 Genetics of RDs

According to RetNet, more than 69 genes are associated with different modes of RP. These genes encode proteins which act as molecular components of different pathways like, photo-transduction, retinoid cycle, etc. There are some proteins which are part of interactomes, structural components of photoreceptor cells, act as transcription factors, components of splicing machinery and are involved in the intracellular transport/ trafficking (Maubaret and Hamel, 2005, Naz et al., 2010). The first RP gene was discovered in 1990, which encodes Rhodopsin (*RHO*) protein (Dryja et al., 1990).

The worldwide frequency of autosomal recessive RP is very high, and it varies according to different genes like; *USH2A* (12%), *ABCA4* (8%), *PDE6B* (7%), *CNGB1* (6%) and *PDE6A* (5%). A study conducted on 150 families of Saudi Arabia presenting RP showed majority of mutations in genes including; *TULP1, RPGRIP1, RP1* and *CRB1* (Abu-Safieh et al., 2013). A review of published data of 103 Pakistani families having retinal dystrophies were most commonly mutated for *AIPL1* and *CRB1* (Khan et al., 2014).

The underlying genetics of LCA is vastly heterogenous; currently 25 genes are known to be associated with LCA pathogenesis. Most of the genes implicated in LCA are solely or predominantly expressed in the retina or the underlying RPE. The proteins encoded by these genes may function in phototransduction cascade, retinoid cycle, photoreceptor ciliary transport, photoreceptor morphogenesis and integrity (den Hollander et al., 2008, Wang et al., 2015, Astuti et al., 2016). These known genes account for most (70-80%) of the LCA cases (Kumaran et al., 2017). The most common genes are found to be *CEP290, GUCY2D, CRB1, RPE65* and *RDH12* (den Hollander et al., 2008).

NMNAT1 mutations were observed in patients with coloboma-like macular atrophic lesions whereas, patients who had grossly normal presentation of the retina had mutations in *CEP290, GUCU2D* and *CRX* (Han et al., 2017). The first gene discovered for LCA was *GUCY2D* and mutations in this gene accounts for approximately 10-20% cases (Perrault et al., 2000). This gene encodes retinal guanylate cyclase-1 (RetGC1) enzyme which is present in outer segments of photoreceptor cells (Dizhoor et al., 1994, Liu et al., 1994). RetGC1 functions in the phototransduction cascade and its main role is in the recovery of photoreceptor cells (Pasadhika et al., 2010, Jacobson et al., 2013).

Thirty-five genes are known for CRD, and of these 23 genes are linked with arCRD (RetNet). Approximately 25% of disease cases are solved and rest have to be identified yet (Roosing et al., 2014, Nash et al., 2015).

ABCA4 is the gene that is mainly linked with Stargardt disease but at the same time, mutations in *ABCA4* are involved in the pathogenesis of 30-60% arCRD cases. Other genes which are also part of this pathogenesis include; *RPGRIP1, ADAM9, CDHR1* and *HRG4*. RPGR that is an interacting protein with RPGRIP1 is found to be mutated in X-linked form of CRD. Usually CRDs are non-syndromic but have been reported in

syndromic forms as well including BBS and Spinocerebellar ataxia type 7 (Michaelides et al., 2004).

Mutations in 16 different genes have been known to cause CSNB and many cases are yet to be solved (Zeitz et al., 2015). Major genes known for above mentioned common types of RDs are summarized in table 1.1 and these genes are grouped on the basis of function or the mechanism in which they are involved.

Table 1.1: Genes associated with recessive forms of common RDs

S. No	Function	Genes	Phenotype
1	Phototransduction	PDE6A (Pittler et al., 1990), PDE6B (Weber et al., 1991), PDE6G (Tuteja et al., 1990), SAG (Ngo et al., 1990), ARL2BP (Sharer and Kahn, 1999), NEUROD1 (Naya et al., 1995), ZNF513 (Li et al., 2010), CNGA1 (Kaupp et al., 1989), CNGB1 (Sugimoto et al., 1991), RHO (Nathans and Hogness, 1984)	RP
		GUCY2D (Shyjan et al., 1992), AIPL1 (Sohocki et al., 1999), RD3 (Chang et al., 1993)	LCA
		CNGA3 (Biel et al., 1994), CNGB3 (Kohl et al., 2000), PDE6C (Piriev et al., 1995), PDE6H (Shimizu-Matsumoto et al., 1996), GNAT2 (Morris and Fong, 1993)	CRD
		GNAT1 (Lerman and Minna, 2000), *SLC24A1* (Tucker et al., 1998), *GRK1* (Khani et al., 1996), *SAG* (Saga et al., 2004)	CSNB
2	Ciliogenesis/ Ciliary transport	*BBS1* (Mykytyn et al., 2002), *BBS2* (Nishimura et al., 2001), *C2orf71* (Nishimura et al., 2010), *C8orf37* (Estrada-Cuzcano et al., 2012), *FAM161A* (Langmann et al., 2010), *IFT140* (Nagase et al., 1998), *IFT172* (Hirosawa et al., 1999), *KIZ* (El Shamieh et al., 2014), *TTC8* (Ansley et al., 2003), *SPATA7* (Zhang et al., 2003)	RP
		TULP1 (North et al., 1997), *CEP290* (Nagase et al., 1998), *RPGRIP1* (Roepman et al., 2000), *LCA5* (Den Hollander et al., 2007a), *SPATA7, CLUAP1* (Takahashi et al., 2004), *IQCB1* (Otto et al., 2005), *IFT140* (Xu et al., 2015)	LCA
		C8orf37, C21orf2 (Scott et al., 1998), *IFT81* (Masuda et al., 1997), *POC1B* (Hames et al., 2008), *RPGRIP1* (Beryozkin et al., 2021)	CRD
3	Photoreceptor morphogenesis/ maintenance	*CRB1* (den Hollander et al., 1999b), *EYS* (El-Aziz et al., 2008), *IMPG2* (Kuehn and Hageman, 1999), *MERTK* (Graham et al., 1994), *NR2E3* (Kobayashi et al., 1999), *NRL* (Yang-Feng and Swaroop, 1992), *RP* (Pierce et al., 1999) , *RP1L1* (Conte et al., 2003), *TULP1, USH2A* (Eudy et al., 1998), *PROM1* (Miraglia et al., 1997), *REEP6* (Saito et al., 2004), *SAMD11* (Inoue et al., 2006)	RP
		CRX (Freund et al., 1997), *CRB1, GDF6* (Chang et al., 1994), *PRPH2* (Travis et al., 1989)	LCA

		CDHR1 (Rattner et al., 2001), *CEP78* (Andersen et al., 2003), *CNNM4* (Wang et al., 2003), *TTLL5* (He and Simons, 2007)	CRD
4	Visual Cycle	*ABCA4* (Allikmets et al., 1996), *LRAT* (Ruiz et al., 1999), *RBP3* (Liou et al., 1987), *RGR* (Jiang et al., 1993), *RLBP1* (Crabb et al., 1988), *RPE65* (Hamel et al., 1993)	RP
		LRAT, RPE65, RDH12 (Haeseleer et al., 2002)	LCA
		ABCA4, RDH5 (Simon et al., 1996)	CRD
		RDH5	CSNB
5	Metabolism	*CYP4V2* (Li et al., 2004), *DHDDS* (Endo et al., 2003), *HGSNAT* (Fan et al., 2006), *IDH3B* (Kim et al., 1999), *MVK* (Schafer et al., 1992)	RP
		NMNAT1 (Emanuelli et al., 2001)	LCA
		RAB28 (Brauers et al., 1996)	CRD
6	Protein trafficking	*ARL6* (Chiang et al., 2004), *SPATA7* (Wang et al., 2009)	RP
		SPATA7 (Wang et al., 2009); (Perrault et al., 2010)	LCA
		KCNV2 (Ottschytsch et al., 2002)	CRD
7	Miscellaneous/ Unknown	*DHX38* (Ajmal et al., 2014a), *AGBL5* (Kastner et al., 2015), *BEST1* (Petrukhin et al., 1998), *CERKL* (Tuson et al., 2004), *CLRN1* (Joensuu et al., 2001), *GPR125* (Fredriksson et al., 2003), *KIAA1549* (Nagase et al., 2000), *MAK* (Matsushime et al., 1990), *NEK2* (Nishiguchi et al., 2013), *POMGNT1* (Yoshida et al., 2001), *PRCD* (Zangerl et al., 2006), *SLC7A14* (Nagase et al., 2000), *TRNT1* (Nagaike et al., 2001), *ZNF408* (Collin et al., 2013), *AHR* (Ema et al., 1994), *ARHGEF18* (Blomquist et al., 2000), *EMC1* (Nagase et al., 1995)	RP
		CABP4 (Haeseleer et al., 2000), *CCT2* (Won et al., 1998), *DTHD1* (Abu-Safieh et al., 2013), *KCNJ13* (Krapivinsky et al., 1998)	LCA
		ADAM9 (McKie et al., 1996), *ATF6* (Kohl et al., 2015), *CACNA2D4* (Qin et al., 2002), *CERKL* (Littink et al., 2010), *RAX2* (Wang et al., 2004)	CRD
		CABP4 (Zeitz et al., 2006), *GNB3* (Levine et al., 1990), *GPR179* (Bjarnadóttir et al., 2005), *GRM6* (Hashimoto et al., 1997), *LRIT3* (Kim et al., 2012), *TRPM1* (Duncan et al., 1998)	CSNB

1.6 Role of Genomic Techniques in RD Gene identification

Analysis of the previously published data from different studies shows the identification of very few genes till 1990. However, like other Mendelian disorders RD genes were also identified at the rapid pace with the popularisation of Sanger sequencing approach. This is obvious from the data available on RetNet which shows that approximately 50 genes were known for RDs till 2000. It is important to note that there is gap between number of mapped loci and the number of identified genes for RD. This suggests that RDs are not completely solved at the genetic level and many cases remain unsolved (den Hollander et al., 2010). Reasons suggestive to this research data could be that the mutations present in the unsolved cases may be present in the genes involved in the syndromic forms and therefore, skipped during the analysis of non-syndromic patients or in genes that are linked with the major types of RDs and are not included in sequencing panels or it may also be possible that the gene is yet to be known (Chiang et al., 2015, Chiang and Trzupek, 2015).

The utilization of consanguineous families and homozygosity mapping approach has resulted in the mapping and identification of many RD genes. As consanguineous matings result in genomic fractions which are identical by descent and the mutant alleles because they descend from a common ancestor (Bittles and Neel, 1994). Homozygosity mapping is the most suitable technique for such populations for the identification of candidate genes involved in recessive disorders (Lander and Botstein, 1987). In case of outbred populations, the success rate of this technique is much less and the better way to uncover the underlying causative variants is to screen the known genes which are found to be frequently mutated in literature. This step is necessary before moving towards high throughput technologies like, next-generation sequencing (NGS) (Maria et al., 2015).

Homozygosity mapping, also known as autozygosity mapping, is considered a proficient gene mapping procedure in recessive disorders/traits in inbreed families (Gholipoorfeshkecheh et al., 2020). The goal of homozygosity mapping is to identify contiguous stretches of DNA that are homozygous in all affected individuals for the specific trait and are heterozygous in healthy individuals. Studies on several human recessive diseases have shown that the chances of identifying the candidate gene within the mapped homozygous genomic region are over 90% (Soorni et al., 2017).

Homozygosity mapping has been used to identify RD genes in families with autosomal recessive mode of inheritance. Mapping based on homozygosity defines the specific genomic regions where the causative gene can be present that could be either previously described or a novel gene (den Hollander et al., 2007b). These homozygous regions help in the identification of genes and their mutations in the NGS data by shortening the regions to be explored (Bocquet et al., 2013).

Next generation sequencing (NGS) is a high-throughput technology which enabled researchers to generate large amount of sequence data in a short period of time. This enhanced data generation capacity has resulted in significant changes in DNA sequencing and its applications (McCombie et al., 2019). It has several advantages over traditional sanger sequencing (Bahassi and Stambrook, 2014). With the advancement of NGS technologies, the third-generation sequencing platforms have gained more attention because of their ability to provide longer read lengths and real-time single-molecule sequencing (Van Dijk et al., 2018). Different NGS based strategies are available but a researcher can choose appropriate method from targeted panel sequencing, exome sequencing (ES), and whole genome sequencing (WGS) depending on the nature of disease and sample volume. Targeted gene panels have lower cost than ES or WGS and provide excellent coverage depth, but some genes of interest may not be included. ES has lower cost than GS, but the coverage of some exons might be suboptimal, especially in GC-rich regions (Belkadi et al., 2015) (Meienberg et al., 2016).

Another breakthrough in RD genetic was witnessed with the advent of targeted sequencing of selected panel of genes (Teer and Mullikin, 2010). The combination of microarray based genotyping and targeted sequencing have identified RD genes in 50% of total cases (Daiger et al., 2013). The success rate of identification of actual variants in different populations increased with the emerging technologies of NGS and this reached upto 70% in specific populations (Fu et al., 2013, Chen et al., 2013, Jinda et al., 2014).

Then comes the whole exome sequencing (WES) that covers all the exonic regions of genome. This also falls under the category of targeted sequencing as it mainly focuses on protein coding regions. WES became the most widely used technique for monogenic mendelian disorders. It not only discovers exonic variants but also identifies splice variants through a sequence load of only 2% (protein coding region of human genome).

Previously used methods of sequencing like Sanger sequencing were time consuming as the candidate genes need to be selected before being sequenced but WES covers all the genes and is kind of unbiased technique. According to an estimation, human exome includes 85% of mutations that can be related with disease phenotypes (Majewski et al., 2011). Mutational analysis of most of the monogenic disorders also shown to have causative variants in protein coding regions (Kuhlenbäumer et al., 2011). Within these exonic mutations, there is a high frequency of missense and nonsense mutations accounting for 60% of disease related variants (Botstein and Risch, 2003). High percentage (70%) of previously unidentified mutations shows remarkable heterogeneity in arRP (Bocquet et al., 2013). In another study the identification rate was around 63% and they identified novel variants in a cohort of Chinese patients presenting arRP via targeted next generation sequencing (NGS) (Fu et al., 2013).

Another advantage of WES is that it could reveal all the potential variants within the protein-coding regions apart from the actual causative mutation and sometimes such variants may act as modifiers for the pathogenic variant (Maranhao et al., 2015).

In case of RDs, the rate of mutation detection differs depending upon the phenotype. For example, in a study with large percentage of individuals having RP were solved with a rate of 50%. But 84% and 29% cases with USH and CD, respectively were also solved in this study which indicate the variation in diagnostic yield based on the phenotype. The detection rate of variants is also influenced by ethnicity (Carss et al., 2017). The rate of mutation detection in different ethnicities was also estimated by whole genome sequencing (WGS). Three different ethnic groups i.e. one from African ancestry, another from European ancestry and third includes individuals from South Asian ancestry showed mutation detection rate of 30%, 55% and 57% respectively. The task of variant interpretation becomes more challenging in African populations due to increased genetic diversity (Genomes Project et al., 2015, Lek et al., 2016). Individuals of South Asian ancestry presents 66% of homozygous pathogenic variants as compared to 18% homozygous variants detected in individuals from European ancestry. This is probably attributed to the high rates of consanguinity in the South Asian populations (Carss et al., 2017).

1.7 RDs in Pakistan

In Pakistan, the prevalence of RD is not well defined but according to a study conducted in hospitals, RP is the most frequent of the RD phenotype present in the Pakistani population (Adhi and Ahmed, 2002). Prevalence of autosomal recessive disorders largely depends upon consanguinity. As consanguineous marriages are common norm in Pakistan, therefore it is probable to have high prevalence of recessive RDs though comprehensive studies are still lacking. Rate of consanguinity is more than 60% in Pakistani population and within this frequency 80% are first cousin marriages (Bittles, 2001).

Families with autosomal recessive forms of RD has been used to map genes since 1990s and loci mapped in many of these families later helped to identify the underlying genes. For example, an arRP locus was mapped on chromosome 1 in a large family by using microsatellite markers (Leutelt et al., 1995). Similar approach was applied for the mapping of additional loci in RD families with RP (Hameed et al., 2001; (Hameed et al., 2001); (Naz et al., 2010) and CRD (Khaliq et al., 1999); (Khaliq et al., 2000) and BBS (Beales et al., 2001). Later mutations were identified in PCDH15 (Ahmed et al., 2001) gene in two families with USH, AIPL1 (Damji et al., 2001) gene in four families with LCA. In another study (Khaliq et al., 2003) performed genetic analysis of several LCA families and reported CRB1 mutations in three Pakistani families. Similarly, mutations were identified in CNGB1 (Zhang et al., 2004), RP1 (Khaliq et al., 2005); (Riazuddin et al., 2005), PDE6A (Riazuddin et al., 2006), PROM1 (Zhang et al., 2007) and Opsin (Azam et al., 2009) genes in families with arRP. Additionally, mutations were identified in CNGA3 and CNGB3 (Azam et al., 2010) gene in achromatopsia (ACHM) families, MERTK (Shahzadi et al., 2010), EYS (Khan et al., 2010), CLRN1 (Khan et al., 2011), PDE6B (Ali et al., 2011), TULP1 (Iqbal et al., 2011); (Ajmal et al., 2012) genes in arRP families. Several additional studies have identified mutations in the RD families of Pakistani origin (Branham et al., 2018); (Sultan et al., 2018); (Chen et al., 2018); (Li et al., 2018); (Sheikh et al., 2019); (Albarry et al., 2019); (Khan et al., 2020); (Nadeem et al., 2020); (Noman et al., 2020); (Ahmed et al., 2021); (Yousaf et al., 2022); (Tehreem et al., 2022)

Meanwhile, genome wide genotyping with 500K array and Sanger sequencing of candidate genes present within the genomic region mapped in a family with arRP and intellectual disability results in the identification of mutation in CC2D2A gene (Noor et al., 2008). But another study from a different population showed the involvement of

CC2D2A gene in JS (Gorden et al., 2008). In another study exome sequencing identified mutations in BBS5 and INPP5E genes in two families with BBS (Khan et al., 2019). An additional study identified a pathogenic mutation in CNNM4 gene in a family with Jalili syndrome (Parveen et al., 2019).

First comprehensive study was performed on 57 Pakistani families (Northern regions of Pakistan) with visual impairment, but later analysis showed that approximately 67% families have inherited forms of RD. This study further showed that LCA is more common among Pakistani RD families, but authors were able to identify loci/gene in only 23 families (Adhi et al., 2009). Another study recruited 15 families with different types of RDs, but genetic analysis could identify underlying mutations in 9 families. This study identified some known mutations, but four novel mutations were detected in CRB1, CNGB1 and PDE6B genes (Azam et al., 2011). Another study used homozygosity mapping to identify mutation in two families with RD (Hussain et al., 2013). An additional study on three families with RD identified mutations in RPE65 gene (Kabir et al., 2013). Mutation have been identified in DHX38 gene in multiple Pakistani families presenting early onset RP and macular coloboma (Ajmal et al., 2014b); (Latif et al., 2018).

