CHAPTER IV

RESULTS

IV. 1. RESULTS

In the present study, 28 families consists of 35 affected individuals and 40 unaffected relatives were included based on the inclusion criteria. These probands data were obtained from Dr. Kariadi hospital, Central Java Eye Center, Semarang Eye Center and Low Vision Unit Yogyakarta. This study included 24 sporadic cases of RP and 4 families with multiplex cases. Three of the families were consanguineous, whereas the other families were coming from a relatively isolated region in Java Island.

Table 3. Mode of inheritance of all collected samples in this study

No.	Mode of Inheritance	Number of family
1.	Autosomal recessive	5
2.	Sporadic cases	23

Based on the pedigree analysis, 5 families show autosomal recessive inheritance and 23 are sporadic case; none of the families displays an autosomal dominant mode of inheritance. Most of these families are Javanese (89%, 25 families from total of 28 families), 2 families are Chinese (7,1%) and 1 family has a Minang ethnicity (3,6%).

In this study, not all relatives of every patients samples were available, because they were not agree to join in this research.

These families were sent for SNP microarray analysis 700K and candidate genes residing the homozygous region were sequenced using direct Sanger sequencing.

Table 4. Current location and ethnicity of the patients that were collected in this study

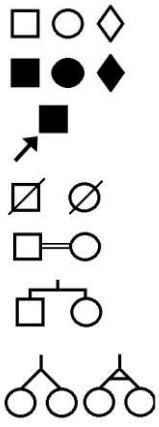
No	Code family	Residence	Ethnicity
1	W10-1985	Semarang	Javanese
2	W10-1986	Bandung	Chinese
3	W10-1987	Pekalongan	Javanese
4	W10-1988	Semarang	Javanese
5	W10-1989	Semarang	Javanese
6	W10-1990	Wonosobo	Javanese
7	W10-1991	Semarang	Javanese
8	W10-1992	Semarang	Javanese
9	W10-1993	Semarang	Javanese
10	W10-1994	Semarang	Javanese
11	W10-1995	Semarang	Javanese
12	W10-1996	Banjarmasin	Minang
13	W10-1997	Semarang	Javanese
14	W10-1998	Semarang	Javanese
15	W10-1999	Semarang	Javanese
16	W10-2000	Semarang	Javanese
17	W10-2001	Ungaran	Javanese
18	W10-2002	Ungaran	Javanese
19	W10-2738	Pemalang	Javanese
20	W10-2739	Kendal	Javanese
21	W10-2740	Demak	Javanese
22	W10-2741	Demak	Javanese
23	W10-2742	Kudus	Javanese
24	W10-2743	Batang	Javanese
25	W10-2744	Tegal	Javanese
26	W10-2745	Demak	Javanese
27	W10-2746	Semarang	Javanese
28	W11-1541	Bandung	Chinese

IV. 1. 1. Pedigree

The pedigree analysis were obtained from the history taking with the patients and family members. Pedigrees were drawn including three generations of the family. However, some pedigree were drawn in two generations due to the lack of data available.

This study included total of 3 consanguineous families in the cohort which came from different region in Java Island. A total of 25 families in this cohort are non-consanguineous of which 23 are sporadic cases; 2 families are considered to display autosomal recessive inheritance.

In the pedigree, affected individuals are indicated with filled symbols, whereas unaffected indicated by open symbols. Probands are indicated by arrows and affected individuals that underwent SNP microarray analysis are marked in asterisk. Deceased family member are marked with slash symbol. Below are symbols that commonly used in pedigree analysis:



Unaffected male, female, unknown

Clinically affected

Proband

Deceased

Consanguinity

Children

Twins, dizygous, monozygous

Below are the pedigree of the families that were involved in this study, probands marked with arrow and samples that were underwent SNP array analysis marked with asterisk.

Consanguineous families

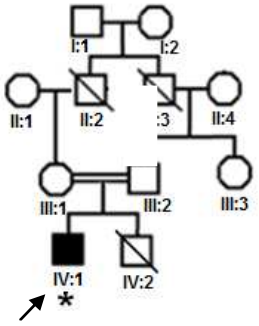


Figure 9. Family W10-1989 with arRP

Consanguineous marriage with single affected family member

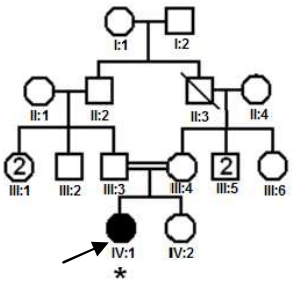


Figure 10. Family W10-2743 with arRP

Consanguineous marriage with single affected family member

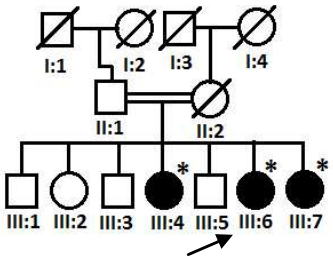


Figure 11. Family W10-2744 with arRP

Consanguineous marriage with single affected

Non Consanguineous families:

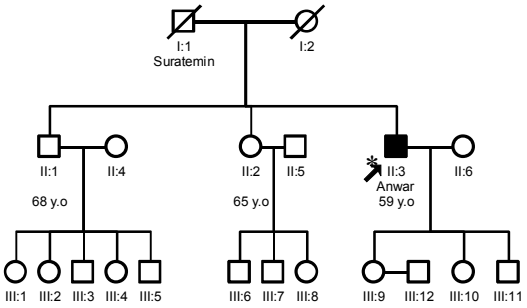


Figure 12. Family W10-1985 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-Linked

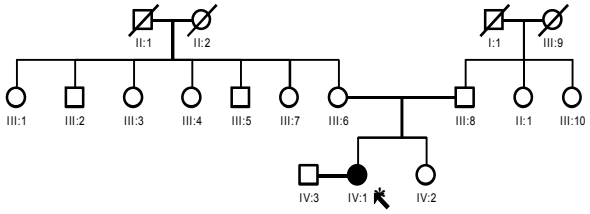


Figure 13. Family W10-1986 with single isolated case

From the pedigree it is suspected that the mode of inheritance is autosomal recessive

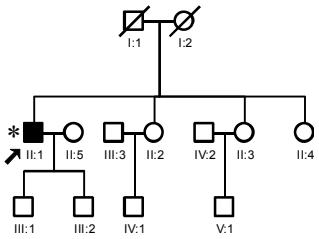


Figure 14. Family W10-1987 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be autosomal recessive or X-linked manner

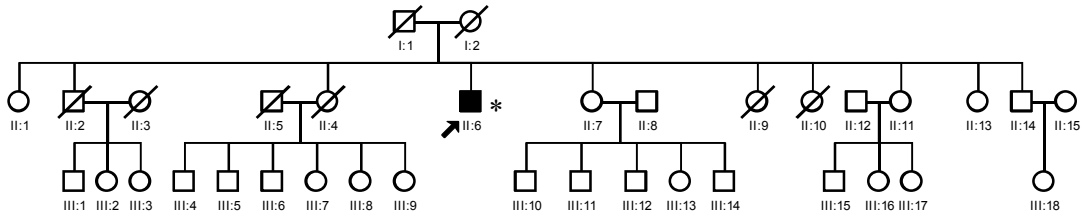


Figure 15. Family W10-1988 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-linked

