IV. RESULTS AND DISCUSSION

IV.I. RESULTS

There are 16 families with 36 affected and 29 unaffected members of families can be included in further analysis based on the inclusion and exclusion criteria. A total of 65 samples were obtained from subject with Retinitis Pigmentosa from three hospitals in Semarang. The mode of inheritance of RP is determined by family history. Most of those families are from Java's ethnics (81.25 %, 13 out of 16) and 1 family from Chinese, Bugis and Sundanese consecutively (see table 10). In 14 families with an apparent autosomal recessive (ar) mode of inheritance, homozygosity mapping was conducted using Illumina 6k or Affymetrix 5.0 SNP arrays. Known arRP genes residing in homozygous regions were sequenced for mutations. In two families segregating adRP, the most frequently mutated adRP genes were sequenced (Rhodopsin).

For three consanguineous families (W09-0041, W09-0042 and W09-0046), the 'low-resolution' Illumina HumanLinkage-12 SNP array (Illumina, San Diego, USA) was used, whereas the patients from the remaining eleven families were genotyped on a high-resolution Affymetrix 5.0 array (Affymetrix, Santa Clara, USA).

Three consanguineous families using the low resolution array because from this tool the chance to have a homozygous region was quite big compare with non consanguineous families.

Table 10: Total amount of families obtained from several places in Indonesia.

No	Code family	Location	(ethnic)
1	W09-0035	Semarang	(Javanese)

2	W09-0036	Semarang	(Chinese)
3	W09-0037	Semarang	(Javanese)
4	W09-0038	Jakarta	(Javanese)
5	W09-0039	Semarang	(Javanese)
6	W09-0040	Makassar	(Bugis)
7	W09-0041	Semarang	(Javanese)
8	W09-0042	Jogjakarta	(Javanese)
9	W09-0043	Bogor	(Sundanese)
10	W09-0044	Semarang	(Javanese)
11	W09-0045	Semarang	(Javanese)
12	W09-0046	Semarang	(Javanese)
13	W09-0047	Semarang	(Javanese)
14	W09-0048	Semarang	(Javanese)
15	W09-0049	Semarang	(Javanese)
16	W09-0050	Semarang	(Javanese)

Not all family members of every patients were available for sampling, a total of 16 unrelated Indonesian families affected with RP were clinically examined at the Kariadi Hospital, William Booth Hospital and Panti Wilasa Citarum Hospital.

IV.1.1 Pedigree

The figures below indicate all the families among Indonesian population that participated on this study.

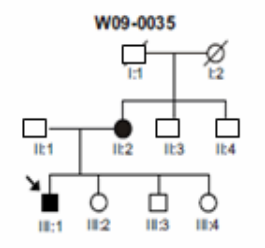


Fig 4: W09-0035 family demonstrating adRP
This is shown two affected persons on the family with the same phenotype.

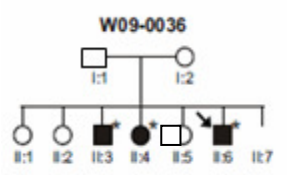


Fig 5: W09-0036 family with arRP

Multiple affected individuals (males and females) on the family, the parent are normally unaffected.

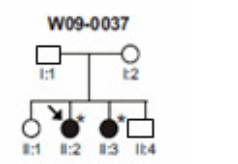


Fig 6: W09-0037 family with arRP

Simple family with two affected person which is not has the history of the consanguineous marriage.

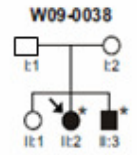


Fig 7: W09-0038 family with arRP

Two affected individuals (male and females) with no history of consanguineous marriage, but the parent was from the same village.

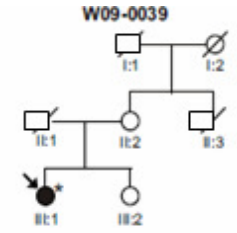


Fig 8: W09-0039 family with arRP

Isolated case of RP which has only one affected person (III.1) with no homozygous region more than 3 Mb.