In 2014, Khan et al., (2014) nicely compiled the data of 103 RD families and showed that RP is most common type of RD in Pakistan followed by LCA, CRD and CSNB. They also showed that majority of RD families have autosomal recessive inheritance and only two families showed autosomal dominant RP. Authors further showed that out of 132 RD genes known at that time mutations in only 36 genes were detected in Pakistani families (Khan et al., 2014). Another study on 13 families with different types of RDs identified known and novel variants by using targeted sanger sequencing approach (Maria et al., 2015). Two studies by our group have also identified known and novel variants in RD families by using homozygosity mapping and sanger sequencing (Saqib et al., 2015); (Ravesh et al., 2015). Another study conducted on a cohort of 144 families resulted in the identification of known and novel variants in large number of families (Li et al., 2017). Most recent study conducted on 108 unrelated families included 15 families from Pakistan and led to the identification of known and novel variants in different RD genes (Biswas et al., 2021).

In a study conducted on Pakistani population from the province of Punjab, 5 out of 26 families were found to have same mutation in *RPE65* gene. But, at the same time there

were 7 mutations being reported for the first time in Pakistani population and 6 were novel pathogenic variants found in known genes. The novel mutations may be seen as a result of, heterogeneity factor and to some extent the understudied nature of this population (Maranhao et al., 2015).

1.8 Aims and Objectives

This study was initiated with an aim to explore the underlying genetic factors of currently unsolved families presenting different types of RDs. As unsolved families show autosomal recessive inheritance, therefore homozygosity and exome sequencing will be used to identify RD causing mutations. Specific objectives of this study will be;

- To identify and recruit families with different types of retinal dystrophies.
- To apply genomic techniques for mutational screening in RD families to discover the genes and their causative variants

MATERIALS AND METHODS

2. Materials and Methods

This study was initiated after seeking the prior approval from Bio-Ethics Committee (BEC) of Faculty of Biological Sciences, Quaid-I-Azam University as per the guidelines specified in the Declaration of Helsinki regarding the protection of human subjects. The experiments were mainly carried out in a research laboratory at Department of Biochemistry, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad except where specified otherwise. This study was also approved by Intuitional Review Board, Quaid-I-Azam University, Islamabad.

2.1 Families Recruitment:

For this study, fifteen families (Family A-O) representing retinal dystrophies (RDs) were identified and recruited from different provinces of Pakistan. During family recruitment only families with two or more RD patients, presenting similar phenotype, were selected for further studies. A detailed history of participating family members and the different aspects of retinal dystrophies was collected from respective heads and normal individuals of each family while visiting their places of residences. The gathered information was then used to construct pedigrees of respective families which helped to infer the mode of inheritance of RD phenotype. In each pedigrees males and females were represented by squares and circles, respectively. The consanguineous marriages between parents were indicated by a double marriage line between the symbols. Similarly, the filled symbols denote family members with RD phenotype.

2.2 Clinical Evaluation:

A detailed clinical evaluation of all affected members of fifteen families was done before the collection of blood samples. The individuals were interviewed about the disease status, time of onset of vision loss, rate of progression and if any affected individual is showing any extra-ocular features which may include; limbs abnormalities, facial dysmorphism, obesity, delayed development of motor skills and hearing loss. After investigating the families, one affected member of each family was examined by a local ophthalmologist. Fundoscopy, slit-lamp examination optical coherence tomography was performed for ophthalmological evaluation of affected members of those families which such healthcare facilities were available in the nearby regions.

Affected individuals of the fifteen recruited families had apparently normal eye morphology but were experiencing variable degree of vision loss along with some additional features. Close up photographs of both eyes of each recruited patient were taken and to document additional ocular features present in these patients. Each patient was asked a set of structured question to gather information about night vision, day vision, response to sunlight and ability to discriminate colours. However, these questions were skipped for those patients which have early onset RP or LCA or have complete vision loss. After gathering all this information recruited families were divided into RP (Family A-E), LCA (Family F-L), Bestrophinopathy (Family M) and STGD (Family N and O).

2.3 Blood collection:

Blood samples were obtained by a phlebotomist from the available individuals of each family. Blood was drawn by using 10 ml sterile disposable syringe (Becton Dickinson) and 21G needle by following standard safety procedures. After drawing the blood, it was immediately transferred into vacutainers containing K2 Potassium salt of EDTA (Becton, Dickinson and Company, USA) which works as an anticoagulant. Each tube was assigned a family and individual specific code and the contents were mixed to prevent blood clotting. The properly labelled vacutainers were transferred to a research laboratory in Department of Biochemistry, Quaid-I-Azam University, Islamabad for storage at 4˚C.

2.4 DNA Extraction:

Genomic DNA isolation was performed by using either organic method (also known as Phenol-Chloroform method) or by using commercially available genomic DNA extraction kits. For both methods blood samples stored at 4˚C were initially taken out and kept at room temperature and slightly tapped to thoroughly mix the contents. Then appropriate amount of blood was taken from these tubes to proceed for genomic DNA isolation, as described below;

2.4.1 Organic method:

The composition of solutions used for organic isolation of genomic DNA are summarized in table 2.1. For this method, 750 µL of blood was poured in a sterile 1.5 mL tubes and an equal volume of solution A was added. After mixing the solution A with blood, tubes were kept at room temperature for 10-15 minutes before

centrifugation at 13,000 rpm for one minute. The centrifugation was performed in a microcentrifuge (EB21; Hettich, Germany. After centrifugation, supernatant was discarded by carefully inverting the tubes on the waste bin. The pellet of cells left at the bottom of tubes was resuspended in 400 µL of solution A. At this step special care was taken to completely dissolve the pellet before proceeding for centrifugation as described above. Again, the supernatant was discarded by inverting the tubes and the pellet settled at the bottom of tubes was resuspended in solution B (500 µL), 20% sodium dodecyl sulphate (12 µL) and proteinase K (5 µL) enzyme. The tubes were placed in an incubator (B28; Binder, Germany) at 37˚C for overnight and contents were mixed several times during incubation to completely dissolve the pellet. After incubation, freshly prepared mixture (500 µL) consisting of solution C and D in volume ratio of (1:1) was added and the contents were thoroughly mixed by repeatedly inverting tubes. Afterwards tubes were placed in the centrifuge at 13,000 rpm for 10 minutes which separates the contents in two layers. The upper aqueous layer was collected in a new 1.5 ml tube with the help of a micropipette. Tubes containing the lower layer were discarded. Then solution D (500 µL) was added in tubes containing the upper layer and contents were again mixed by inverting the tubes several times. Once again, the tubes are centrifuged at 13,000 rpm for ten minutes which again separates contents in two layers. The upper aqueous layer containing gDNA was transferred in a 1.5 mL tube and tubes containing lower layers were discarded. In order to precipitate genomic DNA, aqueous layer was mixed with chilled solutions of isopropanol (500 µL) and sodium acetate (55 µL; 3M) and mixed by inverting tubes for 5-6 times. Centrifugation was repeated at 13,000 rpm for 10 minutes and the supernatant was discarded carefully, whereas DNA pellet was washed by 70% ethanol (250 µL). At the end centrifugation was given at 4000 rpm for 5 minutes and the supernatant was discarded and tubes containing pellet were left for few minutes to dry at room temperature. The isolated genomic DNA pellet was dissolved DNA buffer (250 µL) and stored at -20˚C before proceeding for subsequent studies.

2.4.2 Genomic DNA isolation by NucleoSpin Kit:

NucleoSpin® Blood kit (Macherey-Nagel, Germany) was used for genomic DNA isolation from some low volume blood samples as per recommended manufacturer's protocol. This kit uses 200 µL of blood rather than 750 µL blood used for genomic DNA isolation by organic method. Initially, blood (200 µL) was decanted into 1.5 mL tubes and B3 buffer (200 µL) and proteinase K (25 µL) were added and vigorously mixed for few seconds. The lysate obtained by this treatment was incubated at 70˚C for 10-15 minutes and then 100% ethanol (210 µL) was added and mixed. This sample was loaded into the column containing silica membrane (NucleoSpin® Blood Column) followed by centrifugation at 11,000 g for one minute. After discarding the flow-through, column was shifted in a new collection tube. First washing of silica membrane of the column was done by adding Buffer BW (500 µL) and centrifugation at 11,000 g for one minute. The flow-through was again discarded and column was transferred in the tube for second washing with Buffer B5 (600 µL). After adding buffer B5 tubes were centrifuged again as described for earlier wash. After discarding the flow through empty column was centrifuged again for the same speed and time to dry the membrane. The column was then placed in a 1.5 mL microcentrifuge tube and elution of gDNA was done by adding preheated (at 70˚C) Buffer BE (100 µL) and centrifuged at 11,000 g for 1 minute. The DNA collected after this step was stored at -20˚C.

DNA extracted by both the methods was checked on 1% agarose gel and the concentration of each sample was determined by using microvolume spectrophotometer.

2.5 Genetic Analysis of RD Families:

Genetic analysis of fifteen RD families was carried out by using three different approaches. Firstly, 11 out of 15 families were subjected to whole genome scan which employs single nucleotide polymorphism (SNP) microarray. In twelve families, whole exome sequencing (WES) was performed. But four out of twelve families (Family E, I, L and N) were directly subjected to WES but remaining eight families underwent WES after genome wide scan. Only Family G was scanned through targeted Sanger sequencing (TSS) of candidate genes after performing genome wide scan.

2.5.1 SNP Genotyping and Homozygosity Mapping:

DNA samples of family members of all families, except family B, C, K and M, were subjected to genome wide scan by using Infinium® HumanCoreExome BeadChip (Illumina, USA) which contains approximately 550K SNP markers. The genotyping was performed by following standard protocols at the University of Washington Center for Mendelian Genomics.

The concentrations were determined by quantifying DNA samples using spectrometer (Titertek Perthold, Germany). All samples were diluted such that they have concentration of 50 ng/µL. Quality control testing was carried out for quality assurance of DNA samples and gender testing was done for confirmation of pedigree information. DNA (4 µL) of the samples which passed the above-mentioned criteria were poured in 96 well plate. After denaturation, these samples were initially amplified and later subjected to controlled enzymatic fragmentation. Isopropyl alcohol was used to precipitate the fragmented DNA followed by centrifugation at 4˚C. The precipitated DNA was dissolved in hybridization buffer and were loaded onto the bead chip. This bead chip was containing locus specific oligonucleotides, to which DNA fragments were annealed after incubating at 48˚C in hybridization oven. Bead chip was washed to remove the unhybridized DNA fragments. Single-base enzymatic extension was done for incorporation of fluorescent nucleotides that were detectable during laser scan imaging done by Illumina iScan (Illumina, USA). High resolution images were generated by iScan and analysed with the help of software, Genome Studio 2.0 (Illumina, USA).

Initial analysis of the resulted genotype was performed by using PedCheck to detect Mendelian incompatibilities and MERLIN for genotyping error detection. Later, another online tool, HomozygosityMapper (HM) was used to analyse the genotype data available for members of each family. While analysing genotype data on HM, homogeneity analysis option was selected to identify homozygous regions shared by all genotyped affected individuals of respective family. The homozygous regions represented in bar chart were selected based on homozygosity score greater than 80%. The homozygous genomic regions mapped in each family were explored for the identification of known genes associated with RDs. Many families mapped to novel regions of several Mbs harbouring a large number of genes and some families with multiple homozygous regions were subjected to whole exome sequencing and only one

family was explored by targeted sanger sequencing based on candidate genes found in the mapped region of homozygosity.

2.5.2 Whole Exome Sequencing (WES):

DNA samples of two members from family B, C and K and DNA sample of one member from the rest of the families except three families (Family D, H and L) were subjected to WES. These experiments were performed at University of Washington Center for Mendelian Genome Sequencing through a research collaboration with Prof. Dr Suzanne M Leal. For WES, 36.6 Mb target region was captured by using Human Exome Library v.2.0 (Roche NimbleGen) by following the manufacturer recommended protocol. Sequencing of all submitted DNA samples was carried out on HiSeq platform (Illumina, USA) and the obtained fastq files were subjected to Burrows-Wheeler Aligner (BWAv0.5.9) for alignment with human reference genome assembly 19 (hg19), to produce BAM files. Subsequent analysis was performed by using Genome Analysis Toolkit (GATK) before generating Variant Call Format (VCF) files. The data was further analysed by focussing on the regions of homozygosity, mapped by genome wide SNP genotyping. Analysis of exome data aimed to find coding variants and variants affecting splicing which were not found in dbSNP and were present with a minor allele frequency (MAF) of <0.05 in the gnomAD.

2.5.2.1 Filtration of exome data

First of all, VCF files were annotated using either Seattle Seq 137 (http://snp.gs.washington.edu/SeattleSeqAnnotation137/) or wANNOVAR (http://wannovar.wglab.org/) with default parameters. The filtration process was based on the following parameters;

1. It focussed on variants which were present in the coding regions or near the splice sites.
2. Synonymous variants were excluded during filtration.
3. Variants with a MAF of less than 0.01 in different public databases like, 1000 Genome Project (TGP), NHLBI, ESP and gnomAD were included.
4. Variants which were predicted to be pathogenic by various pathogenicity tools.
5. Lastly, homozygous variants were selected on preferential basis.

The filtration process successfully shortlisted variants in each family which were further checked for segregation in the respective family. Sanger sequencing was done by using primers for specific genomic regions carrying variant in each family.

2.5.3 Detection and Analysis of Large Genomic Deletion:

Family A ended up with a homozygous genomic region but without any strong variant in the VCF file. After exhausting all possible options to identify variant in the exome data of this family it was decided to reanalyse the genotype and exome data.

Initially we reanalysed all regions of homozygosity mapped in this family to look for any inconsistency. While looking at the homozygous region mapped on chromosome 14 a region of no calls was noticed in all affected members of this family. Reanalysis of genotype data of this region showed that three SNPs are missing indicating the involvement of large deletion. In order to map this deletion different sets of primers, (Table 2.2) were designed from the sequence in between the deleted SNPs and intact SNPs present both upstream and downstream of the deleted region. Graphical presentation of primer sets with respect to deleted region is shown in Figure 2.1. The primers were amplified using one normal and one affected member of respective family. Fine mapping was done using primer sets flanking the deleted region and the amplified product of the affected sample was Sanger sequenced to map the exact break points. In addition to the flanking primers, a primer set for exon 5 of *SPATA7* was also amplified in whole family.

2.5.4 Expression analysis at RNA level

In order to confirm the deletion of SPATA7 gene in the patients of family A, which harbours large deletion on chromosome 14, fresh blood samples were collected from affected individual and a single carrier parent. Blood sample were collected in Tempus RNA tubes (ThermoFisher Scientific, USA) and RNA was isolated by using Tempus Spin RNA isolation kit (ThermoFisher Scientific, USA) by following recommended protocol, which is briefly described below;

Fresh blood was drawn directly into Tempus™ Blood RNA tubes, shaken vigorously for few seconds and RNA extraction was done using Tempus™ Spin RNA Isolation kit. Stabilized blood from RNA tube was transferred to 50 mL falcon tube and diluted with 3 mL of 1X PBS (Ca^{++} and Mg^{++} free Phosphate Buffer Saline). Tubes were vortexed vigorously for 30 seconds and centrifuged at 3000 g at 4˚C for 30 minutes.

Supernatant was discarded carefully without disturbing transparent RNA pellet and tubes were inverted on the absorbent sheet for 1 to 2 minutes. RNA pellet was resuspended in RNA purification resuspension solution (400 μL) and kept on ice. Th filter membrane was pre wet with wash solution 1 (100 μL) and centrifuged at 3000 rpm for 30 seconds. Afterwards resuspended RNA (400 μL) was poured on purification filter followed by centrifugation at 6000 g for 30 seconds. Liquid waste was carefully discarded, and filter was washed with wash solution 1 (500 μL) followed by centrifugation as explained above. This step was repeated but this time washed with wash solution 2 (500 μL) followed by centrifugation at 6000 g for 30 seconds. The filter was dried by centrifugation and transferred to a new labelled collection tube followed by the addition of elution buffer (100 μL) and incubated at 72˚C for 4 minutes. After centrifugation, purification filter was discarded and upper 90 μL of RNA elute was transferred to a new labelled collection tube.

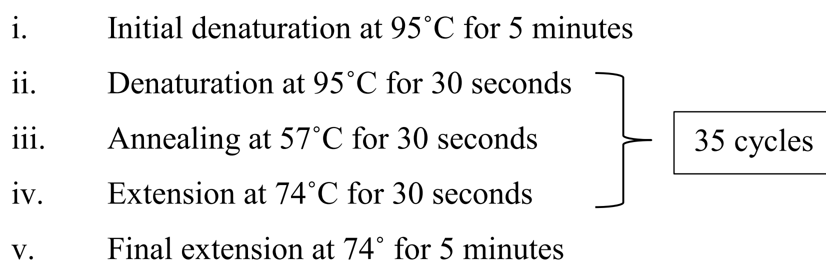
RNA isolated from these samples was used for cDNA synthesis by using Oligo dT (ThermoFisher Scientific, USA) primers. The flanking region of exon 8 – exon 12 of *SPATA7* gene was amplified from the cDNA sample obtained for two affected members (III-4 & III-6) of family A and one related and one unrelated control member. *GAPDH* was simultaneously amplified in these individuals and served as positive control.

2.5.5 Targeted Sanger Sequencing

Targeted Sanger sequencing of candidate genes selected based on homozygous regions was used for family G and for the co-segregation of specific variants identified in rest of the families. Primers were designed from the flanking regions of variants or coding exons of selected genes by using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/).

2.5.5.1 Exon amplification

Polymerase chain reaction (PCR) was done for the amplification of specific exonic parts of the selected genes. PCR reaction was prepared by adding 4 μL of PCR water, 1 μL of gDNA, 6 μL of 2X PCR Master Mix (Fermentas, Thermofisher Scientific, USA) and 1 μL of each primer (forward and reverse). A 13 μL reaction was prepared in a PCR tube and placed in thermocycler (thermocycler or T100) under following conditions:



The amplified PCR product of each sample was resolved on 2% agarose gel and purified by Exo-SAP-IT kit. For purification, 8μL of amplified product is added to another PCR tube and into this tube, 2 μL of Exo-SAP was added. These tubes were incubated at 37˚C for 15 minutes and then at 80˚C for another 15 minutes.

2.5.5.2 Sequencing PCR

Sequencing PCR was started with a mixture of 10 μL reaction using BigDye® Terminator v3.1 Cycle Sequencing Kit (Part No 4337455; Applied Biosystems™, USA). The reaction mixture contained the following reagents.

- BigDye® Terminator v3.1 Cycle Sequencing Kit — 04 μL
- Sequencing Buffer — 01 μL
- Primer (10 nM) — 01 μL
- Purified PCR product (2-10 ng) — 1-2 μL
- Nuclease-free water — As required

This mixture containing PCR tubes were placed in thermocycler for initial denaturation at 96˚C for 5 minutes. The profile used for 30 cycles to amplify single stranded DNA fragments as follow; (i) denaturation at 96˚C for 20 seconds, (ii) annealing at 60˚C for another 20 seconds and extension at 60˚C for 4 minutes. Lastly, final extension at 65˚C for 10 minutes was done. After completion, the sequencing reaction was stopped by adding stop solution (5 μL) followed by addition of absolute ethanol (45 μL). This solution was prepared by mixing 2 μL sodium acetate (3M, pH 5.2), 2 μL sodium EDTA (100mM, pH 8.0) and 1 μL of glycogen (20 mg/mL). The tubes were vortexed to thoroughly mix all the contents and placed in centrifuged at 13000 rpm for 20 minutes keeping the temperature at 4˚C. After discarding the supernatant pellet was washed by chilled 70% ethanol (150 μL) and centrifuged at 13000 rpm for 8 minutes and temperature was again maintained at 4˚C. Supernatant was again discarded and pellet was dried in a Concentrator Plus (Eppendorf, Germany) and resuspended in Hi-Di-

Formamide before loading on capillary DNA sequencer (ABI 310; Applied Biosystems, USA).