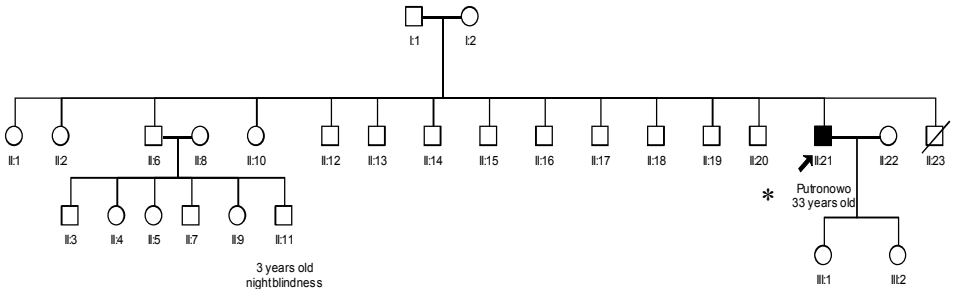


Figure 16. Family W10-1990 with single isolated case

The mode of inheritance can be either autosomal recessive or X-linked

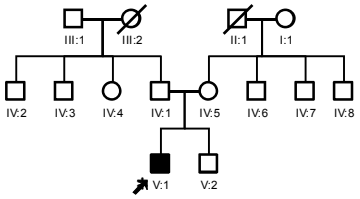


Figure 17. Family W10-1991 with single isolated case

The mode of inheritance can be either autosomal recessive or X-linked

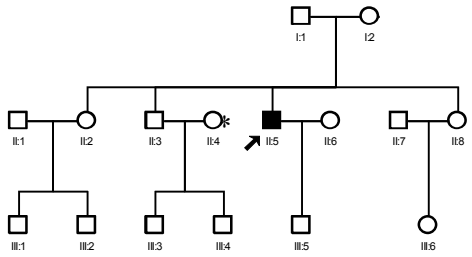


Figure 18. Family W10-1992 with single isolated case

The mode of inheritance can be either autosomal recessive or X-linked

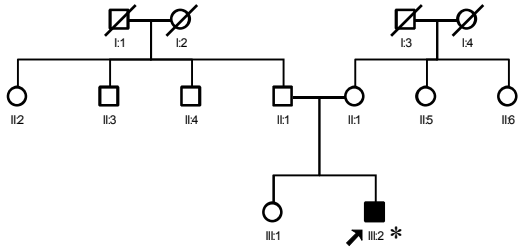


Figure 19. Family W10-1993 with single isolated case

The mode of inheritance can be either autosomal recessive or X-linked

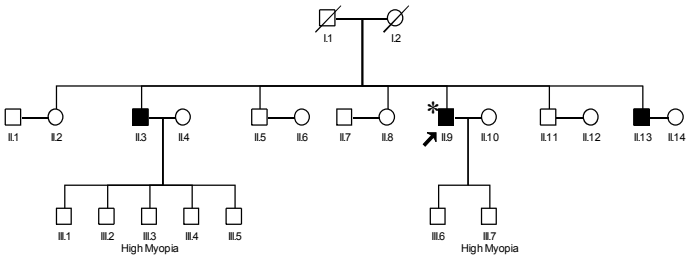


Figure 20. Family W10-1994 with suspected as xlRP

Three affected in this family, there is no consanguinity

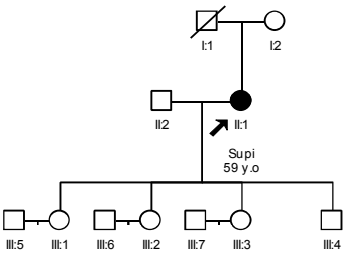


Figure 21. Family W10-1995 with single isolated case

The most probable mode of inheritance in this family is autosomal recessive

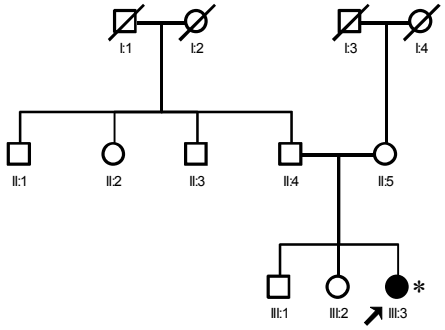


Figure 22. Family W10-1996 with single isolated case

The mode of inheritance is expected as autosomal recessive

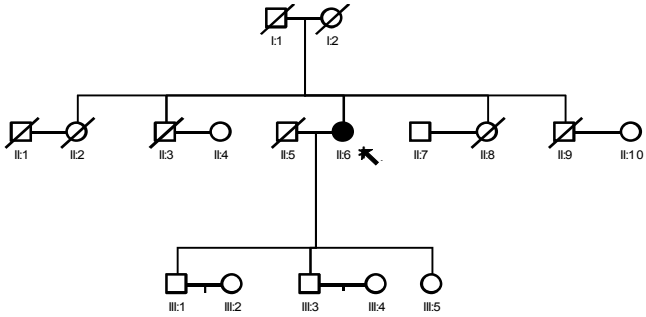


Figure 23. Family W10-1997 with single isolated case

The mode of inheritance is suspected as autosomal recessive

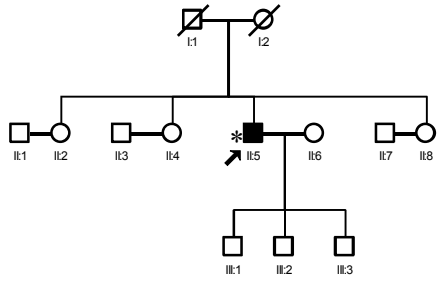


Figure 24. Family W10-1998 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-linked

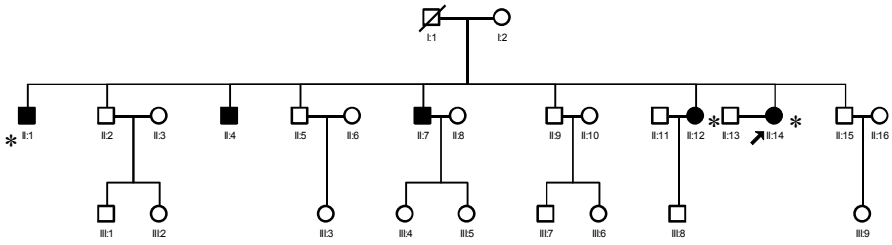


Figure 25. Family W10-1999 with arRP

From the pedigree it is suspected that the mode of inheritance is autosomal recessive RP. There is no consanguinity in this family

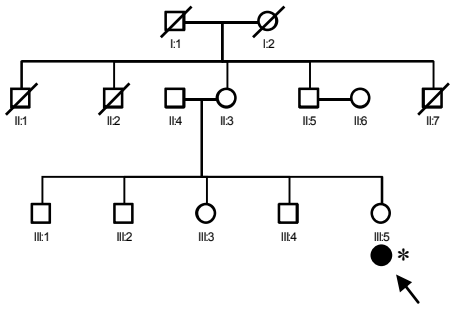


Figure 26. Family W10-2000 with single isolated case

The mode of inheritance is suspected as autosomal recessive

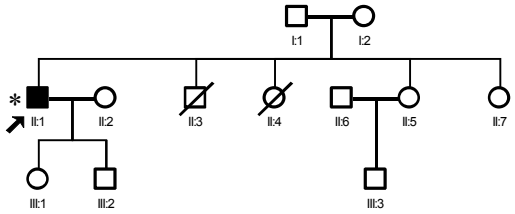


Figure 27. Family W10-2001 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-linked

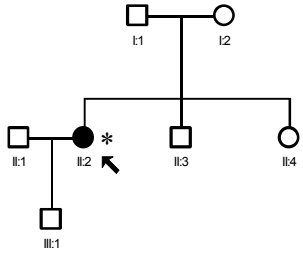


Figure 28. Family W10-2002 with single isolated case

The mode of inheritance is suspected as autosomal recessive

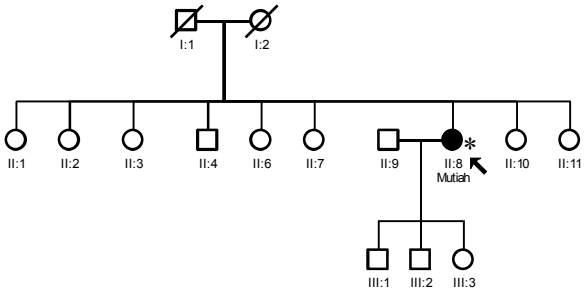


Figure 29. Family W10-2738 with single isolated case

The mode of inheritance is suspected as autosomal recessive

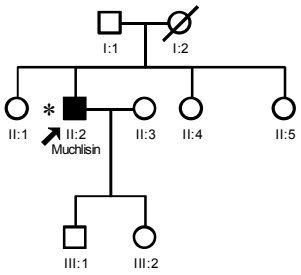


Figure 30. Family W10-2739 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-linked