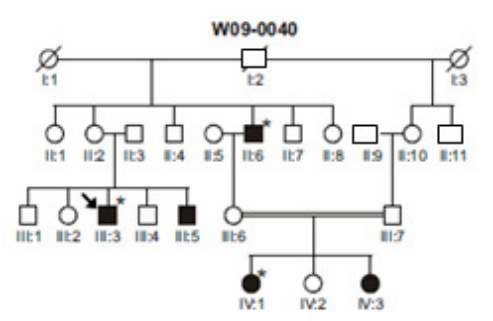


Fig 9: W09-0040 family with arRP.
Consanguineous marriage on the family with several affected persons.

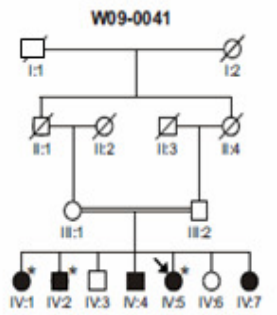


Fig 10: W09-0041 family with arRP
Consanguineous marriage with several affected persons with the same phenotype.

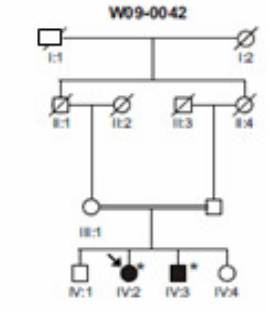


Fig 11: W09-0042 family with arRP
Consanguineous marriage with two affected persons.

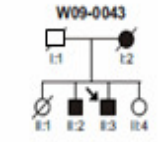


Fig 12: W09-0043 family with adRP

Typical vertical pedigree pattern, with multiple generation affected, male and female are equally affected.

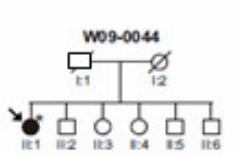


Fig 13: W09-0044 family with arRP
One affected person on the family.

Fig 14: W09-0045 family with arRP
Multiple affected individuals on the family with one non-identical twin.

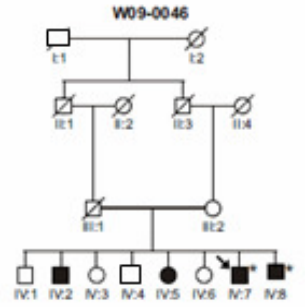


Fig 15: W09-0046 family with arRP
Typical consanguineous marriages with multiple affected individuals.

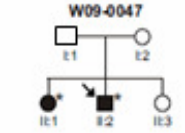


Fig 16: W09-0047 family with arRP
Two affected offspring with the same phenotype

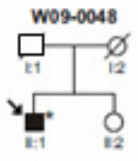


Fig 17: W09-0048 family with suspected arRP/X-linked RP

Only one affected offspring on the family with no history of consanguineous, assuming the type of inheritance are arRP or X-linked RP

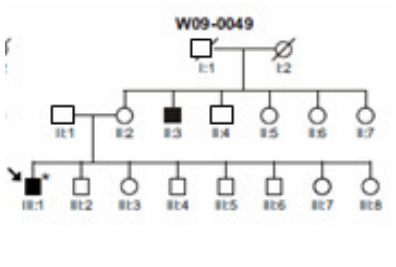


Fig 18: W09-0049 family with suspected arRP/X-linked RP.

No history of consanguineous marriage, the type of inheritance could be arRP or X-linked RP.

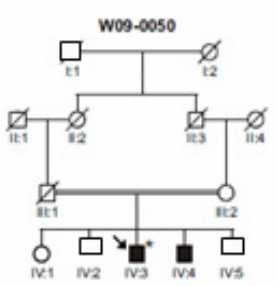
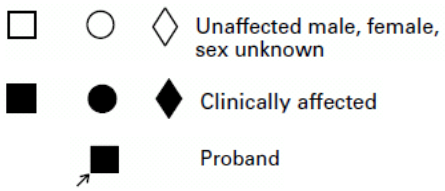


Fig 19: W09-0050 family with arRP.

Consanguineous marriage with two affected individuals with same phenotype.

Overview of the pedigree structures of the Indonesian families used in this study. Affected individuals are indicated with filled symbols, whereas unaffected relatives are indicated by open symbols. Probands are indicated with arrows, and individuals that were genotyped on genome-wide SNP arrays are marked with asterisks. Symbols with a slash depict deceased individuals. Because of the lack information from the family there were several pedigree with just only drawn with two generation

Pedigree symbols that commonly used for pedigree analysis.