2.5.6 Data analysis

Sequencing data was analysed by using BioEdit 7.0.5 (http:/www.mb~o.ncsu.edu/bioedit.htm) for the identification of variants by comparing with reference sequences obtained from UCSC genome browser (hg19). The alignment of both sequences helped to find out the variants.

2.5.7 *In-silico* analysis

Different variants detected in each family were analysed for the degree of pathogenicity by using various online tools of pathogenicity. The variants were tested by PolyPhen2 (www.genetics.bwh.harvard.edulpph2) (Adzhubei *et al;* 2013), SIFT (sorts intolerant from tolerant amino acid substitutions; (www.sift.jcvi.org/) (Kumar *et al;* 2009) and MutationTaster (www.mutationtaster.org/) (Schwarz *et al;* 2014). Splice variants were analysed with MutPred Splice (http://www.mutdb.org/mutpredsplice/submit.htm), Human Splice Finder version 3.0 (http://umd.be/HSF3/HSF.html) and Skippy (http://research.nhgri.nih.gov/skippy).

Missense variants were analysed for their evolutionary conservation using HomoloGene (http://www.ncbi.nlm.nih.gov/homologene). This analysis was performed by aligning the protein sequences from different vertebrate species.

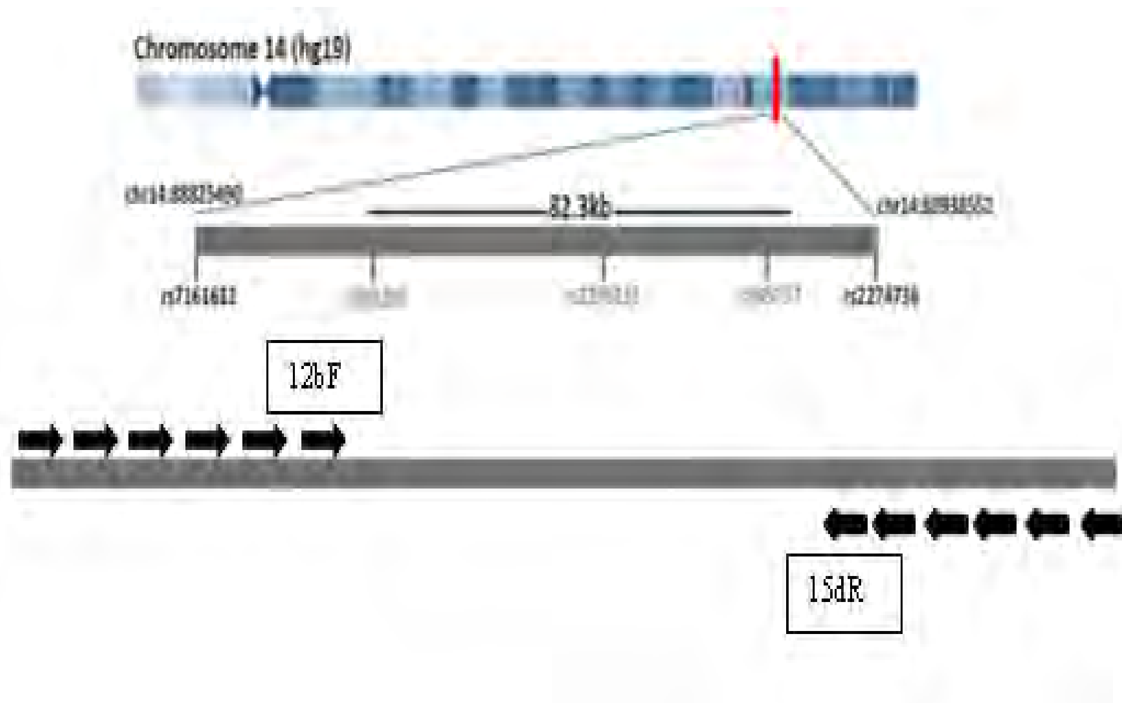


Figure 2.1: Graphical presentation of primer sets used for the determination of deletion breakpoints in family A

Table 2.1 Composition of solutions used for genomic DNA isolation

<table>
<tr><th>Solution A</th><th>Solution B</th><th>Solution C</th><th>Solution D</th></tr>
<tr><td>Sucrose 0.32 M</td><td>Tris 10 mM (pH 7.5)</td><td rowspan="4">Phenol</td><td rowspan="2">Chloroform (24 volumes)</td></tr>
<tr><td>Tris 10 mM (pH 7.5)</td><td>NaCl 400 mM</td></tr>
<tr><td>$MgCl_2$ 1.5mM</td><td rowspan="2">EDTA 2 mM (pH 8.0)</td><td rowspan="2">Isoamyl alcohol (1 volume)</td></tr>
<tr><td>Triton X-100 1% (v/v)</td></tr>
</table>

Table 2.2: Primers used for the mapping of deletion breakpoints

Set		Primer sequence	Tm	GC%	Product size
1	F	AGACTCCTTCCTCAATGAACCA	60.11	45.45	2557 bp
	R	CACTGTGGGTAACTGGAACTCA	60.07	50	
2	F	TGGGGTAGGATAACTGCTGAGT	60.02	50	2793 bp
	R	GCCTGATGGTATTAGACCGAAG	59.99	50	
3	F	CAGTGAAAACTGTGCGGTGT	59.79	50	2313 bp
	R	AACAGGGTTAGAAGGGGAAGTG	60.73	50	
4	F	GTTTGCCTGATGCAGTTTCC	60.65	50	2817 bp
	R	CTGGTGGAACAGAATGAAGAGC	61.18	50	
5	F	ACCAATGTCCTGGTGTTTCC	59.68	50	2839 bp
	R	GATATCCCGGCCTTTGTCTC	60.8	55	
6	F	GCATGTTCTAAACCACTGCAAG	59.81	45.45	3727 bp
	R	CTAGTTCTCCCCACACCGTTTA	60.4	50	
7	F	CAGCCCATATAGTTGAGACAATC	57.79	43.48	2255 bp
	R	ACAAGGCCAGTCTTACTCTGATTC	60.19	45.83	
8	F	ATCCCACTTGGTCATGGTTA	57.71	45	2498 bp
	R	AGAGAGCACCTCAATCAGGAAG	60.02	50	
9	F	ACTTCATCCCCCAGCTTTTT	59.94	45	2392 bp
	R	TGAGCTCTAAGCCCTTTGGA	60.09	50	
10	F	CCAGGAATCCTCTGAATTATGAAA C	61.31	40	3680 bp
	R	TTGCTACTTACCAGCTTGATCTTG	59.97	41.67	
11	F	GGATGAGAACTCTCCTTCATGG	60.07	50	2383 bp
	R	CCTAGGTTGGTGAAGTGTCAGA	59.25	50	
12	F	CCAGTATCATTCTTTCCTCGAGTC	60.48	45.83	3719 bp
	R	TCTGAGAATGAAGCCTACACAGTC	59.95	45.83	
13	F	CAAGTCACTACCTCTGTCCACTTT	58.9	45.83	3273 bp
	R	GACGTGTGGCTGTATTCATGTT	59.93	45.45	
14	F	GAAGTGAGTTAAGGAGTGACATGG	59.21	45.83	3266 bp

	R	AAGGGAGAAGAGCTTTAGGTACTG	58.73	45.83	
15	F	GAGGACATATAAGTGGCAACAGG	59.91	47.83	3959 bp
	R	TGACTAGGACAAGAGTAGGTGGAG	58.99	50	
16	F	CTCAAACTCCTTTCTGGGAGAACC	63.68	50	2386 bp
	R	CCTGAAAGGTAAGCAGTGGTACAG	61.39	50	
17	F	TTTAACCCTTCAAGCCTTTTTGC	62.73	39.13	5677 bp
	R	GTTCTCCCAGAAAGGAGTTTGAGA	62.72	45.83	
12a	F	TTACTATCCCATGGTAGGGATG	57.84	45.45	2541 bp
	R	TAAGTGACAGCTGTGAACAAGG	58.09	45.45	
12 b	F	CTTCATCACCGAGGAGTGATAT	57.84	45.45	1733 bp
	R	ATATCACTCCTCGGTGATGAAG	58.15	45.45	
12c	F	TTACTATCCCATGGTAGGGATG	57.84	45.45	1142 bp
	R	GTCCAGTGAGGAAAGATGAAAG	57.91	45.45	
15a	F	GTACCACTGCTTACCTTTCAGG	57.95	50	2518 bp
	R	CCCTCTCCATGGTACGTTTT	58.91	50	
15 b	F	CAGAGGCAGTAAACCTATGAACC	59.21	47.83	2478 bp
	R	GTGTACAGAGTCCTCATCCAGTTC	59.19	50	
15c	F	GGATGAGGACTCTGTACACAAATG	59.93	45.83	2229 bp
	R	GAGAGATAGGGGGTCCAGTTTT	59.11	45.83	

RESULTS

3. RESULTS

3.1 Families Description

This study was conducted on fifteen different families (Family A-O), which were recruited from different provinces of Pakistan along with complete family history. All fifteen families have minimum of two individuals presented same type of retinal dystrophy. Pedigree analysis show autosomal recessive pattern of inheritance of RD in each family.

These fifteen families were divided into three groups based on the clinical presentation of affected individuals and type of RD phenotype. Eight families (Family A to H) were placed in Leber Congenital Amaurosis (LCA) group, four families (Family I to L) were placed in Retinitis Pigmentosa (RP) group and last three families (Family M to O) were placed in other retinal dystrophies group. Affected members of two families from the last group were presented with Stargardt disease, but members of the third family were presented Best disease.

3.1.1 Clinical Description of LCA families

As mentioned above LCA group has eight (Figure 3.1 to 3.8) families (Family A to Family H) and affected members of these families show complete vision loss since birth or early childhood. Affected individuals of these families have grossly normal eyes without any additional extra ocular feature unless specified otherwise.

3.2.1 Family A

Family A contains three affected male members (III-3, III-4 and III-6) out of seven members included in this study. These three male affected individuals are born to parents II-1 and II-2 and despite our repeated attempts healthy members of the family could not explain exact relationship between II-1 and II-2. However, both parents belong to the same ethnic group and have remote relatedness, but they could not explain the exact nature of relationship. The affected members were representing severe loss of vision. Visual acuity was restricted to light perception with no colour perception at all. Nystagmus and keratoconus were also observed in affected members. Fundoscopy of one affected individual III-6 revealed the pigment clumping in the peripheral areas of retina (Figure 3.9).

3.2.2 Family B

Family B contains five affected members out of ten members. Five affected members include three males (V-3, V-5 and V-6) and two females (V-7 and V-8). The affected members of this family have severe loss of vision from early childhood.

3.2.3 Family C

Family C consisted of eleven members but four are affected male members (V-2, V-6, VI-4 and VI-5). These members have severe vision loss since early childhood.

3.2.4 Family D

Family D contains four affected members (III-1, III-2, III-4 and III-7). All the affected individuals initially had slight vision loss in early years of life, but it worsens with age. Reduced day and night vision was observed in these individuals ultimately resulting in congenital vision loss before the age of 7 to 8 years.

3.2.5 Family E

Family E comprises of five individuals but only two are affected including one female (IV-1) and one male member (IV-2). The affected members were presenting severe vision loss indicating severe form of retinal dystrophy i.e; LCA. Nystagmus was also observed in these individuals.

3.2.6 Family F

Family F consists of six individuals and three affected individuals, one male (IV-1) and two female individuals (IV-2 and IV-4). The affected members were displaying congenital vision loss resulting in complete blindness.

3.2.7 Family G

Family G is also comprised of total six individuals and three affected individuals, two female (IV-4 and IV-5) and one male (IV-6). The affected individuals were presenting day and night vision loss. There was slight vision in the initial years of life, but later progressive vision loss was observed.

3.2.8 Family H

Family H comprises of five members in total; two females (II-1 & III-3) and three males (II-2, III-1 & III-2) and two of them are affected (III-1 & III-3). The affected individuals were presented with complete blindness. A male and a female affected individuals are born to parents II-1 and II-2 which informed about the existence of remote relationship, but they could not explain exact relationship. A normal family member informed that both belong to the same ethnic group.

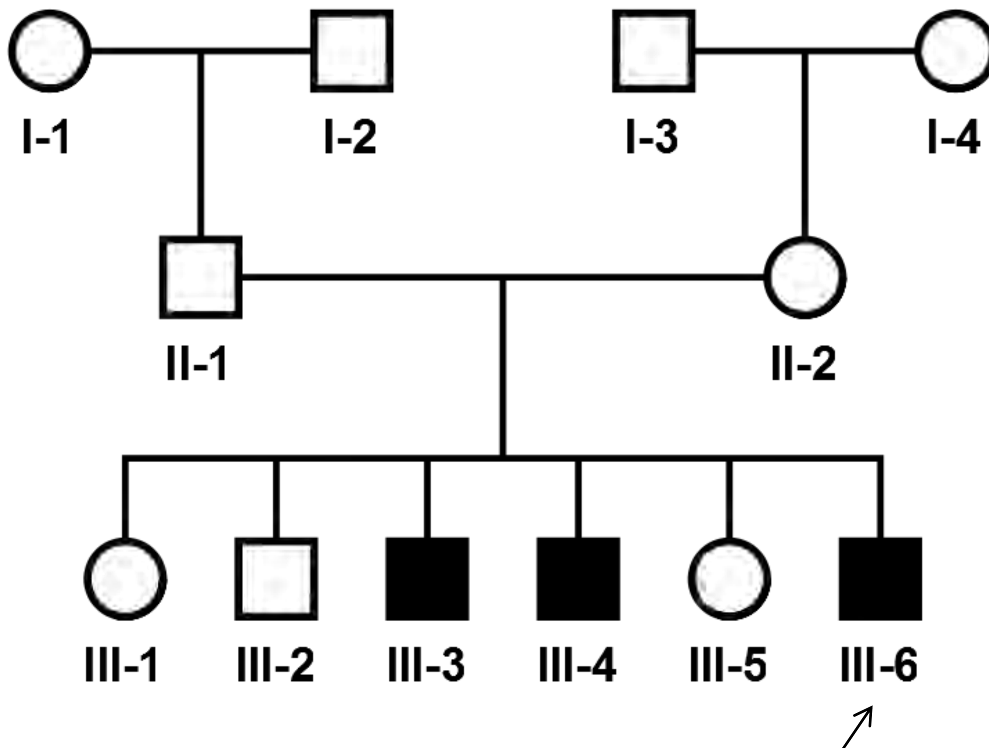


Figure 3.1: Family A pedigree show three affected individuals.

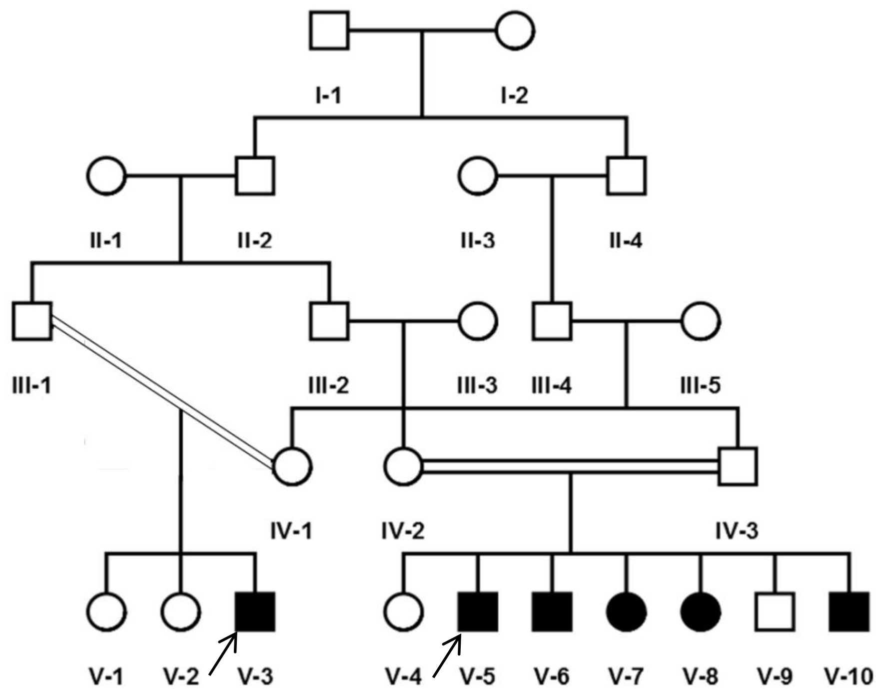


Figure 3.2: Family B pedigree show six affected individuals in two loops.

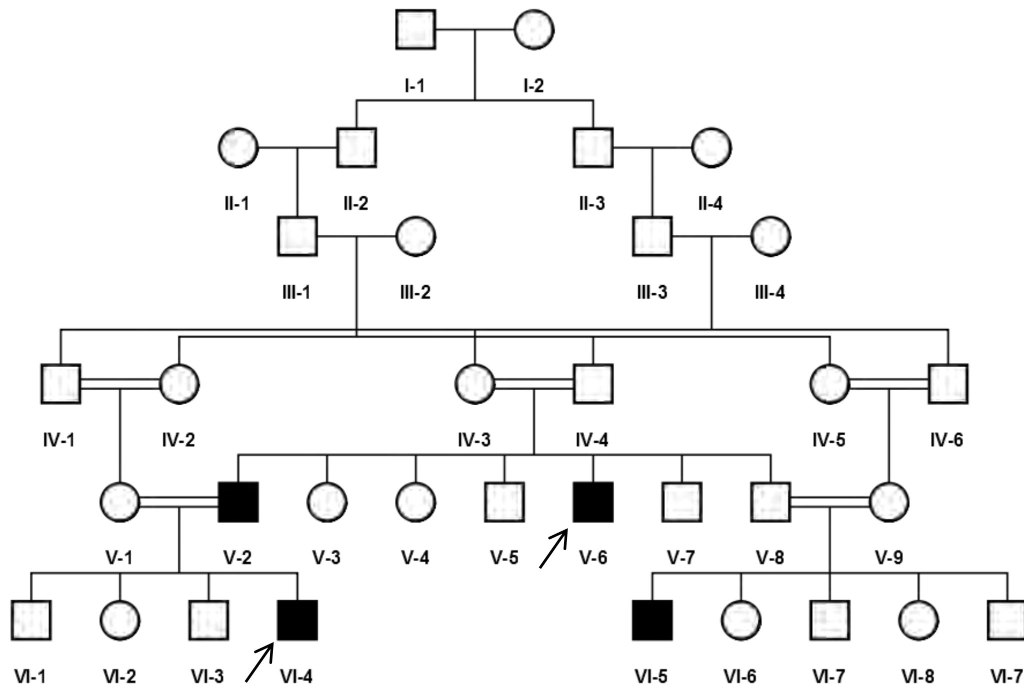


Figure 3.3: Family C pedigree show four affected individuals present in three different loops.