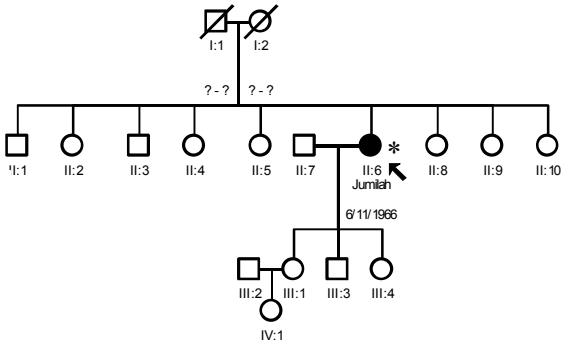


Figure 31. Family W10-2740 with single isolated case

The mode of inheritance is suspected as autosomal recessive

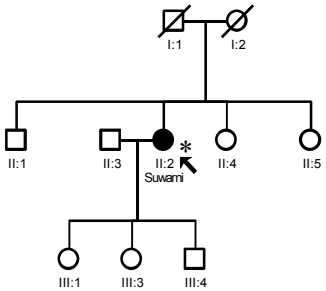


Figure 32. Family W10-2741 with single isolated case

The mode of inheritance is suspected as autosomal recessive

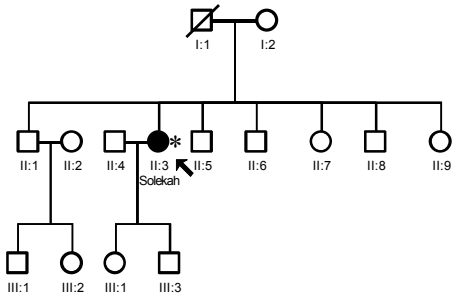


Figure 33. Family W10-2742 with single isolated case

The mode of inheritance is suspected as autosomal recessive

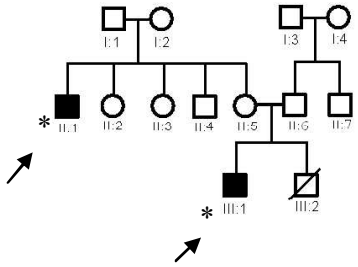


Figure 34. Family W10-2745

From the pedigree it is suspected that the mode of inheritance is X-linked but the possibility autosomal recessive RP still cannot be excluded. There is no consanguinity in this family

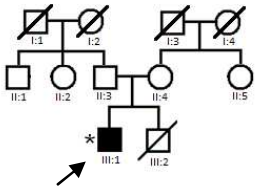


Figure 35. Family W10-2746 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-linked

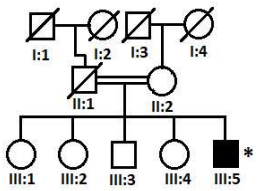


Figure 36. Family W11-1541 with single isolated case

From the pedigree it is suspected that the mode of inheritance can be either autosomal recessive or X-linked

IV. 1. 2. Clinical features

The diagnosed of RP in this study was based on the informations obtained from the history taking and ophthalmic examination. Clinical evaluation of probands was conducted in Dr. Kariadi hospital, Semarang Eye Center and Central Java Eye Center/ William Booth Hospital. The probands displayed varying symptoms. All of the patients had complaints of night blindness as the first symptom, followed by visual field constriction and decrease of visual acuity in the later stage.

The onset of disease and its severity are diverse between each patient. Some of the patients had the first symptom at an early age; for others, the onset of the disease was in adulthood. Most of the patients were diagnosed in the late stage when the constriction of peripheral vision and decrese in the visual acuity has started. There are only a few of patients were diagnosed in the early stage of RP when night blindness was the only symptom occur.

In the majority of patients, fundus examination revealed a typical features of RP, such as mid periphery bone spicule pigmentation, arteriole attentuation and a waxy, pale optic disc.

Table 5. Clinical characteristics of RP patients

No.	Family Code	Patients Code	DOB	Age of Onset	Initial Symptoms	Visual Acuity		Fundus Appearance
						OD	OS	
1.	W10-1985	057825	20/04/1951	39	NB	4/60	4/60	AA,BS
2.	W10-1986	057826	04/12/1978	5	NB	1/300	1/300	AA,BS,POD
3.	W10-1987	057830	07/07/1979	20	BV	1/300	1/300	AA,BS (central)
4.	W10-1988	057832	17/11/1940	11	NB	1/300	1/300	AA,BS,POD
5.	W10-1989	057835	15/11/1994	7	NB	1/60	2/60	BS,POD, AA
6.	W10-1990	057836	28/12/1976	6	NB	1/60	1/300	BS, AA
7.	W10-1991	057840	19/06/2002	6	NB	2/30	3/9,5	BS
8.	W10-1992	057843	06/11/1979	12	NB	20/400	1/300	BS, AA POD
9.	W10-1993	057849	18/07/1996	11	NB	20/20	20/20	BS
10.	W10-1994	057853	07/05/1957	35	BV	20/80	20/200	BS, AA, POD
11.	W10-1995	057854	28/08/1952	40	NB	1/300	0,5/60	BS, AA, POD
12.	W10-1996	057855	12/06/1978	12	NB	1/60	20/200	BS,AA,POD
13.	W10-1997	057859	17/08/1957	25	BV	1/~	1/300	BS,AA,POD
14.	W10-1998	057861	52 years old	30	BV	1/300	1/300	BS, AA
15.	W10-1999	057865	29/11/1978	12	NB	1/60	1/60	BS,AA
16.	W10-2000	057872	59 years old	20	NB	1/300	1/60	BS,AA,POD
17.	W10-2001	057873	27/09/1979	7	NB	6/30	6/30	BS, AA
18.	W10-2002	057877	01/05/1984	15	NB	1/300	0,5/60	BS, AA
19.	W10-2738	058396	10/03/1963	20	NB	1/300	1/300	BS,AA
20.	W10-2739	058397	12/06/1969	8	NB	4/60	4/60	BS,AA,POD
21.	W10-2740	058398	06/11/1966	34	NB	1/300	1/~	BS,AA,POD
22.	W10-2741	058401	11/04/1959	25	NB	1/~	1/~	BS,AA,POD
23.	W10-2742	058402	12/07/1979	30	NB	1/300	1/60	BS,AA,POD
24.	W10-2743	058405	01/01/1995	4	NB	6/30	6/30	AA,POD
25.	W10-2744	058409	06/10/1975	9	NB	1/300	1/300	BS,AA
26.	W10-2745	058414	28/05/2004	4	NB	20/20	20/20	BS
27.	W10-2746	058423	29/03/1978	19	NB	1/60	2/60	?
28.	W11-1541	058458	01/08/1971	22	NB	1/300	1/300	BS,AA,POD

OD:oculus dextra (right eye) OS : oculus sinistra (left eye)

NB: night blindness BV: blurred vision

BS: bone spicules POD : pale optic disc

AA: attenuated arterioles

Patients with several entities, such as syndromic RP, congenital stationary night blindness, vitamin A deficiency, Stargardt disease, cone-rod dystrophy, drug intoxication and inflammation reaction were not included in this study. Patients with cataract were referred to undergo cataract removal surgery before the diagnosis was made. Retinal electrophysiology data were not available due to the lack of electroretinography equipment in Semarang. An overview of probands' clinical data is presented in Table 5.

IV. 1. 3. Molecular genetic analysis

IV.1.3.1. SNP array analysis

SNP microarray is a genotyping approach that was used in this study. This technique is based on a rapid genotyping using 700,000 single nucleotide polymorphisms (SNPs) markers spread across the genome. Single nucleotide polymorphism is a variation at a single site in the DNA and the most common type of variation in the human genome (10 million SNPs) which is extremely conserved throughout evolution and within a population. Homozygous regions were determined by utilizing PLINK software. Table 6 presents an overview of the number and sizes of the homozygous regions for each individual in consanguineous cases and non-consanguineous cases.

Table 6. Homozygous regions of the probands and the candidate genes.