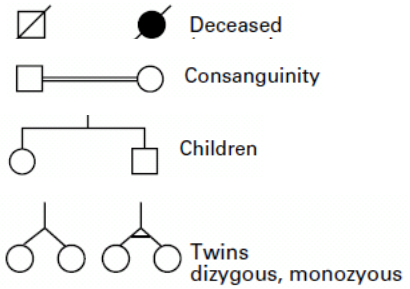


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Table 11: Mode of Inheritance of All families

Mode of inheritance	Amount of family
Autosomal dominant	2
Autosomal recessive	12
X-linked	2

IV.1.2. Clinical features.

Varying symptoms may presents on RP patients even many patients fail to recognize the manifestation of their condition until it has progressed become very severe. In our study all the patients has a chief complaint with the night vision difficulty or night blindness. Some of the patients, onset in the childhood and the rest was on adult period. Patients are extremely slight in their symptoms especially during the early stage and after arriving at the severe stage they had the problem with their vision and visual field as well.

In our study diagnosis of RP is based on the anamnesis (history taking), Visual acuity and also appearance of funduscopy. There are so many masqueraders appearance like syphilitic retinopathy, CMV retinopathy, Rubella retinopathy, toxoplasmosis, cancer associated retinopathy, traumatic retinopathy. By making a good anamnesis some of this

differential diagnosis can be excluded. Attenuated (thinning) of the vessel in the retina is the earliest sign in RP patients. The later stage was Mottling of RPE (hyper plastic formation) and referred to as “bone spicules” which is typically indicative (pathognomonic) of RP. As the disease progresses the broad atrophy of the Retinal Pigment Epithelial might reveal the larger choroid vessels. Optic nerve head in early stage looks normal and then may shows a waxy yellow or pale appearance in later stage. Some patients had a cataract so it is quite difficult to perform the funduscopy. We did not included ERG and Visual field examination in our study because until now in Semarang there is no ERG machine and examination for visual field quite expensive. The table II below was the summary of the clinical features from all affected person.

Clinical characterization that include funduscopy and visual acuity measurements revealed that the majority of affected individuals had poor vision and showed typical hallmarks of RP on funduscopy, e.g. bone spicule pigmentation, attenuated arteries and/or a pale appearance of the optic disc (Table 1). Only in family W09-0036, the visual acuity is not dramatically decreased

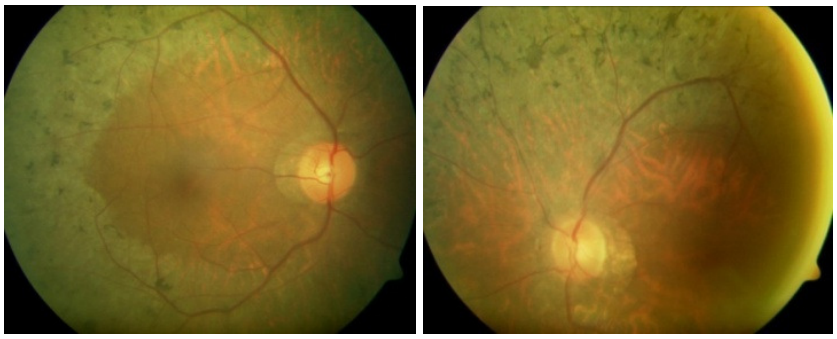
Tables 12: Clinical features from affected individuals