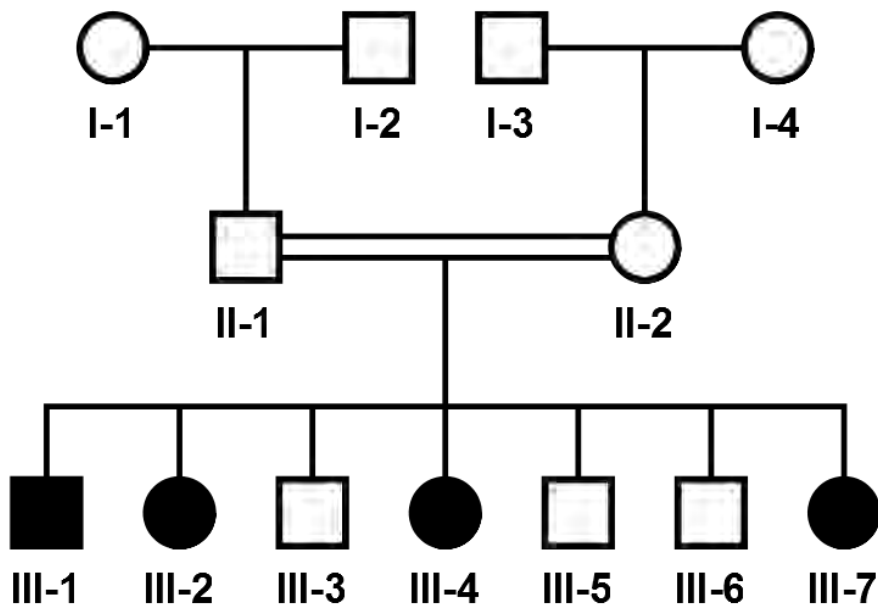


Figure 3.4: Family D pedigree show four affected individuals.

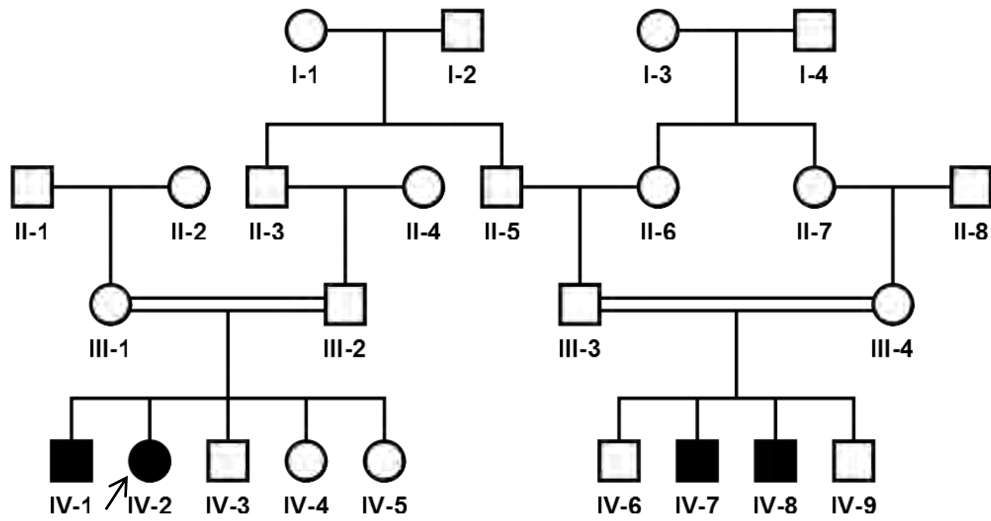


Figure 3.5: Family E pedigree show four affected individuals in two loops.

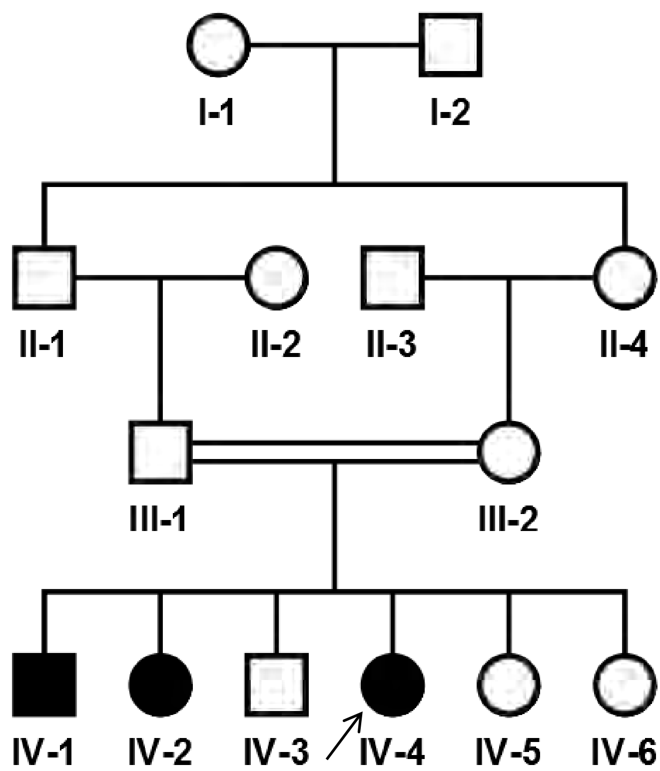


Figure 3.6: Family F pedigree show three affected individuals.

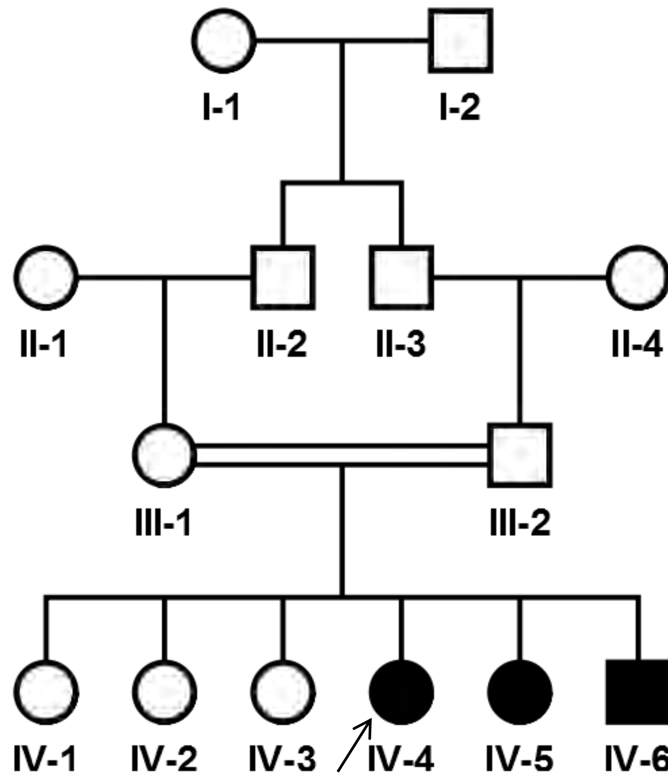


Figure 3.7: Family G pedigree show three affected individuals.

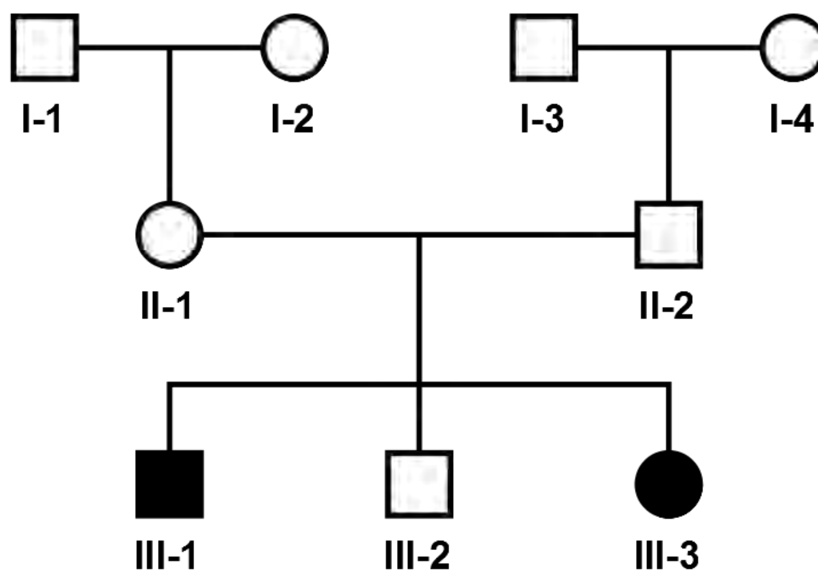


Figure 3.8: Family H pedigree show two affected individuals.

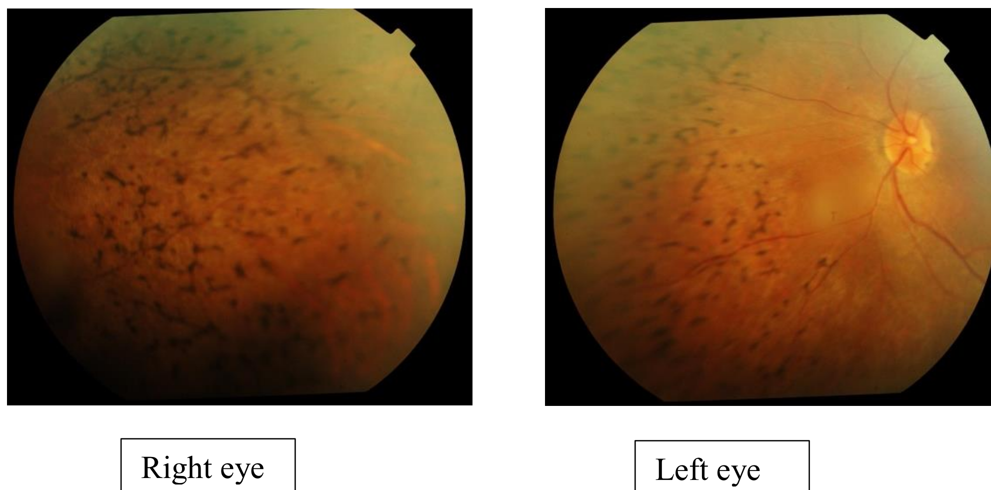


Figure 3.9: Fundoscopic examination of affected member (III-6) of Family A. Both eyes of the proband show scattered pigment clumping in the peripheral retina.

3.3 Genetic Analysis of LCA Families

Available members of all LCA families were anaylzed mainly by genome wide genotyping, homozygosity mapping and whole exome sequencing (WES) to identify the causative variants. Six families (Family A, D-H) were first analysed by genome wide genotyping and homozygosity mapping while two affected member each from two families (Family B and C) were directly subjected to whole exome sequencing. All available members of these families except Family F (5 instead of 6) underwent SNP-based genotyping to find out the homozygous regions. None of the individuals of Family D and H was selected for exome sequencing as mentioned in table 3.1.

3.3.1 Family A

3.3.1.1 SNP based Homozygosity Mapping

Genotype data available from the seven members of family A, including 3 affected individuals, was initially analysed by HomozygosityMapper. Homozygosity mapping analysis detected three regions on three different chromosomes (Figure 3.10). The first bar on chromosome 2 showed a 8.31 Mb region, (Genomic coordinates chr2:224,232,297-232,550,626; GRCh38/hg38), second region of 28.5 Mb was detected on chromosome 14 (hg38:chr14:69,198,531-97,781,834) and the last bar was detected on chromosome 19 (hg38:chr19:57,933,570-59,094,136), but this region has low homozygosity score. As this family has multiple homozygous regions which have large number of genes (some even known to cause RD), therefore we decided to proceed for exome sequencing rather than sanger sequencing of the candidate genes.

3.3.1.2 Exome Sequencing of Family A

Single affected member (III-6) of Family A was subjected to exome sequencing. Further analysis and filtering of variants listed in the VCF, as explained in methods, could not identify any potential variant relevant to the phenotype of this family. The potential pathogenic variants present within three mapped homozygous genomic regions were also checked but none of the short-listed variant was considered as causal variant for this family.

3.3.1.3 Re-analysis of Genotype and Exome Data

After failure to detect potential variant after exome sequencing of individual III-6 of family, the available genotype and exome data was reanalysed to explore different possibilities. Reanalysis of homozygosity mapping data especially the genotype tables of three homozygous regions were checked which revealed the presence of no calls within the homozygous region mapped on chromosome 14. Interestingly three consecutive SNPs (rs865285, rs2295135 and rs845757) were not called in three affected individuals, but they were called in healthy members of this family. This led to the assumption of a large deletion within homozygous region on chromosome 14 that contains a known RD gene i.e. *SPATA7*. The deleted SNPs were flanked by rs7161612 and rs2274736 markers and spans a region of 113,363 bp (Figure 3.11).

Later, the deletion was also confirmed by viewing the BAM file of individual III-6 with Integrative Genomics Viewer (IGV 2.14.1; Broad Institute). This analysis showed the absence of reads from *SPATA7* gene in this affected individual. Therefore, genotype data of 7 members and exome data of individual III-6 confirms the involvement of a large deletion that also involves SPATA7 gene. However, the exact breakpoints of this deletion could not be identified from the BAM file due to noncoverage of deep intronic region.

3.3.1.4 Deletion Mapping

The breakpoint of large deletion was determined by designing primers from the regions between deleted and intact SNPs on each end (Intact SNP rs7161612; Deleted SNP rs865285 /Deleted SNP rs845757; Intact SNP rs2274736). Initially, fifteen sets of primers were designed and amplified in one affected and one normal individual of Family A. For the primer sets from the regions before the breakpoints, the regions were amplified for both members. But after crossing the breakpoints, only the normal member was amplified. Further primer sets were designed within these regions to reach out the exact breakpoint location (Figure 3.12).

Once the region was narrowed down, flanking primers were used to amplify the product which was expected to be amplified in the affected individual and not in the normal individual. But in actual the product was amplified in both the individuals showing that the normal individual must be heterozygous in this case (Figure 3.12). Primer set 12bF,

15dR gave the amplified product with the smallest size and both the primers were concluded to be the closest to the original breakpoints (Figure 3.12). The intact SNPs were covering a region of 113,163 bp (Figure 3.11), but Sanger sequencing of product amplified from affected individuals confirmed the exact chromosomal position of the deletion (Chr14:88855277-88937126) which covers a region of 81,849 bp. The identified deletion region spans a RD gene *SPATA7,* therefore deletion of this gene in affected individuals was confirmed by using primers specific for exon 5. The exon 5 was amplified only from normal individuals which confirmed the deletion of *SPATA7* gene due to the deletion.

3.3.1.5 Expression at RNA level

The effect of deletion on the expression of *SPATA7* gene was further explored to verify that deletion completely removes this gene. Fresh blood samples were collected from two affected individuals (III-3 and III-6) and their mother (II-2) in the Tempus RNA tubes for RNA isolation and subsequent cDNA synthesis. *SPATA7* transcripts were detected in the cDNA samples of earlier mentioned individuals and a healthy control by using primers from exon 8 and 12. The agarose gel clearly shows the absence of SPATA7 specific band in both the affected individuals (III-3 and III-6) of family A (Figure 3.13). However, SPATA7 specific bands are detected in the carrier mother and a healthy control. But band observed in the mother of two affected individuals was faint compared to the healthy control individual used in this study (Figure 3.13). The faint band of the mother is probably due to the heterozygous status of the identified deletion which results in less expression from the single intact copy of *SPATA7* gene.

3.3.2 Family B

Family B was directly subjected to exome sequencing with two individuals V-3 and V-5 as probands. Exome analysis resulted in the identification of a novel variant in exon 7 of *CRB1* gene. A non-sense mutation at genomic location, chr1:197396879 resulted in T to A transition with reference to cDNA position c.2424 and resulted in protein change that inserted a stop codon in place of Tyrosine at 808 position. The segregation analysis of variant within Family B was validated through Sanger sequencing as shown in figure 3.14.

3.3.3 Family C

Family C was directly analysed through exome sequencing using two individuals, V-6 and VI-4 as probands. WES analysis resulted in few variants after filtration process, but a single variant strongly associated with LCA was found in the affected member of this family. The variant involves a 2-bp deletion c.1550_1551delGA in exon 8 of *LCA5* gene. This frame-shift deletion resulted in the protein change by replacing Arginine into Leucine at 517 with early termination just after the addition of three amino acids. The LCA5 variant segregates in family C (Figure 3.15).

3.3.4 Family D & E

3.3.4.1 Homozygosity Mapping of Family D & E

Family D with all the available individuals was subjected to genome wide genotyping and data was analysed to detect homozygous regions shared by affected members of the family. Homozygosity mapping identified multiple regions on two chromosomes that is; 2 and 11. Apparently there were two regions as shown in figure 3.16 but further analysis of genotype tables showed that all detected homozygous regions were actually very small and does not display continuous stretches of homozygosity. Additionally, none of the identified homozygous region has any known RD candidate gene.

Homozygosity mapping in family E detected two genomic regions, one on chromosomes 16 and other on chromosome 17 (Figure 3.17). The genomic coordinates of both homozygous regions are chr16:13,140,622-17,025,608 and chr17:70,317,514-78,923,953 which correspond to regions of 3.88 Mb and 8.6 Mb, respectively. These genomic regions were carefully explored in UCSC genome browser to identify any known RD gene, but could not find any significant candidate.

3.3.4.2 Whole Exome Sequencing of Family E

Single individual (IV-2) from family E was subjected to WES and the exome data was analysed to identify the potentially pathogenic variant. The variant analysis results in the shortlisting of a strong variant in exon 3 of *AIPL1* gene (NM_014336). The variant is present at genomic location; chr17:6331638 but lies outside the homozygous region mapped in this family. The variant found in family E is a missense mutation with cDNA change that is; c.465G>T and a protein change of p.Glu155His. AIPL1 variant segregates in the members of family E (Figure 3.18). This variant was found in gnomAD

with MAF of 0.00002491 and was predicted to be pathogenic by most of the pathogenic tools in in silico analysis.

3.3.4.3 Sanger Sequencing in Family D

Both families (Family D and E) reside in the same village and this prompted us to test the involvement of AIPL1 variant (identified in family E) in the affected individuals of family D. Sanger Sequencing confirms the segregation of c.465G>T variant in the affected members of family D (Figure 3.19). This sequencing result confirmed the presence of same missense variant in exon 3 of *AIPL1* gene in affected members of both families (Family D and E; Figure 3.18-3.19).

3.3.5 Family F

3.3.5.1 Homozygosity mapping of Family F

For family F, five individuals including three affected (IV-1, IV-2 & IV-4) individuals were initially subjected to genome-wide genotyping. Homozygosity mapper generated four regions (Figure 3.20); one on chromosome 1 (hg38; chr1:6,317,963-16,352,937) having a size of 10.03 Mb and containing a long list of known genes. Additionally, two peaks were detected on chromosome 12 (hg38; chr12: 90,081,188-93,806,877 & chr12: 114,868,138-117,153,385) covering 3.72 Mb and 2.28 Mb regions, respectively. Another peak was seen on chromosome 19 (hg38; chr19:50,796,905-51,584,196) which covers a region of 0.78 Mb. As this family has multiple homozygous genomic regions (Figure 3.20), therefore single affected member from this family was subject for WES.

3.3.5.2 Whole Exome Sequencing of Family F

WES of affected member (IV-4) resulted in the identification of a known variant in *NMNAT1* gene. The variant is located at chromosomal location chr1:10032156 and is present within the homozygous region mapped in this family. The variant was found to produce a cDNA change of G to A at position 25 and replaces amino acid Valine to Methionine at position 9. This missense mutation (c.25G>A; p.Val9Met) was neither found in gnomAD nor any other databases. The *NMNAT1* missense mutation (c.25G>A; p.Val9Met) segregates in the additional members of this family (Figure 3.21).