Family ID	Number of affected individuals	# Homozygous regions > 3Mb	Chr.	Start position (hg 18)	End position (hg 18)	Size (Mb)	Rank	arRP gene in the region	Mutation (DNA)	Predicted effect (protein)
W10-1985	1	2	6	137.036.902	143.910.663	6,9	1			
			11	47.813.829	51.447.829	3,6	2			
W10-1986	1	2	1	141.786.422	144.975.398	3,2	1			
			3	47.027.941	50.201.155	3,17	2			
W10-1987	1	20	1	190.478.465	227.907.316	37,4	1	*CRB1, USH2A*	c.3914C>T	p.P1305L
			3	144.737.948	170.791.287	26	2	*CLRN1*		
			4	73.418.004	96.432.844	23	3			
W10-1988	1	0	-	-	-	-	-			
W10-1989	1	18	2	11.713.812	31.318.332	19,6	1	*C2ORF71*		
			4	20.085.898	38.668.700	18,6	2			
			6	72.463.040	88.599.333	16,1	3			
W10-1992		2	6	72.526.393	76.681.799	4,2	1			
			1	141.786.422	145.050.191	3,3	2			
W10-1993		0	-	-	-	-	-			
W10-1995		3	4	42.343.155	48.048.814	5,7	1	*CNGA1*		
			11	46.217.272	51.447.829	5,2	2			
			11	55.171.982	58.184.165	3	3			
W10-1996		1	8	110.925.657	114.857.600	3,9	1			
W10-1998		1	2	21.717.238	26.426.956	4,7	1			
W10-1999		3	5	42.907.219	46.434.850	3,5	1			
			2	208.357.668	211.659.690	3,3	2			
			5	98.504.520	102.781.114	4,3	3			
W10-2001		2	1	141.786.422	144.973.250	3,2	1			
			16	16.133.471	19.148.212	3	2			
W10-2002		0		27.124.217	29.486.822					
W10-2738	1	0	11	62.793.854	66.103.554	2,4	1	*CRB1*	c.3914C>T	p.P1305L
W10-2739	1	0	-	-	-	-	-			
W10-2740	1	1	7	62.793.854	66.103.554	3,3	1			
W10-2741	1	0		-	-	-	-			
W10-2742	1	11	1	19.912.406	106.982.348	87	1	*RPE65, ABCA4*		
			3	13.219.384	60.870.619	47,6	2	*GNAT1*		
			2	115.066.438	141.539.769	26,5	3			
W10-2743	1	27	8	46.994.719	112.822.143	65,8	1	*TTPA,RP1*		
			6	79.880.090	103.011.272	23,1	2			
			12	46.563.979	69.123.679	22,6	3			
W10-2744	3	1	16	77.098.649	80.083.138	3	1			
			8	50.357.738	52.191.973	2	2			
			16	65.737.699	67.289.958	1.5	3			
			8	55.432.038	56.765.392	1.3	4	*RP1*	c.1012C>T	p.R338X
W10-2745	2	7	11	82.848.837	112.264.668	29	1			
			1	63.787.930	88.240.255	24	2	*RPE65*	c.295 G>A c.730G>A	p.V99I p.G244S
			2	134.029.325	155.362.938	21	3			

Red color indicated genes with mutations; grey color indicated genes that have been excluded by sequencing analysis.

In this study, 15 families did not have any homozygous region larger than 3Mb. All of these families are sporadic cases which had relatively small homozygous regions (~2 Mb on average); in these cases compound heterozygous mutations are the most likely disease-causing alterations.

Families with multiple affected individuals and consanguinity had variable number and length of homozygous regions above the threshold. The largest homozygous region of 65.8 Mb with 4,838 SNPs was found in consanguineous family W10-2743. The length of homozygous regions in this family is caused by the small number of meiotic recombination between the ancestor and affected individual due to the second-cousin marriage. In family W10-2744 which has uncertain degree of consanguinity, displayed only one homozygous region larger than 3 Mb which suggested that the distance between affected individuals and ancestor is relatively big.

Four families which did not report any consanguinity also displayed the presence of homozygous regions larger than 15 Mb. This fact can be explained by the origin of the parents of affected individuals who were come from a relatively isolated area where the number of close relatives' marriages is high.

Candidate genes were determined from the homozygous regions using available websites and softwares aforementioned in the Method Chapter. Direct sequencing was subsequently performed on the candidate

genes. Based on the homozygosity mapping data we have sequenced *CRB1, C2ORF71, RPE65, ABCA4* and *RP1* genes. We also performed *LCA5* sequencing on two samples which harbor this gene in their homozygous region. We found *CRB1, RP1* and *RPE65* mutations in three different families (Table 7). Candidate genes residing in homozygous regions have been excluded since no alterations were identified in them. All of the variants found in this study, except for the *CRB1* mutation were novel mutations that have never been reported in any previous study about RP, either in Indonesian population or other ethnicity.

Table 7. Amino acid prediction of the mutations that were found in this study

Family code	Gene affected	Mutation (DNA)	Mutation consequence	SIFT	PolyPhen	Align GVGD	Grantham score	PhyloP
W10-1987	*CRB1*	c.3914C>T	p.P1305L	Tolerated (score: 0.88)	Probably damaging (score 1.000)	Class 0	98	4,32
W10-2744	*RP1*	c.1012C>T	p.R338X	-	-	-	-	-
W10-2745	*RPE65*	c.295G>A	p.V99I	Tolerated (score: 0.09)	Tolerated	Class 0	56	6,26
W10-2745	*RPE65*	c.730G>A	p.G244S	Tolerated (score: 0.15)	Probably damaging	Class 0	29	5,77

Mutation analysis of each family

Family W10-1987

Affected individual of family W10-1987 (32 years) was coming to the hospital with the complaints of blurred vision. The symptoms began with night blindness at 20 years of age, followed by visual field constriction. The patient ignored these symptoms because he could still

function normally in his daily life. Lately, he developed a progressive decrease of visual acuity, which started to influence his activities. Recent examination revealed the visual acuity of 1/300 (hand movement) in both eyes. Color vision was difficult to examine due of his limited sight. Fundus appearance showed bone spicule pigmentation in mid-periphery of retina and narrowing of retinal vessels. No other family member shown in the pedigree (Figure 37a) displayed the same symptoms. The family lived in a relatively isolated area in Pekalongan, where the number of close relative marriages is high.

Candidate gene analysis was performed on the largest homozygous region of 37 Mb on chromosome 1 (Table 6). All the available information regarding genes located within this region was collected to determine the candidate genes. *CRB1, USH2A* and *CLRN1* genes were found within the homozygous region; all of these genes were already known as RP-causative genes. Previous study on Indonesian families has revealed p.P1305L mutation in the *CRB1* gene (13). Therefore, sequencing analysis for *CRB1* gene, starting with aforementioned alteration, was performed in this patient.

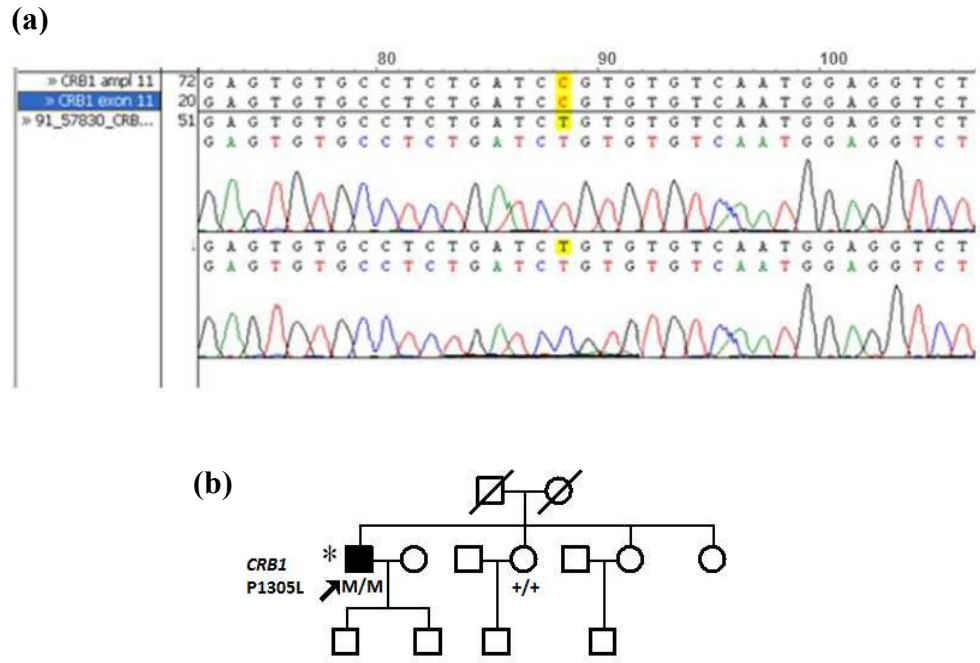


Figure 37. Family W10-1987 with p.P1305L mutation in *CRB1* gene. (a) Sequencing analysis of *CRB1* gene exon 11 shows subtitution from cytosine to thymine which result in Proline change in position 1305 to Leucine; (b) Segregation analysis of the p.P1305L mutation in *CRB1* gene, M: mutated allele and (+): wild type/ normal allele.