No	Family Code	Patient NO	Initial symptoms	Age at presentation	age at onset (Y)	Visual acuity without correction		Fundus Appearance
						OD	OS	
1	W09-0035	50043	NB	44	14	20/40	20/40	aa,bs
2	W09-0035	50089	NB	65	17	1/~	1/~	ND (cataract)
3	W09-0036	50045	NB	46	21	20/40	0.6	aa,bs
4	W09-0036	50053	NB	50	25	20/40	20/40	aa,bs
5	W09-0036	50054	NB	51	25	20/40	20/40	aa,bs
6	W09-0037	50061	NB	32	juvenile	1/300	1/60	aa,bs
7	W09-0037	50064	NB	29	juvenile	1/60	1/60	aa,bs
8	W09-0038	50059	NB	30	17	1/60	2/60	aa,bs
9	W09-0038	50069	NB	32	18	1/60	1/300	aa,bs
10	W09-0039	50074	NB	46	20	1/~	1/~	pod,aa,bs
11	W09-0040	50047	NB	45	Adulthood	0	1/~	pod,aa,bs
12	W09-0040	50049	NB+BV	10	8	1/60	1/60	NA
13	W09-0040	50050	NB	71	Adulthood	1/300	0	ND (cataract)
14	W09-0040	50052	NB	59	Adulthood	1/60	0	pod,aa,bs
15	W09-0041	50077	NB	44	14	20/200	20/200	bs
16	W09-0041	50078	NB	41	13	20/100	20/70	bs
17	W09-0041	50079	NB	49	15	20/200	20/200	bs
18	W09-0041	50080	NB	35	15	20/40	20/40	bs
19	W09-0042	50085	NB+BV	64	childhood	1/~	1/~	pod,aa,bs
20	W09-0042	50102	NB+BV	62	childhood	1/~	1/~	pod,aa,bs
21	W09-0043	50097	NB	53	childhood	1/60	1/60	bs
22	W09-0043	50098	NB	51	childhood	1/60	1/60	bs
23	W09-0044	50075	NB	59	childhood	1/~	1/~	pod,aa,bs
24	W09-0045	50090	NB+BV	55	Adulthood	1/~	1/~	pod,aa,bs
25	W09-0045	50091	NB+BV	37	Adulthood	5/60	5/60	bs
26	W09-0045	50092	NB+BV	35	Adulthood	5/60	5/60	bs
27	W09-0045	50093	NB+BV	48	Adulthood	1/300	1/300	bs
28	W09-0045	50106	NB+BV	39	Adulthood	1/60	1/60	bs
29	W09-0046	50084	NB	46	Adulthood	1/~	`/~	bs
30	W09-0046	50100	NB	40	Adulthood	3/60	3/60	bs
31	W09-0047	50094	NB	39	Adulthood	1/~	1/~	pod,aa,bs
32	W09-0047	50095	NB	33	Adulthood	1/~	1/~	pod,aa,bs
33	W09-0048	50068	NB	59	Adulthood	0	0	pod,aa,bs
34	W09-0049	50044	NB	68	Adulthood	0	0	pod,aa,bs
35	W09-0050	50082	NB	48	Adulthood	1/~	1/~	pod,aa,bs
36	W09-0041	51240	NB	64	13	1/300	1/300	pod,aa,bs

NA : Not available pod : pale optic disc BV: blurred vision
ND : Not done aa : attenuated arterioles NB: Night Blindness
bs : bone spicules

All the affected individuals have the similare fundus appearance like the figure below.

A)



B)

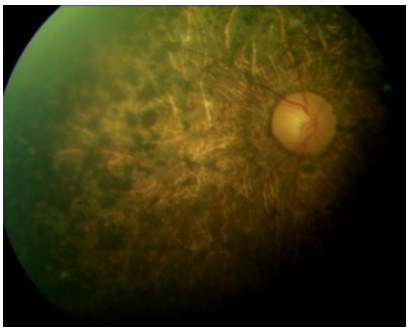


Figure 4.

Funduscopy photographs of (A) individual 50045 of family W09-0036 (affected 46-years old) left and right eye and (B) individual 50044 of family W09-0049 (affected 68-years old) on right eye, matur cataract on left eye

IV.1.3.SNP array analysis

SNP array is actually used to detect polymorphisms in the population. A SNP (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) [80] . SNPs are extremely conserved throughout evolution and within a population. By using 5.0 and 6.0 Affymetrix SNP array we get the results of homozygosity region from the affected family members in each family. From the results we take the three largest regions to be further investigated. The table below was the summary of the three biggest region of each family.