3.3.6 Family G

3.3.6.1 Homozygosity mapping of Family G

Family G consisted of six individuals, including three affected individuals (IV-4, IV-5 & IV-6) were subjected to genome-wide genotyping. Homozygosity mapper found three peaks of homozygosity; one on chromosome 1 (hg38: chr1: 191798964-202146014) covering a region of 10.3 Mb and two on chromosome 16 (chr16: 28826049-49688918) covering 20.8 Mb region (Figure 3.22). The homozygous regions identified in this family also have multiple RD related genes, therefore it was decided to perform WES in this family to identify the underlying mutation.

3.3.6.2 Whole Exome Sequencing of Family G

The affected member (IV-5) of family G underwent WES and data analysis identified a pathogenic variant in *CRB1* gene. The variant is present at chromosomal location chr1:197297588 and lies within the homozygous region mapped in this family. The variant produced a cDNA change at position 107 from C to G and therefore produces a protein change by replacing Serine at position 36 to termination codon resulting in a nonsense mutation. Sanger sequencing confirmed the segregation of above-mentioned *CRB1* nonsense mutation in this family as shown in the figure 3.23.

3.3.7 Family H

3.3.7.1 Homozygosity mapping of Family H

Family H has five individuals with two affected individuals (III-1 & III-3) which were analysed through SNP-based genome wide genotyping. The homozygosity mapping analysis found multiple peaks of homozygosity on different chromosomes including 2, 6, 7, 10 & 17 (Figure 3.24). These regions were analysed for the presence of known RD related genes, but no significant gene was noticed. One affected member of this family was subjected for WES, but it failed. Therefore, at the moment the underlying mutation in this family is unknown.

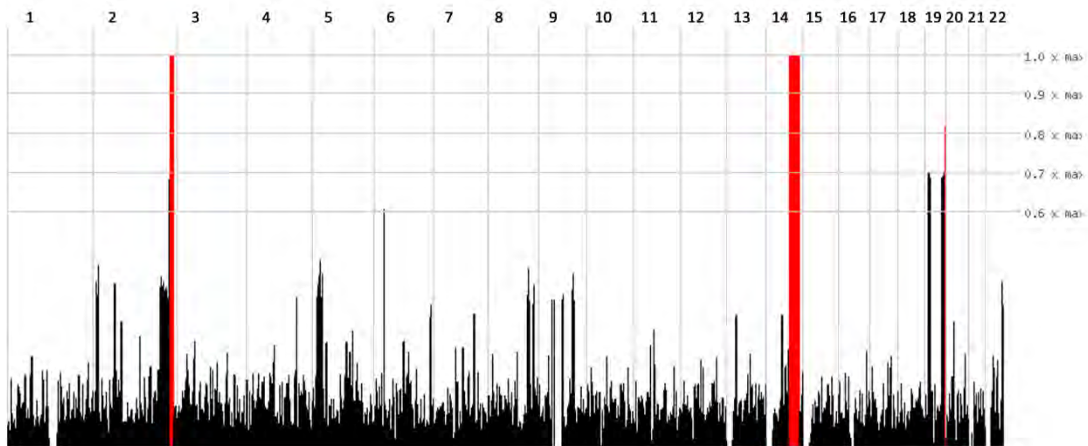


Figure 3.10: Homozygosity mapping analysis of Family A. Red bars represent detected homozygous regions.

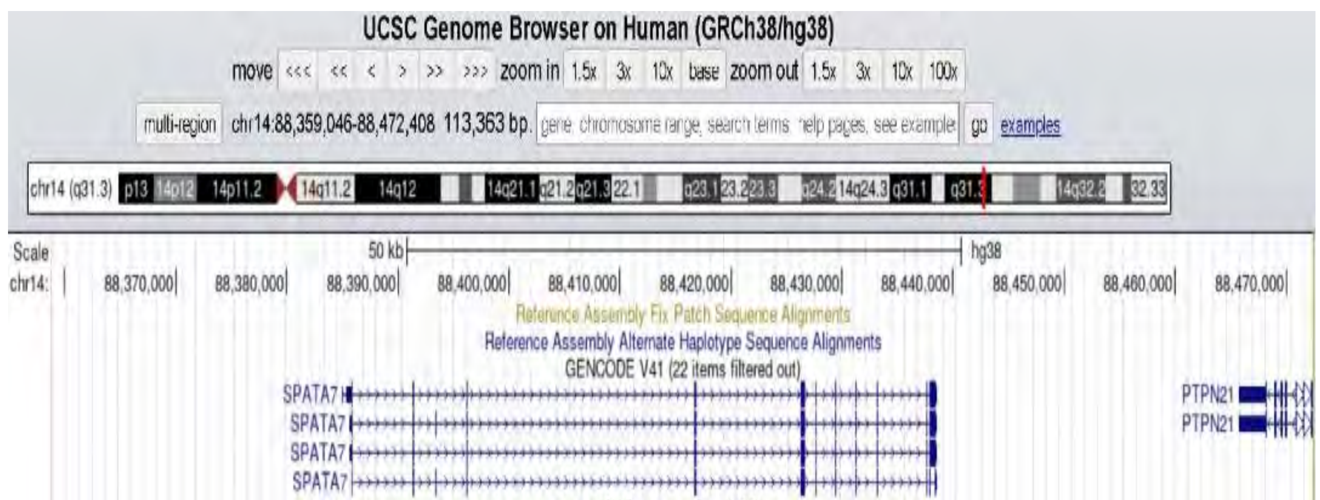


Figure 3.11: Genomic region between rs7161612 and rs2274736 markers which flank the three deleted SNPs detected in affected members of family A. The region contains all exons of SPATA7 gene and some exons of PTPN21 gene.

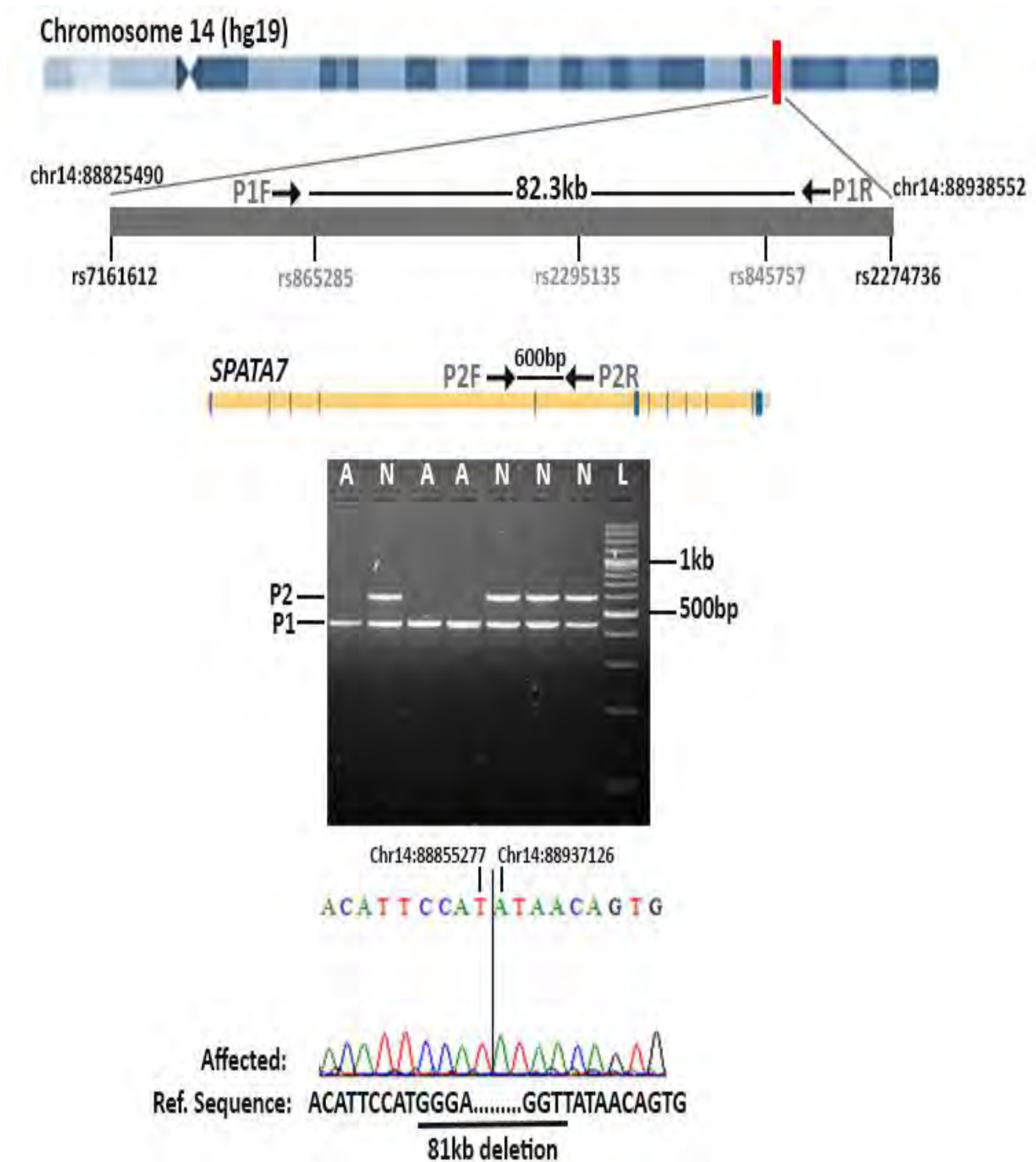
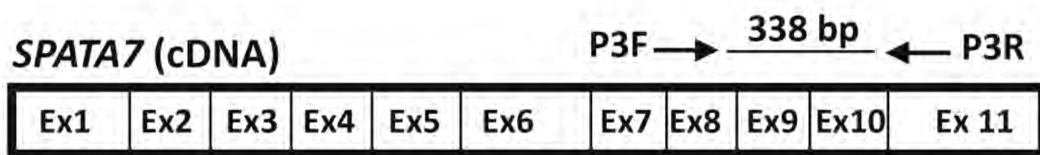


Figure 3.12: Schematic representation of strategy used for deletion mapping. Red line on ideogram shows the cytogenetic position of homozygous region mapped on chromosome 14. Deleted SNPs are shown in grey whereas intact flanking SNPs are shown as bold. P1 and P2 primer approximate location are also shown to which correspond with size of PCR products resolved in agarose gel. The short P1 product was only present in affected members due to the presence of 82.3Kb deletion. P1 product obtained from affected individual was sequenced to identify the exact deletion breakpoints and are shown in sequence chromatogram.



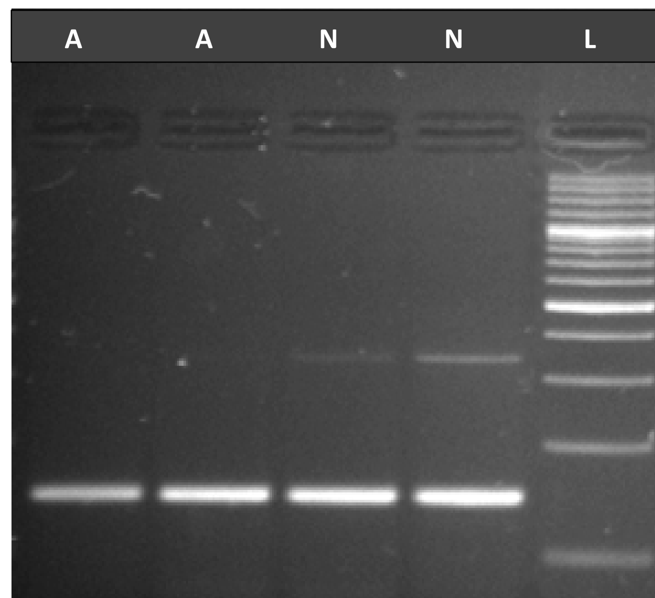


Figure 3.13: Schematic representation of SPATA7 cDNA shows position of primers (P3) used for the detection of SPATA7 transcripts in the blood samples of affected individuals of family A. The upper band specific for *SPATA7* is present in the carrier parent as well as healthy control individual but was absent in both affected individuals (III-3; III-6) of family A. *GAPDH* is used as positive control (lower bands) along with ladder.

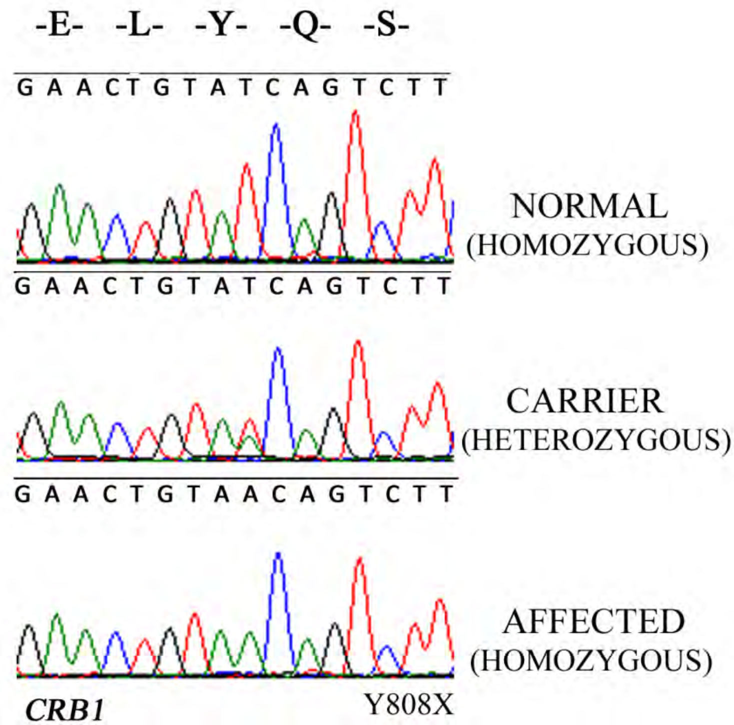


Figure 3.14: Segregation analysis of Family B

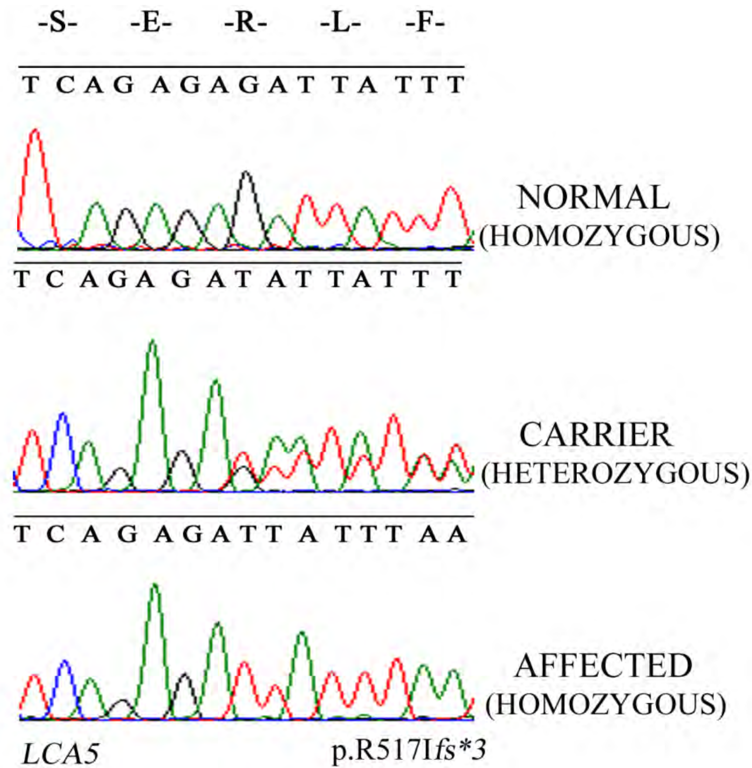


Figure 3.15: Segregation analysis of Family C

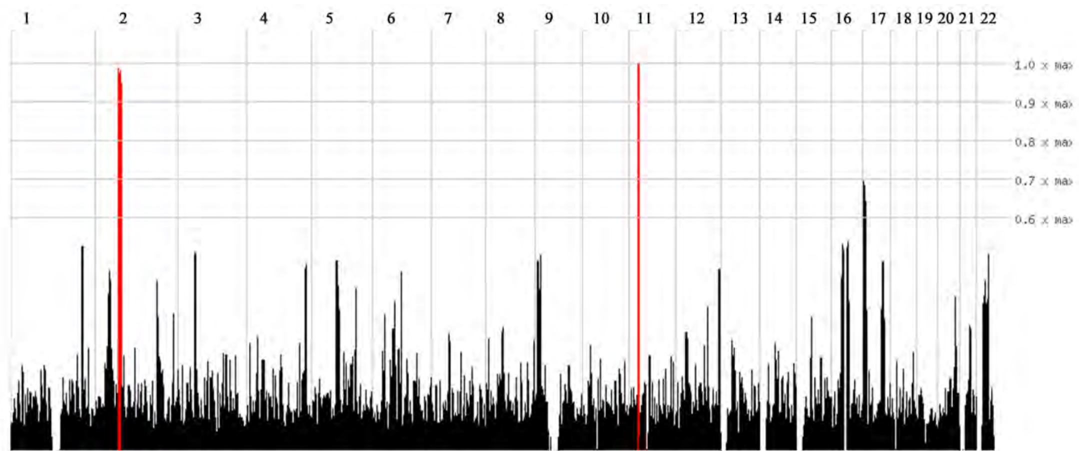


Figure 3.16: Homozygosity mapping analysis of Family D

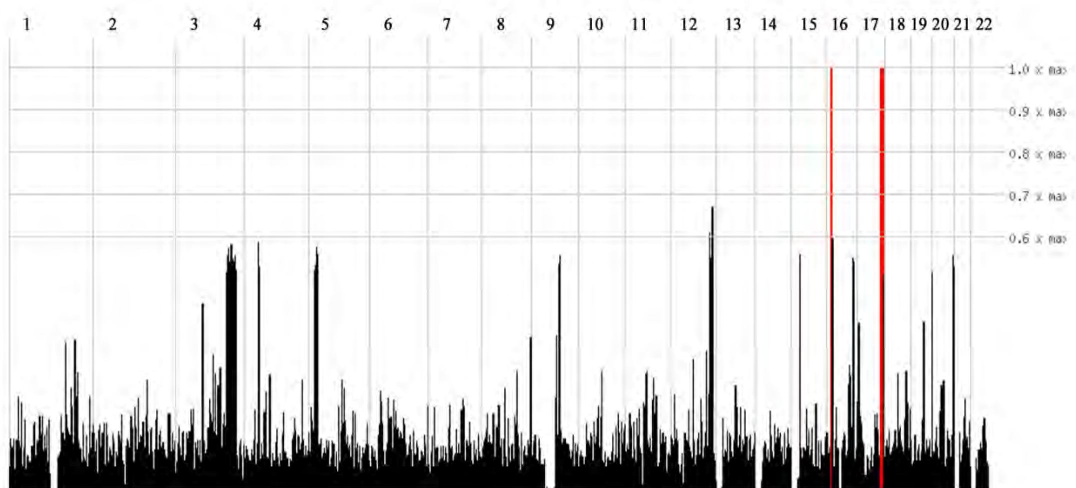


Figure 3.17: Homozygosity mapping analysis of Family E

-L- -L- -Q- ——intron——

C T G C T G C A G G T G G G G

NORMAL (HOMOZYGOUS)