Mutation analysis in the patient led to the identification of a homozygous c.3914C>T substitution (Figure 37b) in exon 11, replacing a proline residue at position 1305 with a leucine (p.P1305L). This mutation was identical with the variant reported in the previyous study in the same population (13).

The homozygous missense substitution affects a proline residue which is highly conserved across several (Figure 38). Moderate physicochemical difference between the amino acids proline and leucine results in a Grantham score of 98 [0-215]. The p.P1305L variant is located in EGF-like type 3 and EGF-like calcium-binding domain.

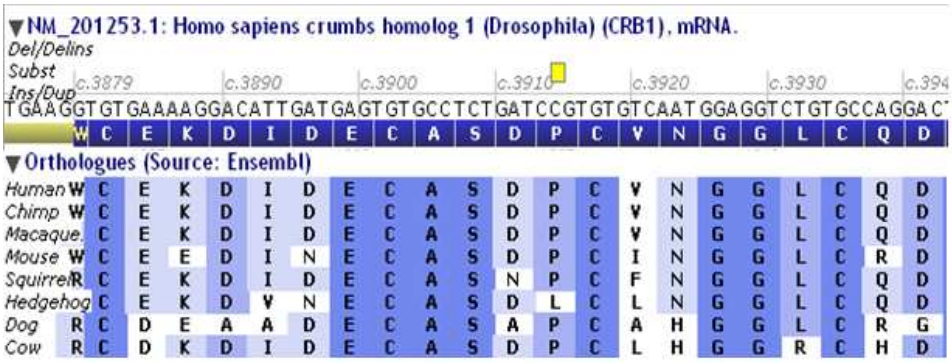


Figure 38. Amino acid conservation of Proline in 1305 position
Proline is highly conserved in this position except for the hedgehog. Mutation is marked with the yellow box

The analysis of the protein sequence, using the project HOPE database, indicates that the size of mutant residue is bigger than the wild-type residue. The wild-type residue was deep in the core of the protein; therefore the mutant will probably disrupt the structure. This variant is predicted to be a possibly damaging change both by SIFT and PolyPhen software and has a PhyloP of 4.32.

To determine whether this substitution is pathogenic we studied the segregation in the family. The missense mutation was not found in unaffected sibling (Figure 37a). Additionally, the mutation was not found in 300 alleles of ethnically matched control individuals. The Crumbs homolog 1 protein is a 1406-amino acid transmembrane protein with 19 EGF domains, three laminin A G-like domains, a single transmembrane domain, and a 37-amino acid intracellular domain. The cytoplasmic domain of CRB1 is highly conserved and functionally related to the cytoplasmic domain of *Drosophila melanogaster* Crb protein.

In this present study, mutation was found in EGF-like 19 domain, which is a subset of EGF domain that has calcium binding-associated residues. Previous study in fibrillin-1 and Notch-1 have implicated that Ca2+ binding is essential in the dynamics of protein, particularly the rigidity of interdomain interface. Mutation in cbEGF 19 (calcium binding EGF like 19) domain are expected to decrease the Ca2+ affinity result in the lower percentage of Ca2+ -bound protein which is caused by disruption of long range interactions between adjacent EGF domains in CRB1 (53). The mutation is expected to affect CRB1 structural integrity in the inter-photoreceptor matrix of retina. Taken together, this missense variant is likely to be pathogenic and can be consider as a population-specific mutation.

Family W10-2745

In family W10-2745, the affected individuals showed typical signs of arRP: night blindness and peripheral visual field loss. Individual III:1 displayed poor night vision since he was 4 years old. He was diagnosed with RP at age of 7 years old. Recent ophthalmic examination revealed that proband's visual acuity and visual field are still within normal limit. Fundus appearance show bone spicule pigmentation, whereas arterioles and optic discs was still relatively normal.

Conversely, individual II:1 noticed night blindness since he was 12 years old followed by peripheral visual field loss and decrease of visual acuity. Funduscopic findings included mid-peripheral bone

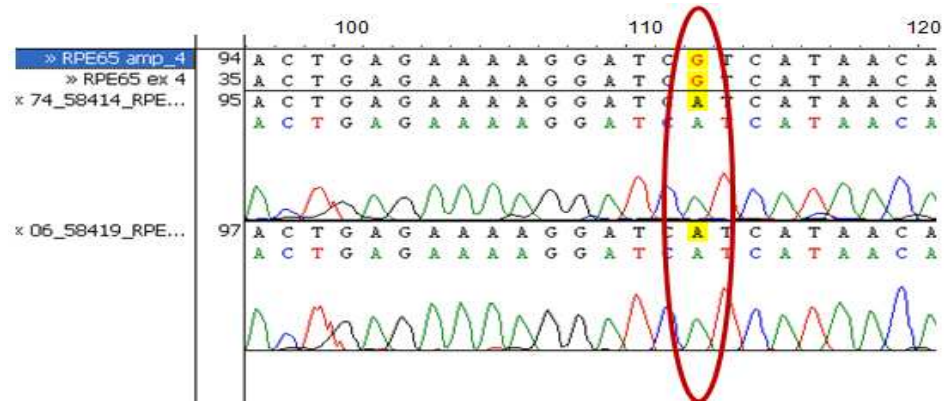
spicule-type pigment deposits, attenuation of retinal blood vessels and pale appearance of optic discs. Other family members does not show any sign and symptom of RP. Most of the family members are living in the same small village in Demak, possibility of close relatives marriage is high.

Genome-wide homozygosity mapping revealed three homozygous regions larger than 21 Mb. The second largest homozygous region of 24 Mb on chromosome 1 comprised *RPE65* (Table 6), a known RP disease-causing gene. No other RP known genes were found within the first and third largest homozygous region.

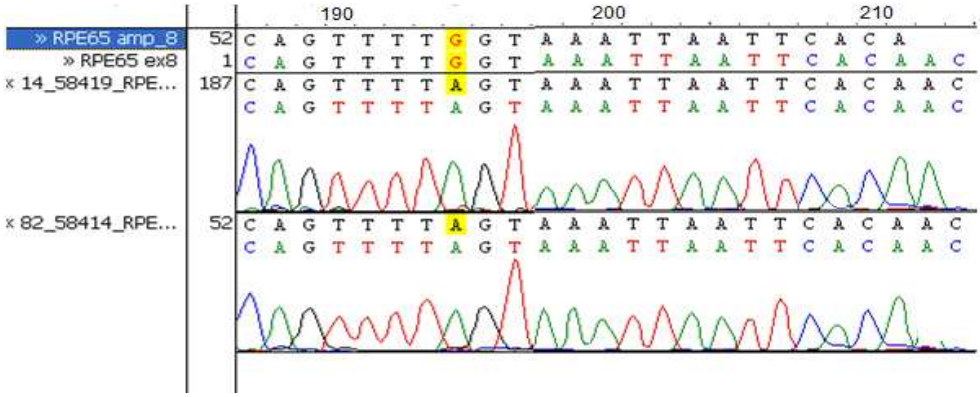
Mutation analysis in both affected revealed two homozygous substitutions, first variant was found in exon 4 and second variants was found in exon 8. The first variant found is a homozygous c.295G>A substitution in exon 4 (Figure 39b)., result in replacement of valine with isoleucine in position 99.

Segregation analysis was performed and this variant was not found in unaffected sibling. The homozygous missense substitution affects valine amino acid which is highly conserved across several species (Figure 40).