Table 13.Homozygous regions and mutations identified in this study

Family	No. of affected	No. of affected on SNP array	SNP array	# homozygous reg. > 3Mb	Rank	Chr	Start position	End position	Size [Mb]	arRP gene in the region	Mutation (DNA)	Predicted effect (protein)
W09-0036	3	3	Aff 5.0	0								
W09-0037	2	2	Aff. 5.0	1	1	1	43.472.727	47.007.137	3.5			
W09-0038	2	2	Aff 5.0	3	1	2	94.722.526	114.958.239	20.2	***MERTK***	complex rearrangement	p.G654AfsX41
					2	2	74.821.929	86.961.722	12.1			
					3	12	46.563.980	50.101.308	3.5			
W09-0039	1	1	Aff 5.0	0								
W09-0040	5	3	Aff 5.0	0								
W09-0041	4	3	Ill. 6k	1	1	15	55.310.834	76.576.278	21.3	***NR2E3***	c.1025T>G	p.V342G
W09-0042	2	2	Ill. 6k	5	1	1	88.202.625	114.480.309	26.3	***ABCA4***	c.302+4A>C	Altered splicing
					2	13	97.294.596	113.158.661	15.9			
					3	9	7.717.818	18.877.591	11.2			
					4	19	795.020	10.879.403	10.1			
					5	17	68.734.433	74.980.093	6.2	*PRCD*		
W09-0044	1	1	Aff 5.0	0								
W09-0045	4	3	Aff 5.0	3	1	7	25.394.592	32.791.157	7.4			
					2	2	109.814.503	114.973.711	5.2	***MERTK***	c.2487-2A>G	Altered splicing
					3	1	49.055.999	52.858.628	3.8			
W09-0046	3	2	Ill. 6k	3	1	5	154.427.149	164.983.908	10.6			
					2	6	74.26.927	76.965.256	69.5	*TULP1,* ***EYS***	c.9082G>T	p.D3028Y
					3	16	37.354	4.720.263	4.7			
W09-0047	2	2	Aff 5.0	2	1	5	139.038.773	163.355.069	24.3	***PDE6A***	c.1675C>A	p.Y558X
W09-0048	1	1	Aff 5.0	2	1	1	188.030.378	207.318.912	19.3	***CRB1***	c.3914C>T	p.P1305L
					2	12	82.720.660	101.627.801	18.9			
W09-0049	2	1	Aff 5.0	5	1	9	14.485.574	27.392.109	12.9			
					2	12	95.081.092	104.387.559	9.3			
					3	2	113.783.799	121.286.558	7.5			
					4	2	60.823.051	64.299.067	3.5	*FAM161A*		
					5	1	48.779.373	52.133.652	3.4			
W09-0050	2	1	Affy 5.0	21	1	1	71.823.794	120.992.603	49.2	*ABCA4, RPE65*		
					2	1	196.164.119	224.123.536	28.0	*USH2A*		
					3	5	106.422.150	131.638.131	25.2			
					4	13	30.509.319	52.819.567	22.3			
					5	4	173.631.572	191.167.888	17.5			
					6	4	121.482.239	136.825.720	15.3			
					7	1	148.152.207	161.819.282	13.7			
					8	16	55.072	12.523.392	12.5			
					9	17	6.888	9.800.824	9.8	*PRCD*		
					10	12	61.880	8.589.738	8.5			

Overview of the homozygous regions per family, and the mutations identified in this study.

From the table above, the smallest region of homozygosity (W09-0049) is 3.4 MB and the largest (W09-0050) with 49.2MB. From the facts mentioned above the possibility of a close relative marriage (consanguinity) could happen, although from history taking (anamnesis) was not found. Unfortunately not all the patients can be taken their blood samples because some families still feel ashamed of this disease. Some families are unwilling to talk about their RP instead of shame if his disease known as hereditary

disease. And to search the candidate gene for this homozygous region, genome browser was used.

Six candidate genes has been screening for sequencing analysis: phosphodiesterase 6A (*PDE6A*), rhodopsin (*RHO*), ATP-binding cassette subfamily A member 4 (*ABCA4*), C-mer proto oncogen tyrosin kinase (*MERTK*), eyes shut homolog *(EYS*), ATP-binding cassette, sub-family A member 4 (*ABCA4*) and *NR2E3* gene. The recognition of homozygosity at a specific locus pooled only by affected individuals and not by unaffected individuals was researched further by sequencing and also using markers at the position for validating homozygosity.