C T G C T G C A T G T G G G G

CARRIER (HETEROZYGOUS)

C T G C T G C A T G T G G G G

AFFECTED (HOMOZYGOUS)

AIPL1 p.Q155H

Figure 3.18: Segregation analysis of Family E

-L- -L- -Q- ——intron——

C T G C T G C A G G T G G G G

NORMAL (HOMOZYGOUS)

C T G C T G C A T G T G G G G

CARRIER (HETROZYGOUS)

C T G C T G C A T G T G G G G

AFFECTED (HOMOZYGOUS)

AIPL1 p.Q155H

Figure 3.19: Segregation analysis of Family D

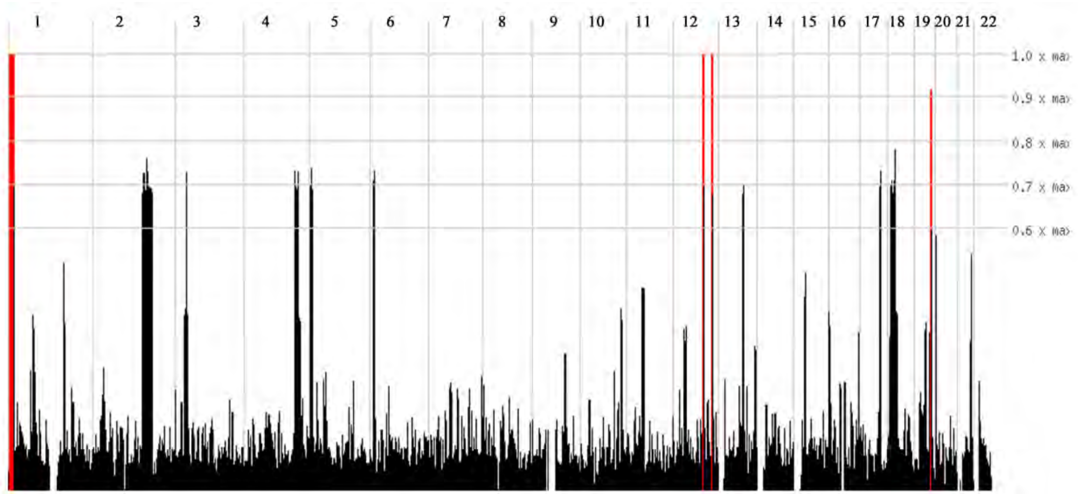


Figure 3.20: Homozygosity mapping analysis of Family F

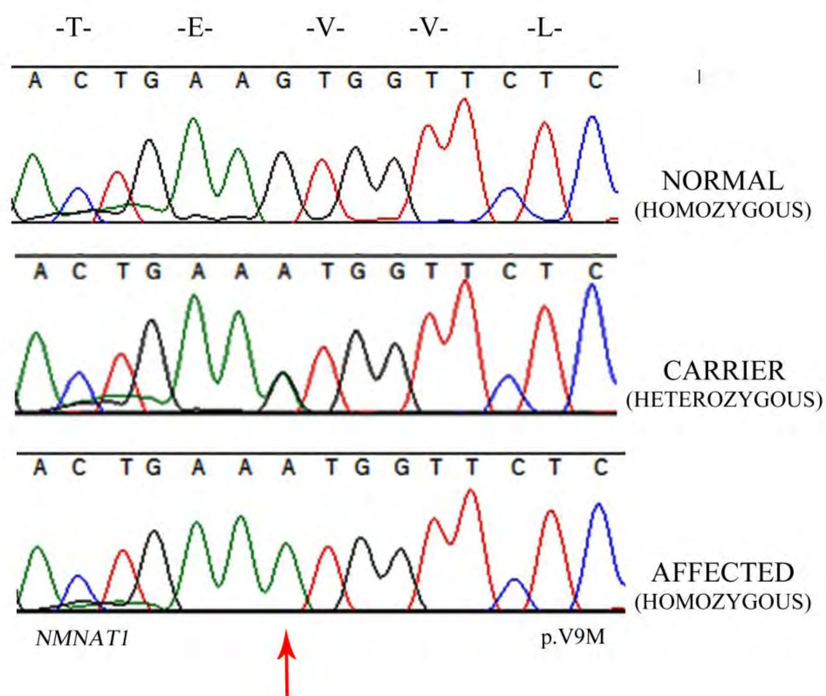


Figure 3.21: Segregation analysis of Family F

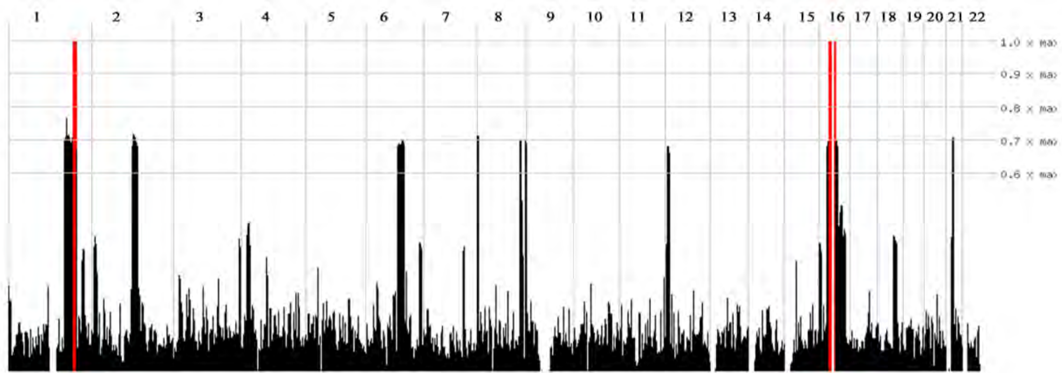


Figure 3.22: Homozygosity mapping analysis of Family G

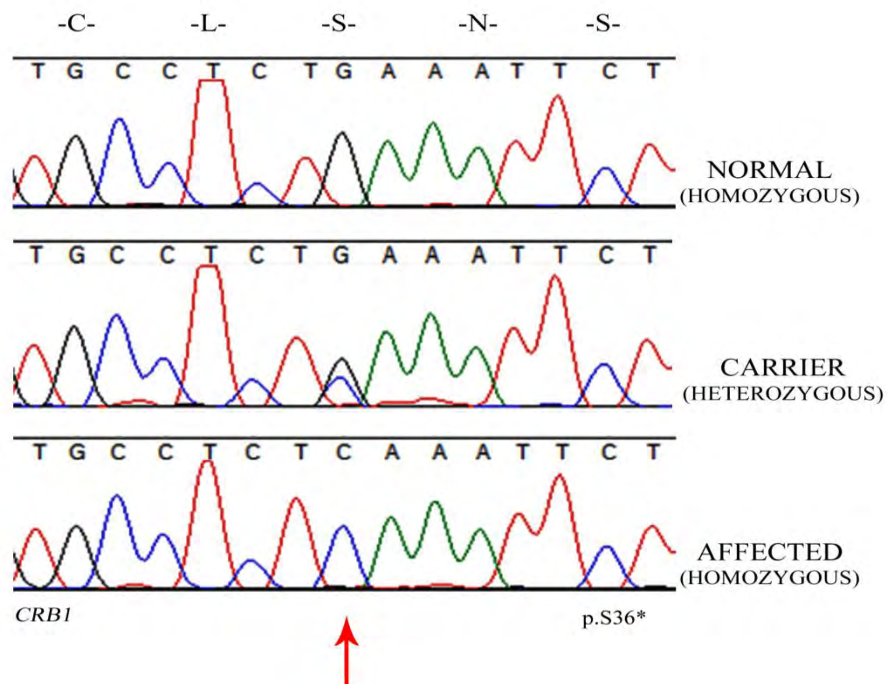


Figure 3.23: Segregation analysis of Family G

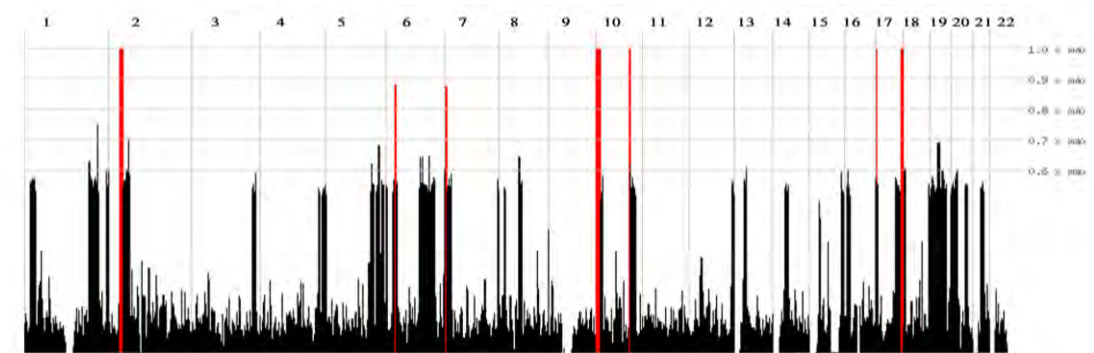


Figure 3.24: Homozygosity mapping analysis of Family H

3.3 Families with RP

The second category comprises of four RP Families: Pedigrees of family I to L are shown in figures 3.25 to 3.28. The clinical description of RP families is described below;

3.3.1 Family I

Family I comprised of five members with two affected (IV-1 & IV-2) individuals in the fourth generation (Figure 3.25). There was only one male (IV-2) and three female members (III-1, IV-1, IV-3 & IV-4) were available for participation in this study. Bilateral cataract was present in the affected individuals of this family, but it was also noted that surgical removal of the cataract in affected individual IV-1 did not improved the vision indicating that vision loss was not primarily due to the cataract. Other features observed in the affected members of this family include photophobia, and nystagmus.

3.3.3 Family J

Family J contains four members in the same generation including three affected (III-2, III-3 and III-4) and one normal (III-1) (Figure 3.26). These three affected individuals are born to parents II-1 and II-2 and despite our repeated attempts healthy members of the family could not explain exact relationship between II-1 and II-2. However, both parents belong to the same ethnic group. There are two affected males and one affected female in this family. Affected individuals of this family initially experienced problems in night vision around the age of 6-7 years. Later they experienced loss of day vision and become legally blind at the age of 15 years. None of the affected individual has additional extra ocular feature indicating the involvement of RP in this family.

3.3.3 Family K

There are seven individuals in family K (Family 3.27). The pedigree depicts a non-consanguineous family but the members of the family were sure of cousin marriage nevertheless their explanation of the fact was not clear. Out of these seven, three males (II-1, II-2 and III-5) were found affected and other four (II-3, II-4, II-5 & III-6) were normal. The clinical presentation of the affected individuals of this family is very much like the affected members of family J, therefore included in the RP group.

3.3.4 Family L

Family L contains three affected male members (III-1, III-2 and III-6) and four (II-2, III-3, III-4 & III-7) normal members (Figure 3.28). These three male affected individuals are born to parents II-1 and II-2 and healthy members of this family could not explain the exact relationship between II-1 and II-2. However, both parents belong to the same ethnic group and are remotely related to each other. The affected members of this family also experienced blindness in first decade of life. The loss of day vision was noticed after 10 years until the elder individuals become legally blind. These affected individuals lack any additional extra ocular defect.

3.4 Genetic Analysis of RP Families

Two RP families (Family I & J) were anaylzed by genome wide genotyping, Homozygosity mapping and WES. DNA samples from all available members of both families underwent genome wide genotyping but WES was performed on the probands. Two individuals of family K were directly subjected to WES. None of the individual of family L was selected for exome sequencing.

3.4.1 Family I

3.4.1.1 Homozygosity Mapping of Family I

Family I was subjected to SNP-based genotyping followed by homozygosity mapping which identified multiple peaks of homozygous regions. Two regions on each chromosome 2 & 3, one on chromosome 8, 16 and 19 were present in this family (Figure 3.29). These genomic regions were quite large and have few known RD genes, therefore one affected member of this family was subjected for exome sequencing.

3.4.1.2 Whole Exome Sequencing of Family I

Whole exome sequencing of individual IV-1 of family I identified a potential variant in *CCDC66* gene at chromosomal location chr3:56605209. The variant alters the cDNA (change A to T) at position 713 resulting in a protein change at 238 position from Aspartate to Valine. This variant (c.713A>T; p.Asp238Val) has a very low MAF of 0.0002728 in gnomAD but was absent in other databases. The variant was also found to be pathogenic by variety of pathogenicity predicting tools. As CCDC66 is not a known RD gene, therefore exome data of this individual was analysed multiple times, but these analyses could not come up with any potential variant related to the phenotype

of this family. The involvement of compound heterozygous variants was also ruled out during these analyses.

The missense mutation (c.713A>T; p.Asp238Val) identified in *CCDC66* segregates within the family (Figure 3.30) which support the identification of a novel RP gene in this family.

3.4.2 Family J

3.4.2.1 Homozygosity mapping of Family J

Homozygosity mapping performed on family J identified two peaks at the same chromosome i.e; chromosome 14. These two peaks were present in genomic regions with coordinates; (hg38: chr14:70,157,548-71,806,727) and (hg38: chr14:81,882,643-89,577,749) (Figure 3.31). This first region was covering a distance of 1.64 Mb, while the second region covers 7.69 Mb region. Both genomic regions were analysed in the UCSC Genome Browser (GRCh38/hg38) for the presence of known RD/RP genes, but none was considered relevant in the smaller region. However, the larger genomic region has *TTC8* gene which is a known RD gene. Considering the mapping of multiple genomic regions one affected member from this family was subjected for WES.

3.4.2.2 Whole Exome Sequencing of Family J

Whole exome sequencing of affected individual III-3 identified a known splice variant in TTC8 gene. The variant (c.115-2A>G) probably alters the splicing pattern and was not found in any of the databases. The segregation analysis confirms the variant segregation in the family (Figure 3.32).

3.4.3 Family K

3.4.3.1 Whole Exome Sequencing of Family K

Two affected members (II-2 & III-5) of family K were directly subjected to WES but data analysis could not identify a potential variant on the autosomal chromosomes. However, analysis of variant present on the X chromosome result in the short listing of a potential variant in in *TENM1* gene. The variant (c.5155C>T) was found at chromosomal location chrX:123539096 and produces a change C to T change at c.5155 from which results change of amino acid Arginine to Tryptophan at position 1719 (p.Arg1719Trp). It was also absent in the databases and declared pathogenic by various

pathogenicity tools. The variant also segregates with RP phenotype in this family (Figure 3.33).

3.4.4 Family L

3.4.4.1 Homozygosity mapping of Family L

Seven members of family L were subjected to genome-wide genotyping and the analysis of homozygous regions resulted in the detection of a region each on chromosomes 3 and 12 (Figure 3.34). These regions were not showing any potential gene that is related to eye disorders. This family still needs further analysis through next generation sequencing techniques to identify the causative gene/variant.

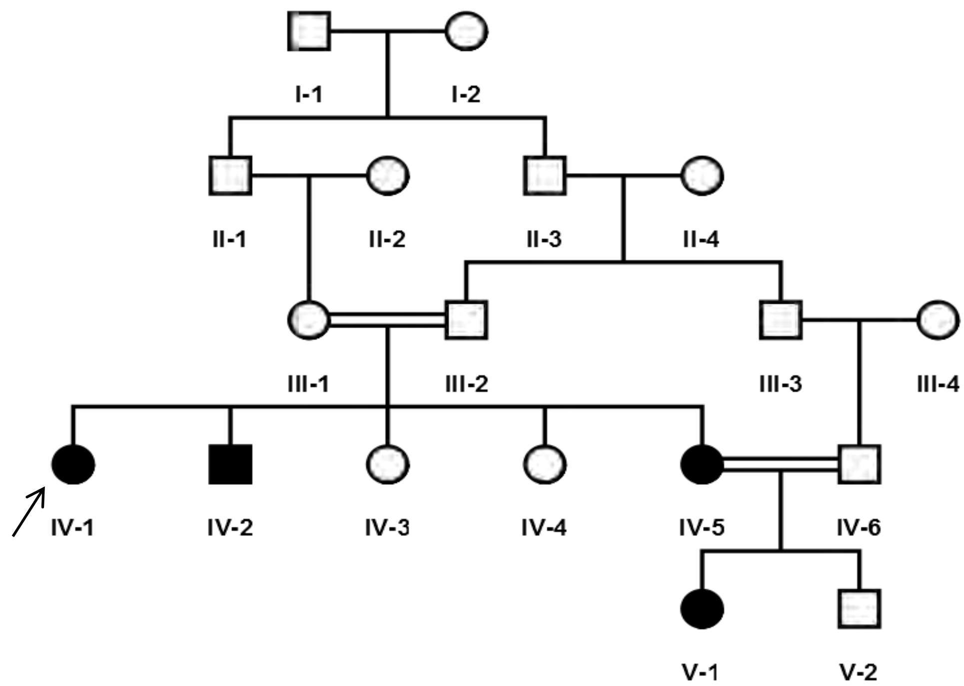


Figure 3.25: Family I show four affected individuals in two loops .

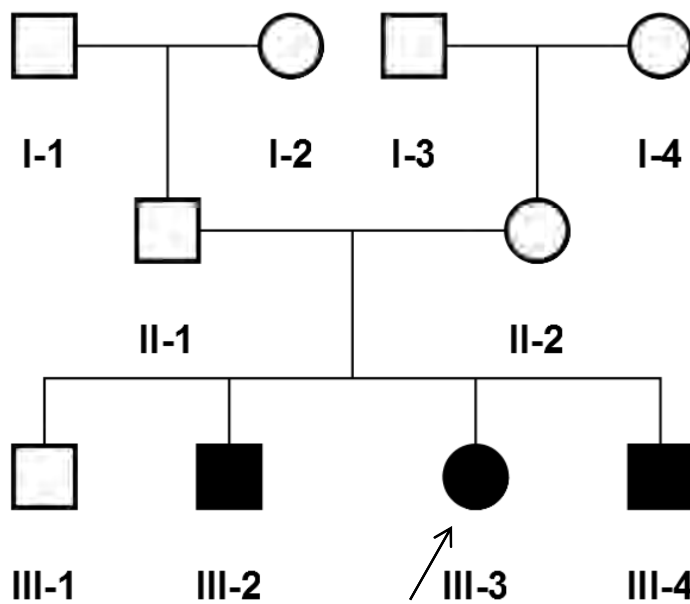


Figure 3.26: Family J showing with three affected individuals in 3rd generation.

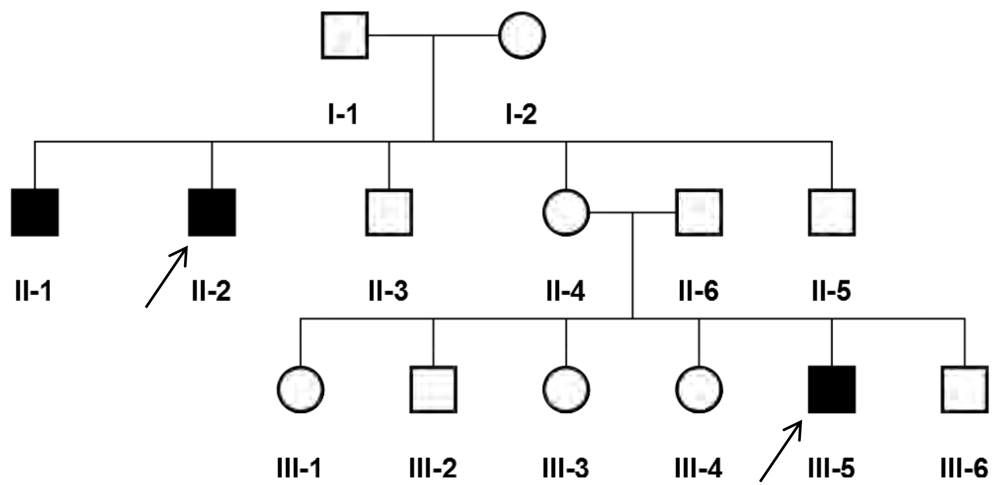


Figure 3.27: Family K showing three affected male individuals in two loops.