(a)



(b)



(c)

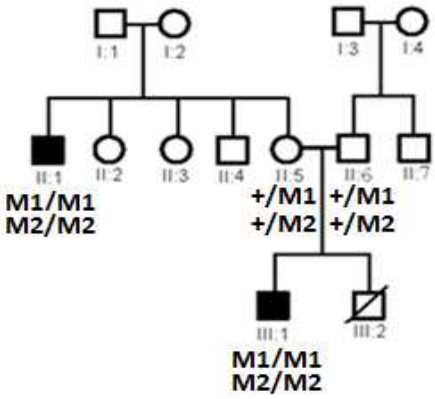


Figure 39. Family W10-2745 with two mutations in *RPE65* gene.
(a) *RPE65* sequencing analysis of mutation p.V99I; (b) *RPE65* sequencing analysis of mutation p.G244S; (c) Segregation analysis of the p.V99I and p.G244S mutations in *RPE65* gene, **M1**: p.V99I, **M2**: p.G244S and **(+)**: wild type/ normal allele;

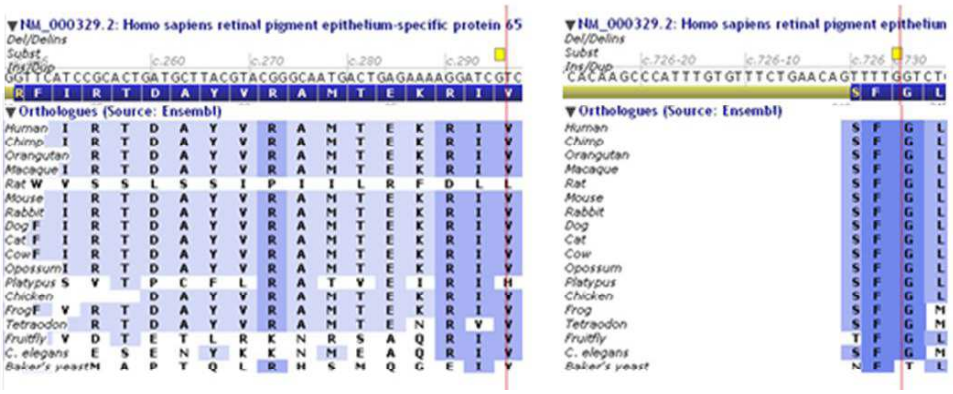


Figure 40. Amino acid conservation of mutations in *RPE65* gene
(a) Amino acid conservation of valine in position 99; (b) Glycine conservation in position 244, mutations are marked with the yellow boxes. Both amino acids that are highly conserved across several species.

Valine and isoleucine have a small physicochemical difference. The mutation is located in the carotenoid oxygenase domain. Functional effect prediction of the missense mutation using PolyPhen and SIFT revealed that this mutation is less likely to be pathogenic (Table 7).

The second missense change found was a homozygous c.730G>A mutation in exon 8 which replaced glycine with serine in position 244 (Figure 39c). It is located in carotenoid oxygenase domain. Segregation analysis was performed and the missense mutation was not found in unaffected sibling. This homozygous missense substitution affects a protein which is highly conserved across several species (Figure 40).

Functional effect prediction of the missense mutation using SIFT revealed that this mutation is tolerated. In contrast, PolyPhen predicted this as a probably damaging missense change and has a phyloP score of 6.26 (Table 7). Glycine is known as the smallest and most

flexible residue of all and this might be necessary for the protein's function. Substitution of glycine with a much larger serine may alter the protein structure and abolish its function. Functional study is needed to prove the pathogeneicity of this mutation. Based on the information above, the second mutation is more likely to be disease-causative variant than the first alteration. Both variants found in this family were novel mutations that never been reported previously.

Family W10-2744

In family W10-2744 two affected individuals had severe symptoms with early onset, and displayed typical fundus changes of RP including a waxy, pale optic disc, attenuation of retinal arteries and bone-spicule pigment deposits in the mid-peripheral retina. The proband (individual II:6) was showing night blindness as the first symptom at 8 years old. Her visual acuity at last follow up at age of 36 was light perception. By indirect funduscopy examination, this proband was diagnosed with RP. Her sister (individual II:4) exhibited classic RP with night blindness at 9 years old, and followed by visual field constriction at 11 years old. Bone spicule-like pigment deposits were present in the mid-peripheral retina along with the retinal atrophy in her eyes.

Individual II:7 has a relatively mild phenotype compared to her siblings, she had nyctalopia at age 11 followed by visual field constriction three years later. She had a reduced vision but still has an

ability to read. The proband's father (I:1) (63-years-old) and one of the siblings (II:2) did not experience night blindness and other symptom of RP.

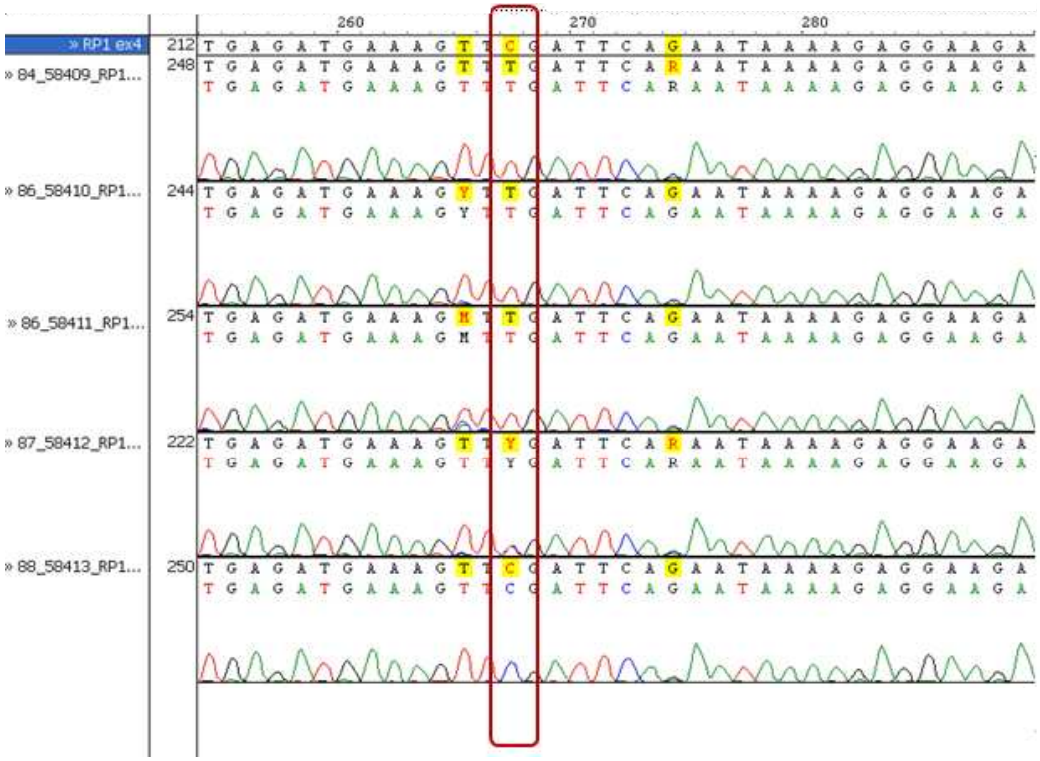
In this family, all three affected family members were analyzed by genome-wide SNP analysis. Subsequent homozygosity mapping revealed four regions larger than 1 Mb are shared in all affected individuals. The fourth homozygous region (1.3 Mb on chromosome 8q12.1) contained *RP1* gene (Table 6). *RP1* gene encodes a photoreceptor microtubule-associated protein that is required for correct stacking of outer segment disc.

Sequence analysis of *RP1* gene identified a homozygous c.1012C>T substitution in the beginning part of exon 4 in all patients of this family, replacing an arginine residue at position 338 by a stop codon (p.R338X). This novel mutation has never been found in any other RP previous studies. Sequence chromatograms of the *RP1* mutations are shown in Figure 41b. From the segregation analysis, this substitution was identified heterozygously in their father who did not reveal any symptoms of RP. In the sibling who is unaffected, this change was not found in both alleles. The R338X mutation was absent in 184 control subjects from Indonesian population.