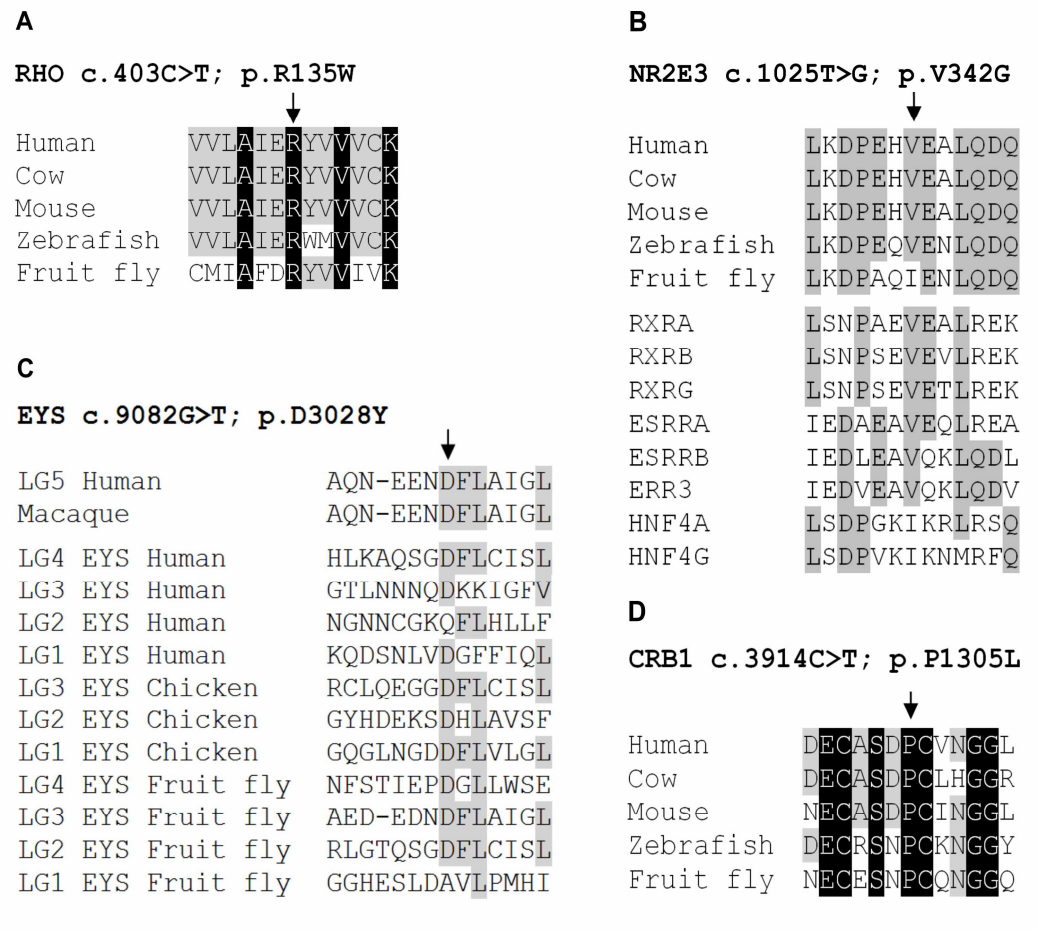


Figure 21:Sequence comparison of amino acids mutated in RP families

Shown are the mutated and flanking amino acids, from orthologous and homologous protein sequences, for A) *RHO*, B) *NR2E3*, C) *EYS* and D) *CRB1*. The arrows indicate the position of the mutated amino acid residue in the alignment. Residues that are conserved in all protein sequences are depicted in white on a black background, whereas residues that are conserved in more than 50% of the analyzed sequences are indicated in black on a grey background.

From that figure above, shown that all that mutations were took place on the very conserved region on the gene. It means that the mutations were quite possible may caused the phenotype of RP