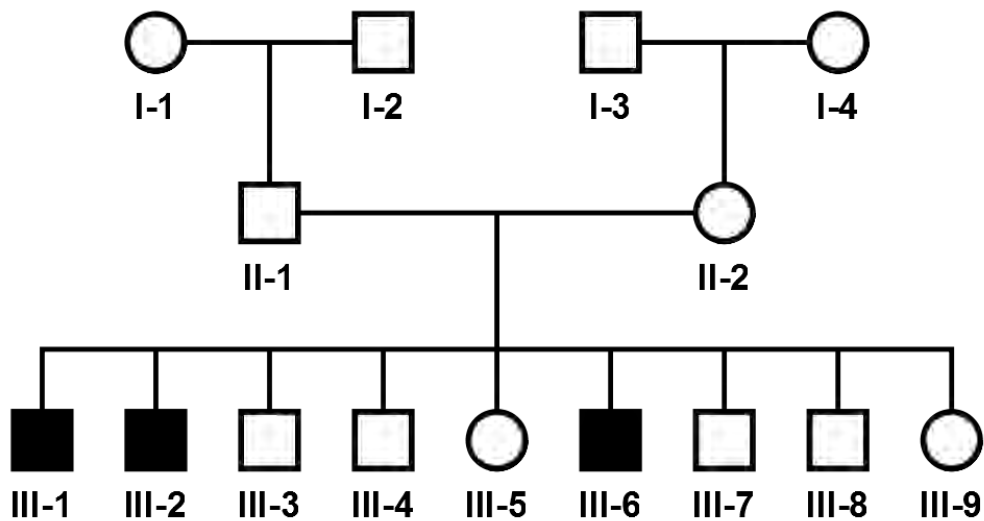


Figure 3.28: Family L with three affected males in the 3rd generation.

Figure 3.29: Homozygosity mapping analysis of Family I

Figure 3.30: Segregation analysis of Family I

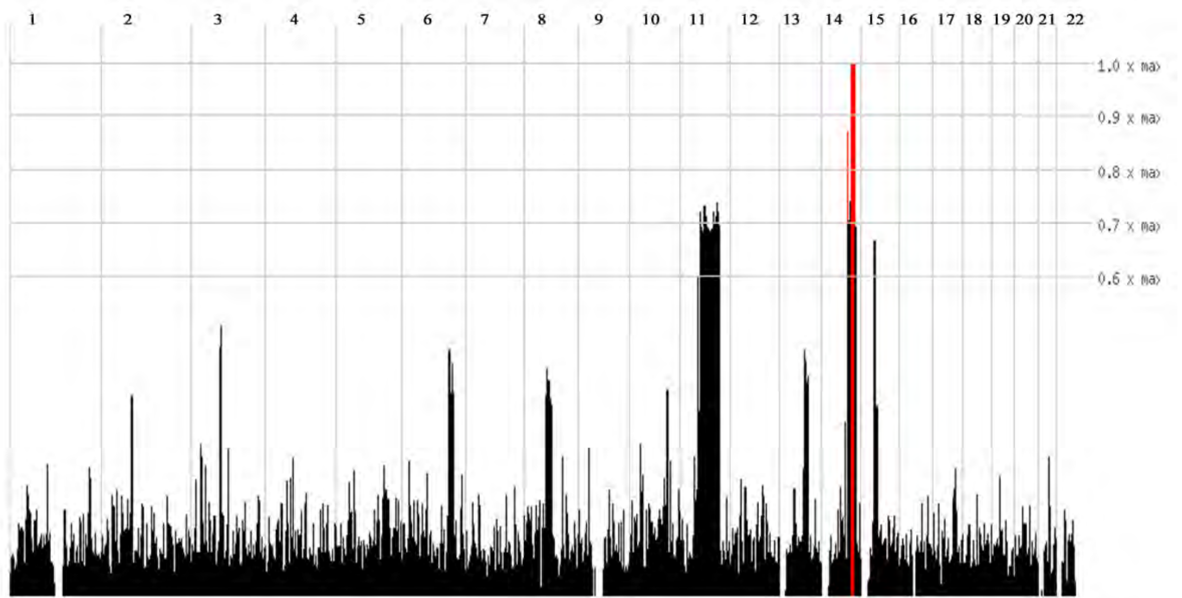


Figure 3.31: Homozygosity mapping analysis of Family J

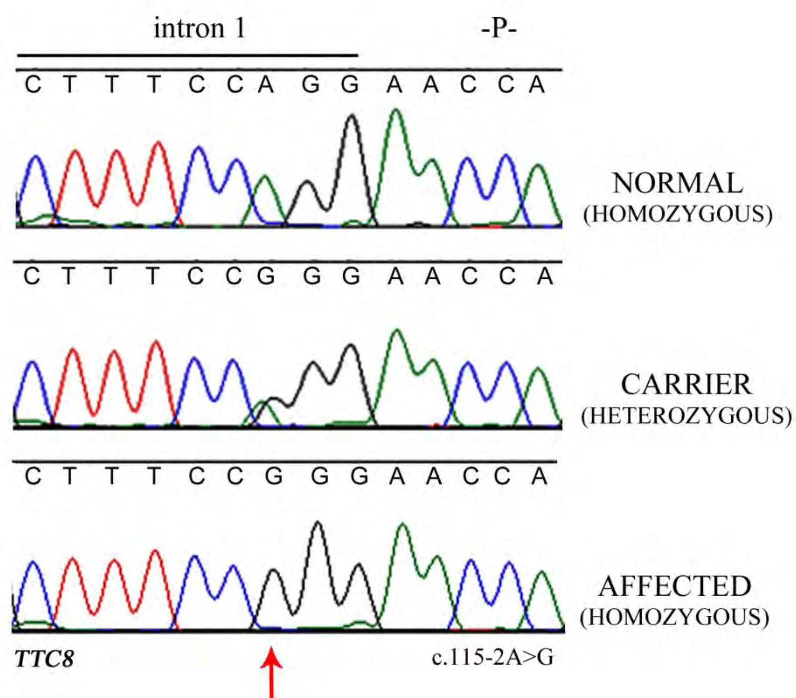


Figure 3.32: Segregation analysis of Family J

Figure 3.33: Segregation analysis of Family K

Figure 3.34: Homozygosity mapping analysis of Family L

3.4 Families with other types of RDs

The third category comprises of three families related to other RDs like Stargardt disease and Bestrophinopathy. Pedigrees of these three families (Family M, N and O) are shown in figures 3.35 to 3.37. The clinical description of these families is as follows;

3.4.1 Family M

Family M comprised if only two members and both were affected (IV-1 & IV-2). These individuals were presenting phenotype of Stargardt disease. Both the affected individuals of this family initially experience night vision loss before years of age. Both individuals were evaluated by an ophthalmologist at the age of 15 and 18 years and these evaluations showed the presence of night blindness, photophobia, reduced central vision, irritation in bright light and difficulty in colour discrimination. Manual fundoscopic analysis of both affected individuals revealed significant macular degeneration which are probable indicative of Stargardt disease in this family.

3.4.2 Family N

Family N comprised of total six members with three affected members (IV-5, IV-6 & IV-7). Affected individuals of this family also showed the presence of photophobia, reduced central vision, epiphora in bright light, and inability to discriminate colours. The clinical presentation of this family bear close similarity with family N, and therefore indicate the diagnosis of Stargardt disease in this family as well.

3.4.3 Family O

Family O consists of four members but three individuals (V-2, V-3 & V-5) are affected. Analysis of medical reports revealed the presence of yellowish lipofuscin deposit and cystoid macular edema in two members of this family. Whereas medical record was not available for the third affected member of this family. The clinical presentation of two affected members of this family supported the diagnosis of Bestrophinopathy.

3.5 Genetic Analysis of Other RD Families

One of the three families (Family M) was directly subjected to whole exome sequencing while other two families (Family N and O) were initially analysed through genome wide genotyping and then subjected to whole exome sequencing. Details of the analysis of these families are illustrated as follows;

3.5.1 Family M

3.5.1.1 Whole Exome Sequencing of Family M

Family M with two members, was directly subjected to whole exome sequencing. WES was performed on affected member IV-1 and exome data analysis resulted in the identification of a strong variant in *ABCA4* gene at chromosomal location chr1:94508316. This variant (c.3328+1G>C) was found to affect splicing of intronic region between exon 22 and 23 and was neither found in gnomAD nor any other database. It was also found pathogenic through different pathogenic tools. The splicing variant also segregates in the family M (Figure 3.38).

3.5.2 Family N

3.5.2.1 Homozygosity mapping of Family N

Family N was subjected to Homozygosity mapping with all the available samples of the family. Homozygosity mapper generated a single region on chromosome 5 (Figure 3.39) with co-ordinates (chr5: 35315860-44901349). This region was analysed on UCSC Genome Browser (hg19) for the presence of any eye RD related gene but could not identify any plausible candidate gene.

3.5.2.2 Whole Exome Sequencing of Family N

Single individual IV-5 of family N was then subjected to whole exome sequencing and analysis of exome data identified a known variant in *ABCA4* gene at chromosomal location chr1:94473278. The variant was not present in the homozygous region mapped on chromosome 5 in family N. This variant (c.5917delG) was a single nucleotide deletion of G at cDNA position c.5917 and resulted in frame-shift mutation resulting in early termination. This variant has MAF of 0.00004985 in gnomAD and was predicted to be disease causing by in silico analysis. The variant also segregates within the whole family (Figure 3.40)

3.5.3 Family O

3.5.3.1 Homozygosity mapping of Family O

Family O was initially checked for homozygous stretches by using Homozygosity mapper and this analysis resulted in the generation of two peaks on chromosome 12 (Figure 3.41). The regions were analysed through UCSC Genome Browser (hg19) for the presence of RD related genes but no potential gene was found.

3.5.3.2 Whole Exome Sequencing of Family O

The affected individual V-5 of family O was subjected to WES and the analysis of exome data resulted in the identification of known variant in *BEST1* gene at chromosomal location chr11:61723252. It is a missense variant (c.310G>C) that causes a cDNA change; c.107G>C and resulted in protein change at position 104 from amino acid Aspartic acid to Histidine (p.Asp104His). It was absent in all the databases and found pathogenic by in silico data analysis. This missense variant (c.310G>C; p.Asp104His) also segregates within the family (Figure 3.42).

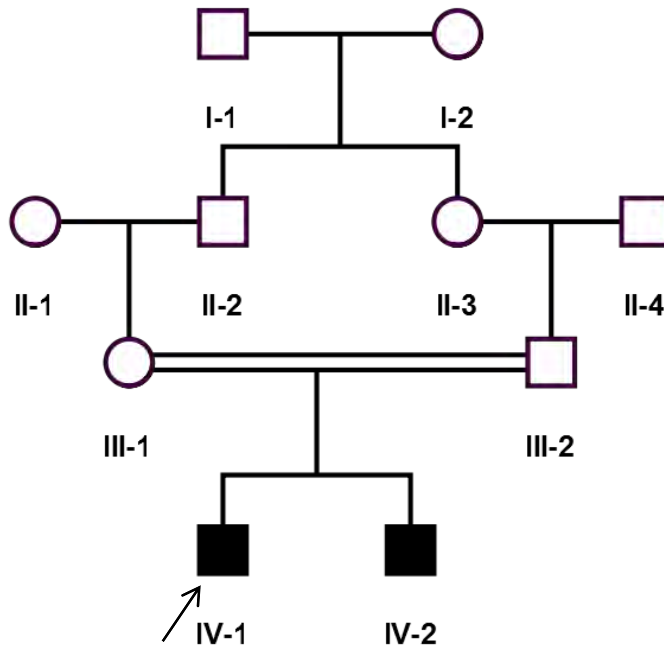


Figure 3.35: Family M with two affected male individuals.

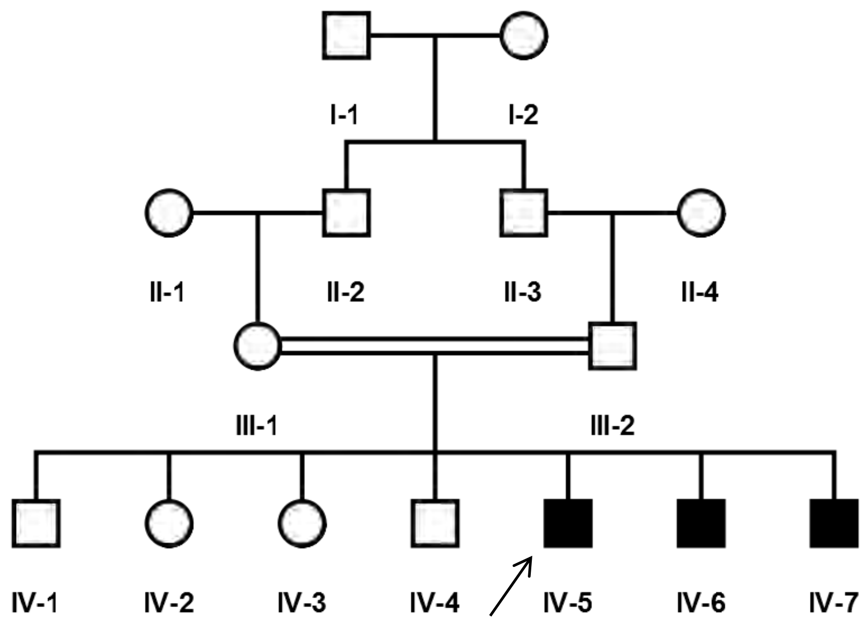


Figure 3.36: Family N pedigree with three affected male individuals.

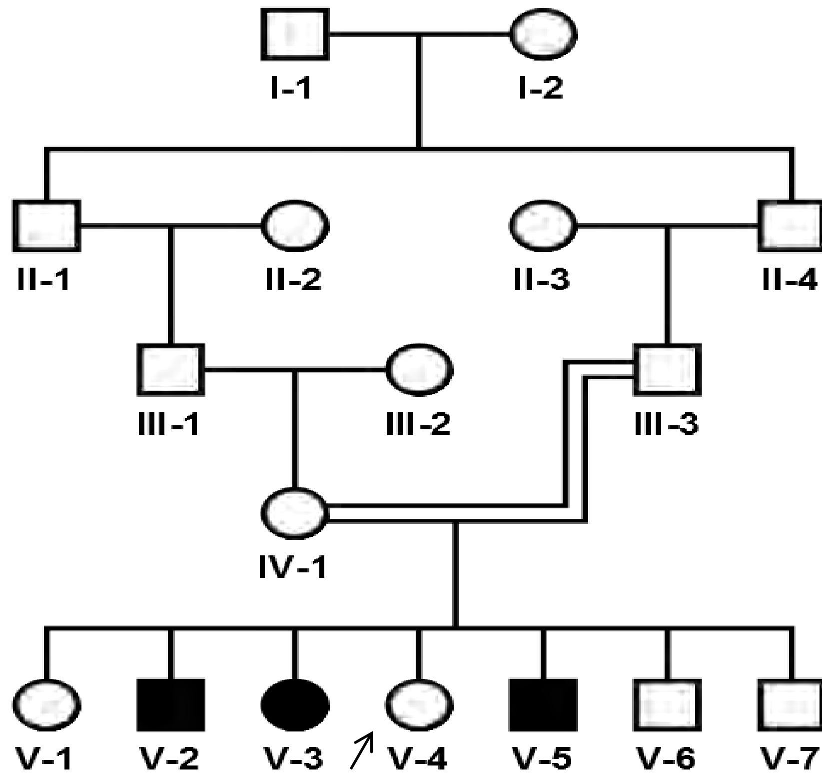


Figure 3.37: Family O pedigree with three affected individuals.