The Retinitis Pigmentosa 1 gene (*RP1*) consists of four exons and gives rise to a transcript of approximately 7.1 kb encoding a protein of 2156 amino acids. This gene encodes a photoreceptor-specific protein

concentrated in connecting cilium and axoneme of photoreceptor sensory cilia (PSC). Previous study by Liu *et al*. reported that murine Rp1 is a photoreceptor-specific Microtubule-Associated Protein (MAP) that regulates the length and stability of the photoreceptor axoneme (54).

(a)



(b)

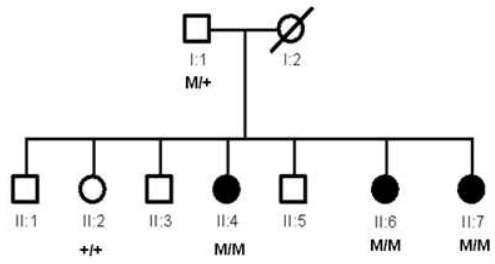


Figure 41. Family W10-2744 with p.R338X mutation in *RP1* gene.
(a) Sequencing analysis of *RP1* gene shows subtitution from cytosine to thymine which result in Arginine change in position 338 into stop codon; (b) Segregation analysis of the p.R338X mutation in *RP1* gene, M: mutated allele and (+): wild type/ normal allele.

To date, 33 mutations are known as disease-causing for RP. These mutations are predominantly frame shift or nonsense mutation which result in a premature stop codons clustered at the beginning of exon 4. Most of the mutations are inherited in autosomal dominant manner, only few cases of autosomal recessive RP caused by *RP1* mutation have been reported (Figure 42) (55-67).

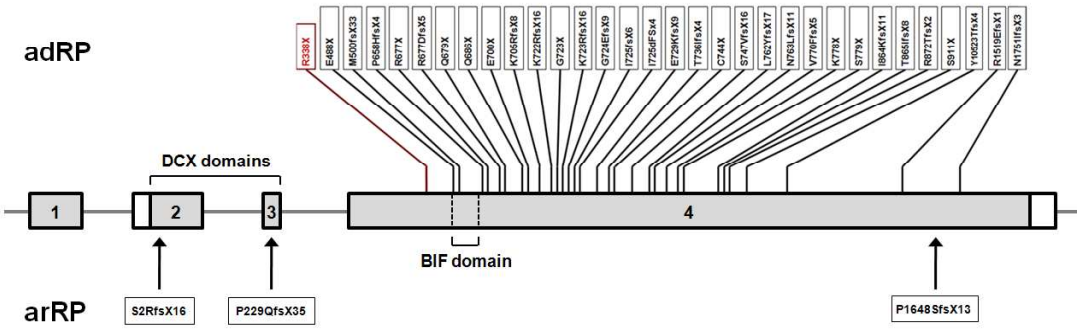


Figure 42. *RP1* gene and all identified mutations
To date, 33 protein-truncating mutations has been reported, all of these mutations are located in the fourth exon of *RP1*gene (55-67).

In the present study, we identified a homozygous novel *RP1* mutation (p. R.338X) in an autosomal recessive RP in an Indonesian family. Mutations in the last exon of *RP1* gene are expected to be insensitive to NMD due to absence of downstream intron resulting in the production of truncated proteins as reported by previous study by Liu *et al*. The Rp1-Q662X knock-in mice show that dominant mutations in *RP1*, which are truncating mutations in exon 4, can produce truncated proteins that retain their microtubule binding domains and localize correctly to the axonemes of PSCs (54). Autosomal dominant mutations

in the hot spot region of the C-terminus (codon 500-1053) are predicted to have an important role in mediating human RP1 function in the correct stacking of the discs in outer segment of the photoreceptor cells. Truncations of this domain are expected to disrupt this function. The truncated protein can bind to microtubule, human wild type RP1 or other protein and exert dominant-negative mechanisms. Therefore, it suggested that dominant mutations in *RP1* cause adRP via a dominant-negative mechanism.

Two mutations located downstream of the hot spot region (p.R1519EfsX and p.N1751IfsX3) have been reported to cause RP in recessive manner. These mutations are predicted to result in truncated protein which retains residual activity due to the minimal loss of the C-terminal portion. The normal allele can compensate this by producing 50% of normal Rp1 protein. Thus, the carrier individual will not display any phenotype of RP.

Conversely, mutations upstream the hot spot region is predicted to result in production of truncated protein which lost the binding domain. This truncated protein molecules lose their ability to localize in the microtubule and does not interfere the wild type protein. On the other hand, the normal allele still produces 50% of the normal Rp1 protein which can still maintain the normal function of photoreceptor cells. Hence, this mutation can only cause disease in the homozygous state.

The variant R338X is the second reported truncating mutation located in the first part of exon 4 which cause RP in autosomal recessive manner. This finding gives a strong evidence to confirm and support the hypothesis about the early truncating mutations in exon 4 of *RP1* gene mechanisms explained above.

Family W10-1989

W10-1989 was one of the families with second degree cousin marriage in the family. The affected individual was a 17 years old male diagnosed with retinitis pigmentosa. First symptom was night blindness at age of 7, subsequently followed by tunnel vision 2 years after the first complaint. Visual acuity was reduced, 1/60 for the right eye and 2/60 for the left eye and proband was complaining of reading difficulty. Fundus photograph shows bone spicule-like pigmentation, attenuation of arterioles and pale optic disc. No symptoms were found in other organs. Other family members do not have any complaints typical for RP.

Genome wide SNPs analysis identified a homozygous region of 19, 6 Mb on chromosome 2 as the largest homozygous region, which contains *C2ORF71* gene. Sequence analysis was performed but no mutations were found.

LCA5 gene was found in the third largest homozygous region of 16, 1 Mb on chromosome 6. Thus, we performed sequencing analysis on this gene and there was no disease-causing mutation found.

Further investigation should be continued for the known RP genes within the homozygous regions. High density SNPs microarray of other unaffected individuals could help to narrow the homozygous region to find the candidate genes.

Family W10-1995

Family W10-1995 is one of the sporadic cases in our cohort of patients. Affected individual is a 59 years old female, complaining her visual acuity impairment. She begin to has night blindness when she was 40 years old, followed by visual acuity impairment. This patient developed mild cataract within the past few years in her both eyes which made the ophthalmic examination was more difficult. Latest visual acuity measurement was 1/300 on the right eye and 0, 5/60 for the left eye. Periphery pigmentation of retina, narrowing of retinal vasculature and waxy pallor optic discs were found in patient's fundus. No other symptoms involving other organ were found. Other family members do not display any symptoms for RP.

SNP microarray recognized a homozygous region of 5, 7 Mb on chromosome 4 as the largest homozygous region, which contains *CNGA1* gene. Sequence analysis was subsequently performed on this gene, there were no variants identified as a pathogenic mutation.

This family only has a few and small size homozygous regions. This is possibly due to the fact that this is a sporadic case, where the mode of inheritance can be either autosomal recessive, autosomal

dominant or X-linked manner. In this study, we focus in autosomal recessive and X-Linked known RP genes, mutation analysis of autosomal dominant RP known genes are also important to be included for the further investigation.

Family W10-2742

This family is a family which lives in a relatively isolated region in the border of Demak and Kudus, proband admits that many of the inhabitants are still familially connected with uncertain degree of consanguinity. Affected individual noticed night blindness at age of 30 years old followed by tunnel vision and impairment of her visual acuity as the disease progress. In the fundus photograph examination, bone spicule pigmentation, narrowing of retinal vessels and pale disc optic were observed in her retina.

Homozygosity mapping in this family identified a large homozygous region of 87 Mb as the first largest region. *RPE65* gene and *ABCA4* gene were found within this region. Subsequent sequencing analysis has excluded these genes because there was no pathogenic mutation found in both genes.

Genome wide SNPs analysis found a very large homozygous region of 87Mb. Consanguineous marriage is very likely in this family although the degree of consanguinity is not clear. Further investigation is needed for the known RP disease causing genes within the homozygous

regions. High density SNPs microarray of other unaffected individuals could narrow the homozygous region to find the candidate genes.