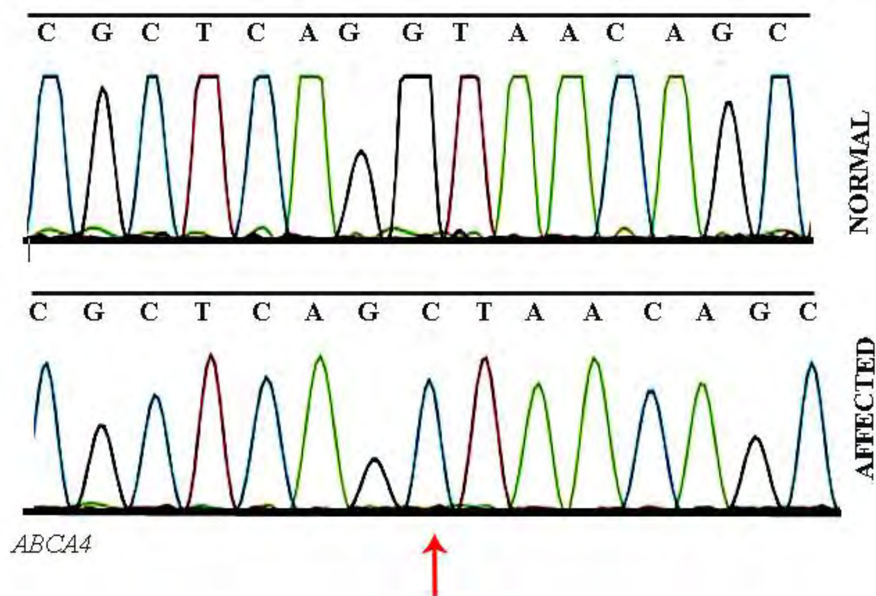


Figure 3.38: Segregation analysis of Family M

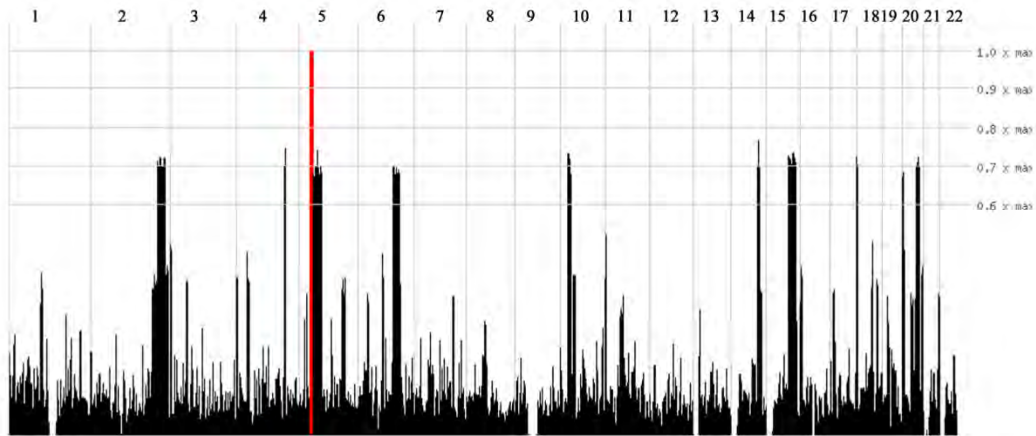


Figure 3.39: Homozygosity mapping analysis of Family N

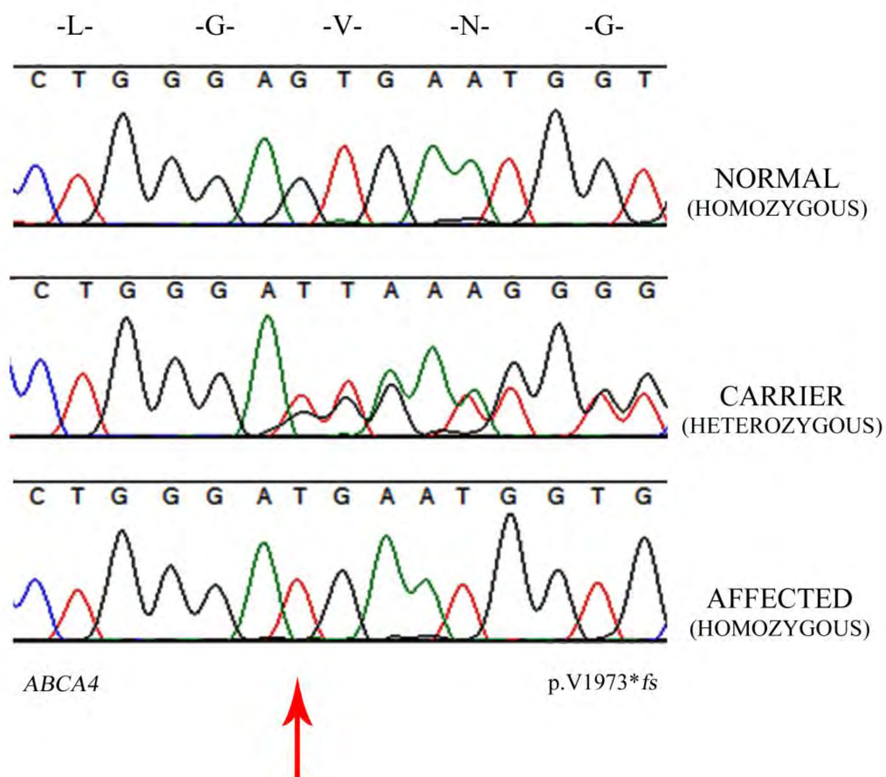


Figure 3.40: Segregation analysis of Family N

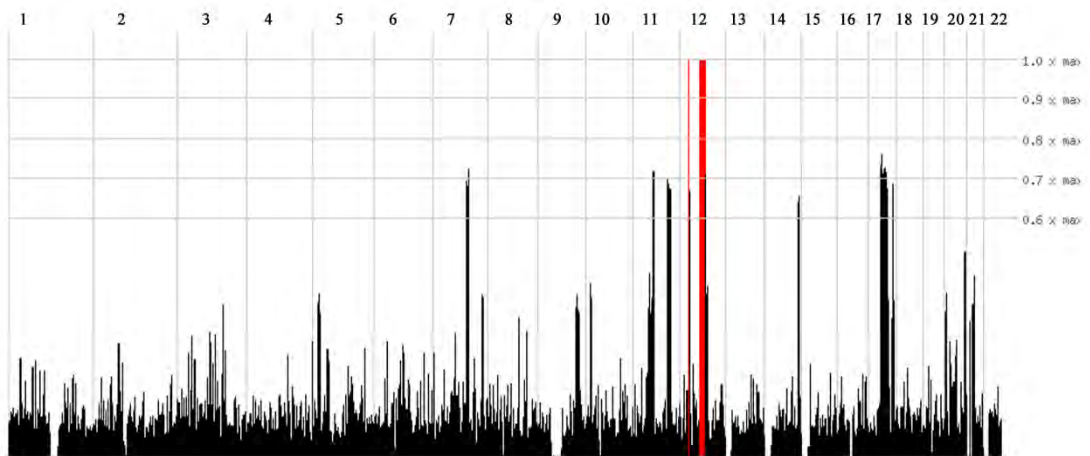


Figure 3.41: Homozygosity mapping analysis of Family O

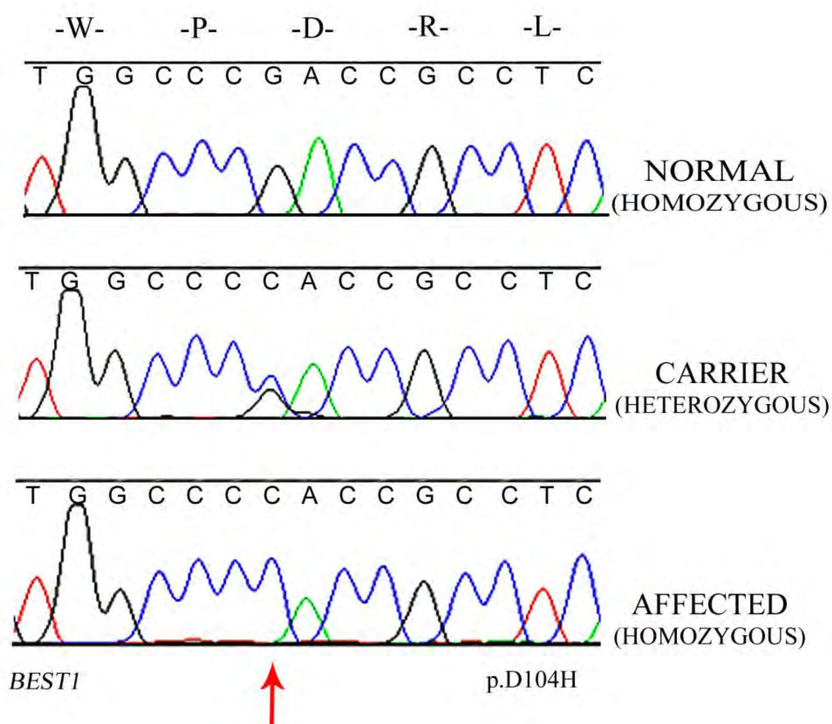


Figure 3.42: Segregation analysis of Family O

3.6 In silico Analysis of Variant Identified in RD Families

The variants identified in 13 families were analysed with mutation taster, Polyphen and SIFT to perform the pathogenicity analysis and the results are summarised in the Table 3.1

Table 3.1: Summary table showing thirteen families solved in this study

Sr.No.	Family ID	Gene	Ref seq	cDNA	Protein	gnomAD	Mutation taster	SIFT	PolyPhen	Known/ Novel
1	**A**	*SPATA7*	NM_018418.5	DELETION	**Deletion of 81849bp on chr.14**					**NOVEL**
2	**B**	*CRB1*	NM_201253	c.2424T>A	p.Tyr808Ter	NF	Disease Causing	Damaging	NA	**NOVEL**
3	**C**	*LCA5*	NM_001122769	c.1550_1551delGA	p.Arg517Ile*fs*3*	NF	Disease Causing	NA	NA	**NOVEL**
4	**D**	*AIPL1*	NM_014336	c.465G>T	p.Glu155His	0.00002491	Disease Causing	Damaging	Probably damaging	KNOWN
5	**E**	*AIPL1*	NM_014336	c.465G>T	p.Glu155His	0.00002491	Disease Causing	Damaging	Probably damaging	KNOWN
6	**F**	*NMNAT1*	NM_022787	c.25G>A	p.Val9Met	NF	Disease Causing	Damaging	Probably damaging	KNOWN
7	**G**	*CRB1*	NM_201253	c.107C>G	p.Ser36Ter	NF	Disease Causing	Damaging	NA	KNOWN
8	**I**	*CCDC66*	NM_001012506	c.713A>T	p.Asp238Val	0.0002728	Disease Causing	NA	Probably damaging	KNOWN
9	**J**	*TTC8*	NM_144596	c.115-2A>G	NA	NF	Disease Causing	NA	NA	KNOWN
10	**K**	*TENM1*	NM_014253	c.5155C>T	p.Arg1719Trp	NF	Disease Causing	NA	NA	KNOWN
11	**M**	*ABCA4*	NM_000350	c.3328+1G>C	NA	NF	Disease Causing	NA	NA	**NOVEL**
12	**N**	*ABCA4*	NM_000350	c.5917delG	p.Val1973Ter*fs*	0.00004985	Disease Causing	NA	NA	KNOWN
13	**O**	*BEST1*	NM_004183	c.310G>C	p.Asp104His	NF	Disease Causing	Damaging	Probably damaging	KNOWN

DISCUSSION

4. DISCUSSION

According to the world health organization (WHO) estimates there are 39 million blind people worldwide which are over 50 years of age. However, there are significant number of young individuals with early onset or childhood blindness but correct estimated of prevalence are not available. Retinal Dystrophies (RDs) are a clinically and genetically heterogeneous group of inherited disorder which affect the vision of large number of individuals worldwide. RDs are the leading cause of vison loss in teens and childhood blindness. RDs are further divided into different types based on clinical presentation of the patients and major types of RD include Leber Congenital Amaurosis (LCA), Retinitis Pigmentosa (RP), Cone-Rod Dystrophy (CRD and Macular Degeneration (MD). The onset, progression and severity of every RD phenotype depends on the nature of gene involved. Despite the great progress on the identification of RD genes, the underlying genetic defect are still unknown in large number of cases/families. Therefore, this study was initiated to recruit families with different RD phenotypes from different regions of Pakistan and perform genetic analysis to identify the pathogenic mutations responsible for early onset phenotypes. The cohort of fifteen families have more LCA families compared to RP and CRD types. This is contrary to the pattern observed in a previous study which showed that arRP is more common in Pakistan compared to LCA (Khan et al., 2014). These authors showed that out of a large cohort of 103 families 59% had arRP whereas 19% have LCA. In our case 53% families (8 out of 15 families) belonged to LCA group and 26% (4 out of 15 families) to RP group. However, this difference is may be due small cohort size of our study, but it can also be due to recruitment bias as in our study preference was given to include those families with early onset vision loss. However, additional large-scale studies will be required to find out the relevant prevalence/contribution of different types of RD in the Pakistani population.

In this study, we identified twelve mutations in thirteen different RD genes. Of these twelve mutations, four were novel and eight were present in the known RD genes. Out of four novel mutations, three were detected in LCA families and one was identified in a family with Stargardt disease.

In family A, a large deletion was detected on chromosome 14 which spans *SPATA7* gene. Interestingly in this family mutation could not be identified by exome sequencing, but reanalysis of genotype data and BAM files of the patient subjected for WES helped

to identify the probable involvement of a large deletion. This finding is significant to solve families for which potential variant could not be detected after exome sequencing. As in case of large deletions, respective genomic regions will not be captured by target/template preparation kits and thus could not called in the reads obtained by exome sequencing. Most of the times variant analysis mainly focus on filtration and evaluation of variants listed in the VCF file but variants from the deleted regions will be absent in this data and therefore can be missed. So, careful analysis of such families is essential before conclusion of unsolved families/cases.

Spermatogenesis associated protein 7 (*SPATA7*) was identified in 2003. In human, this gene spans a genomic region of 52.9 kb and consists of 12 exons. Expression analysis of *SPATA7* gene in the developing and mature retinal layers of mouse suggested its importance in the normal retinal function (Wang et al., 2009). The 81,849 bp deletion detected in the affected members of family A was present in homozygous state and spans *SPATA7* gene. Previously, (Mayer et al., 2015) reported a deletion of *SPATA7* exons 1 to 5 along with 5'UTR and argued that this deletion resulted in the loss of *SPATA7* function and its protein expression. Our results also confirmed the loss of *SPATA7* RNA in the blood samples of two affected individuals homozygous for large deletion. Therefore, it can be concluded that deletion of *SPATA7* gene in affected members of family A causes LCA.

In two families (Family B and C) with LCA, two novel mutations of *CRB1* and *LCA5* were identified, respectively (Table 3.1). The nonsense mutation identified in family B is probably causing an early truncation of *CRB1* protein. This mutation was not found in the databases and was also predicted to be damaging by in silico analysis. Mutations in *CRB1* gene (MIM# 604210) have been reportedly identified by many researchers for families associated with LCA, RP and many other retinal dystrophies (Azam et al., 2011, Vallespin et al., 2007). *CRB1* encodes for a protein crumbs and has its expression in the retinal cells and brain (den Hollander et al., 1999a). The nonsense mutation p.Tyr808Ter is located in second laminin AG-like domain, most likely lead to nonsense-mediated decay. In Family C, a dinucleotide deletion (c.1550_1551 del GA) resulted in a protein change p.Arg517Ile*fs*3*. This variant was also absent in gnomAD. This frame-shift mutation also resulted in introduction of premature stop codon and may lead to nonsense-mediated decay. *LCA5* encodes a protein, Lebercilin that helps in the ciliary function of photoreceptor cells (Den Hollander et al., 2007a). Previous

researches have established that *LCA5* mutations are strongly related to symptoms of early and severe loss of vision along with nystagmus, non-detecable ERGs, abnormal visual acuity and abnormal fundus features showing retinal degeneration (Den Hollander et al., 2007a, Gerber et al., 2007, Ramprasad et al., 2008).

In Family D and E, the LCA phenotypic features were very common and they both belonged to same region of Sindh. The genetic analysis of these families resulted in the identification of same mutation of *AIPL1* gene. This recurrent missense variant (c.465G>T) resulted in protein change of Glutamine residue at position 155 into Histidine. This amino acid residue was found to be highly conserved among the vertebrates. In databases like gnomAD, this variant was found with MAF of 0.00002491 and was also predicted to be disease causing by online pathogenicity prediction tools. Previously, this p.Gln155His variant reported by (Rashid et al., 2020) found to result in the loss of ionic interaction with aspartate residue at position 157, and ultimately resulting in the miss-folding of AIPL1 secondary structure.

In Family F the causative gene found was *NMNAT1.* Another missense mutation was found which resulted in protein change p.Val9Met. This is also a recurrent mutation, frequently found in Pakistani population. (Falk et al., 2012) identified this variant using exome sequencing in a consanguineous Pakistani family. Since *NMNAT1* encodes an enzyme nicotinamide mononucleotide adenylyltransferase involved in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) (Keen et al., 2003). Functional studies of this variant showed a decreased activity of this enzyme.

In Family G, a recurrent mutation of *CRB1* gene was found at cDNA position (c.107C>G) resulting in protein change of Serine residue at position 36 to termination codon. This is a strong variant of *CRB1* gene that is also absent in the gnomAD and other databases and also predicted to be pathogenic by in silico analysis. (McKibbin et al., 2010) identified this variant for the first time in a family from Northern region of Pakistan. According to their study this premature stop codon mutation represents null alleles.

In another Family I, a previously reported variant of *CCDC66* gene was found with cDNA change (c.713A>T) and causes a protein change of Aspartate to Valine at position 238. The MAF of this variant in gnomAD was found to be 0.0002728 and was likely to be pathogenic according to the online pathogenicity prediction tools. A study

has already identified a frameshift mutation in CCDC66 gene in Portuguese water dogs presenting early onset retinal atrophy (Murgiano et al., 2020). Two recent studies have highlighted the role of *CCDC66* gene in cilium assembly (Batman et al., 2022); (Odabasi et al., 2023) and mutations of several genes involved in such processes are already known to cause syndromic and nonsyndromic RDs. Therefore, considering the data from Portuguese dog and role of this gene in cilium assembly, it can be concluded that missense mutation identified in affected members of family I can cause RP. However, additional studies will be required to explore the function of this gene in details.

Family J of this study showed to link RP phenotype with previously reported mutation of *TTC8* gene. This was splice-site variant with cDNA position (c.115-2A>G) and affecting the splicing at the 3' end of the first intron of *TTC8* gene. *TTC8* is a strong candidate of BBS, one of the major hallmarks of which is RP. Previously reported by (Riazuddin et al., 2010), this splice site variant resulted due to a homozygous A to G substitution in intron 1, specifically to the splice acceptor site of exon 2a. this predicted to result in skipping of this exon, causing a 10 amino acid deletion of the specific protein. Moreover, this change segregated with the disease phenotype suggesting it as the causative variant for this family. In Family K, the direct exome sequencing resulted in the identification of an X-linked gene that is; TENM1 that was showing a missense variant at cDNA position (c.5155C>T) and resulting in protein change of Arginine residue at 1719 to Tryptophan.

Family M and N were showing phenotypes of Stargardt disease and for both the families, this study identified mutations in *ABCA4* gene. A novel splice site variant (c.3328+1 G>C) with a very strong pathogenicity score through in silico analysis was identified in Family M. This was also absent in the databases like gnomAD and other frequency databases. A functional index derived from the expression level and ATPase activities of the variants can be used to measure the severity of STGD1 (Molday et al., 2022). The second variant found in Family N was a deletion of single nucleotide (c.5917delG) resulting in protein change of Valine residue at position 1973 to termination codon due to frame shift. This variant was also absent in the databases and was predicted to be pathogenic by in silico data analysis. Nonsense variant found in Family N was previously reported by (Rivera et al., 2000) to be present in homozygous form in one of the affected individual of the cohort under study. Compound

heterozygous mutation pattern is also very much common for the mutation spectrum of *ABCA4* gene for Stargardt disease (Zhou et al., 2014). For autosomal recessive STGD cases, *ABCA4* alone is carrying more than 800 mutations and is most commonly mutated gene (Lee et al., 2016). The frameshift mutation (c.5917delG) previously reported by (Kamenarova et al., 2022) was associated with early disease manifestation and a general photoreceptor dysfunction.

Family O is the last family solved through this study. This family was showing phenotypic resemblance to Bestrophinopathy and was identified with a mutation of *BEST1* gene. A protein change of Aspartate at position 104 into Histidine was observed as a result of G to C change at cDNA position 310. The mutation was absent in online frequency databases and at the same time was predicted to be disease causing by various pathogenicity predicting tools. Human *BEST1* gene was identified in 1998 and it is a calcium-activated chloride channel present in RPE (Yang et al., 2015). This recurrent mutation of *BEST1* was previously reported by (Krämer et al., 2003). Aspartate residue at 104 position is highly conserved among different species. According to another change reported by (Petrukhin et al., 1998) where Glutamic acid replaces Aspartate at the same position 104 indicates that Asp104 is crucial for causing disease.

Most of the families in this study have been solved through genome-wide SNP genotyping leading exome sequencing. These high throughput sequencing technologies have made the identification of causative variant time and cost effective.

This study has utilized advanced genomic techniques to identify 4 novel and 9 known mutations in different RD genes. The identification of majority (70%) of known mutations in this cohort indicate the potential application of targeted sequencing for genetic testing and diagnosis. Whole exome sequencing has successfully identified the underlying mutations in majority (86.6 % families: 13 out of 15 families) of cases but disease-causing mutations could not be identified in two families (Family H and L) with this approach. The underlying mutations in both the unsolved families can be identified by using whole genome sequencing as this method has the potential to identify deep intronic or regulatory mutations.

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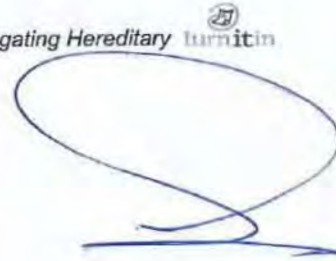

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