Family W10-2743

Affected individual is a 16 years old female diagnosed with retinitis pigmentosa since she was 12 years old. She noticed night blindness as her first symptom, followed by visual field constriction at age of 11 when she often hits objects while cycling. Her visual acuity get worse progressively and she has to sit in the first line of her class in order to read the letter in the black board. Clinical examination revealed that proband has some Marfan-like phenotype, such as reduced upper segment/lower segment ratio (US/LS), increased arm span/height, high arched palate, pectus excavatum, pes planus and mild scoliosis. Other family members did not show any symptoms for RP.

Genome wide SNPs analysis recognized a homozygous region of 65,8 Mb on chromosome 8 as the largest homozygous region, which harbors *RP1* gene. A large homozygous region with 23,1 Mb in size was found on chromosome 6, which contains *LCA5* gene within the region. Subsequent sequencing analysis has excluded both genes since no pathogenic variant was found in any of them.

In an article by Avedikian H et al (68), a similar phenotype was reported. However, since the retinitis pigmentosa seemed to be inherited in an autosomal recessive manner, and Marfan syndrome was passed on

in a dominant manner it was more likely that these are two separate entities with different underlying genetic causes. Therefore, we also hypothesize that the phenotype of the proband in this case were also caused by two different diseases.

IV.1.3.2. Screening of known X-Linked RP genes

Most of the cases in our cohort were sporadic, for which the mode of inheritance can be either autosomal recessive, X-linked or even autosomal dominant. Since half of the sporadic cases in our cohort involved males, X-linked inheritance could not be ruled out in these cases. Mutations in *Retinitis Pigmentosa GTPase (RPGR)* and *Retinitis Pigmentosa (RP2)* gene are the most common causes of xlRP (69). A new exon of *RPGR*, ORF15, has recently been identified as a mutation hot spot for xlRP (70). In this cohort, a screening for *RPGR* and *RP2* genes was performed for all male isolated cases to exclude XLRP. No potentially pathogenic variants were detected in *RP2* gene. In the *RPGR* screening, we found c.3219C>T substitution in 2 affected individuals from different families (family W10-1985 and W10-2001), which is expected to alter the exonic sequence creating an alternative splice site, as predicted by the software.

Subsequently, 186 of ethnically matched control individuals were checked and the mutation was found in 22 control individuals. This finding shows that the c.3219 C>T is a common variant in Indonesian

population and it is not likely to be a disease-causing mutation for RP in Indonesian population.

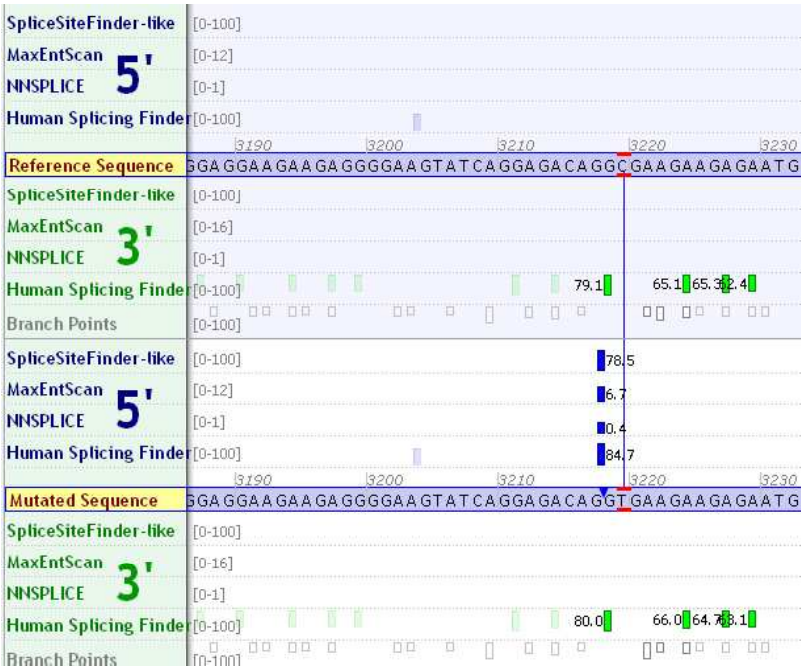


Figure 43. Splicing prediction of from Alamut 2.0 software

This program is used to predict altered splicing caused by a variant, reference sequence shown in the upper panel and mutated sequence shown in the lower panel

IV.1. 3.3 Screening of Known RP disease-causing genes for Indonesian families

Previous study by Siemiatkowska, et al (13), has identified several mutations as causative for RP in Indonesian families. Most of the solved cases were consanguineous families from different regions in Indonesia.

Seven mutations in six different genes have been identified using homozygosity mapping approach (Table 8). In this present study, a screening of these mutations was performed in all of the unsolved cases from Indonesian families.

Table 8. Known RP disease-causing mutations in Indonesian families from the previous study by Siemiatkowska, et al (13)

No.	Family code	Gene affected	Mutation (cDNA)	Mutation (protein)	Screening method
1	W09-0038	*MERTK*	complex rearrangement	exon 15 absent; p.G654AfsX41	Breakpoint PCR
2	W09-0041	*NR2E3*	c.1025T>G	p.V342G	RFLP (*HpyCHIV4)*
3	W09-0042	*ABCA4*	c.302+4A>C	Altered splicing?	RFLP (*Afl II)*
4	W09-0045	*MERTK*	c.2487-2A>G	Altered splicing	ARMS PCR
5	W09-0046	*EYS*	c.9082G>T	p.D3028Y	ARMS PCR
6	W09-0047	*PDE6A*	c.1675C>A	p.Y558	ARMS PCR
7	W09-0048	*CRB1*	c.3914C>T	p.P1305L	ARMS PCR

Using this approach, we found the p.P1305L mutation in *CRB1* gene in one individual (family W10-2738). This is the third case of p.P1305L mutation in Indonesian population. Therefore, we expect that this altertion may be founder mutation in Indonesian population. Screening in larger cohort is needed to support this hypothesis.

Family W10-2738

Family W10-2738 has only one affected individual with retinitis pigmentosa. She noticed night blindness when she was 20 years old followed by a progressive tunnel vision. Her visus was 1/300 in the latest ophthalmic examination when she was 57 years old. Fundus photograph displays bone spicule pigmentation and attenuation of arterioles. Other family members do not show any symptoms for RP.

The patient's sample was screened for known mutations that have been reported in the previous study (Table 8). For the p.P1305L

mutation screening in *CRB1* gene was performed using ARMS PCR method.

Specific ARMS primers were designed for the p.P1305L mutation. In this patient, a positive band was found only in the reaction with the "mutant" primer. This suggested that the proband has the p.P1305L mutation in both alleles. Sanger sequencing was performed to verify this finding, and the p.P1305L was confirmed in the proband. Segregation analysis was not possible since the samples of relatives are not available.

Table 9. Haplotype comparison between three probands with p.P1305L mutations in *CRB1*

dbSNP	Chr	Position	58396	58730	50068
rs1009188	1	195,523,713	AA	AA	BB
rs2786107	1	195,577,400	BB	BB	BB
rs949571	1	195,579,812	AA	AA	BB
rs2111932	1	195,797,173	AA	AA	BB

Probands 58396 and 57830 were analyzed using SNP microarray 700K, proband 50068 was analyzed using SNP array 5K

This is the third case that has been reported for the p.P1305L mutation in Indonesian RP families. All of them are sporadic cases which originate from different regions of Central Java Province. The haplotype comparison of all three probands shows that two of them display a stretch of 700 Kb of the same haplotype, whereas the third one has a different haplotype (Table 9).

To date, 25 mutations have been reported in *CRB1*. The p.P1305L mutation has not been described in any other ethnic background. The finding in this study suggests that this mutation can be a very old founder mutation for RP in Central Java region. The difference of haplotype that was found in the third proband can be explained by several hypotheses. Firstly, this proband was analyzed using a different platform than other two probands which has different marker density and might miss a small shared haplotype. Secondly, this mutation is a transition (substitutes cytosine to thymine) which are known as the most common mutation due to spontaneous deamination of 5-methylcytosine, a methylated form of cytosine. Hence, it can be considered as the *CRB1* mutation hot spot in Indonesian population rather than as a founder mutation.