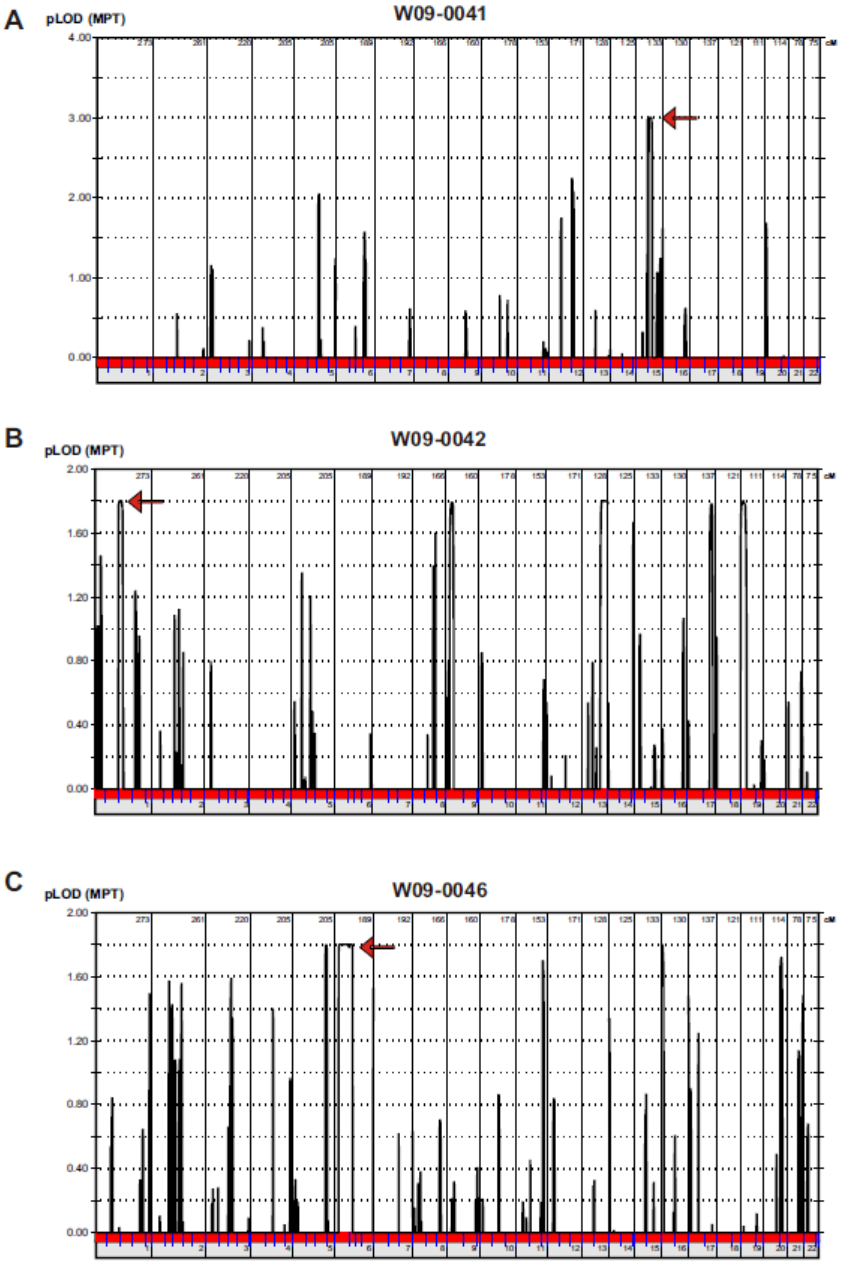


Figure 22:Overview of linkage plots for the three consanguineous Indonesian families, W09-0041 (A), W09-0042 (B) and W09-0046 (C) that were analyzed with Illumina 6k arrays. The peaks that correspond to the regions harboring the genes, in which mutations were identified, are indicated with red arrows.

From the figure 6 above, shown that the bigger region of homozygosity was the more probable that the region contain the causative gen of RP

Family W09-0035 and W09-0043

Two families with autosomal dominant RP (W09-0035 and W09-0043) inheritance was screening for *RHO* gene. The mutation on this gene are the most common cause of Retinitis Pigmentosa.[81] *RHO* gene have 5 exon and located on 3q21-25 coding for the seven transmembrane plasma membrane *RHO* protein and play a prominent role in photo transduction, that encodes the photoreceptor-specific protein. At least 54 genes sharing Rhodopsin photo transduction cascade in retina that plays a role of Ca2+ transport with *RHO*. A mutation in *RHO* is the most common gene that may implicate in adRP and it accounts for 25% to 30% of all cases in most population. More than 100 different nonsense or missense mutation that cause RP have been identified.[82] Mutation in *RHO* gene found in family W09-0035 on c.403C>T;p.R135W (heterozygous) and located in the exon 2. This mutation already described before in other studies. Individuals with this mutation (R135W) had only cone-mediated vision, because outer nuclear layer (ONL) thinned and was not detectable within 3 to 4 mm away from the fovea. The demyelanization of RPE may also occur so this condition may contribute the severity of the vision as well.[83]

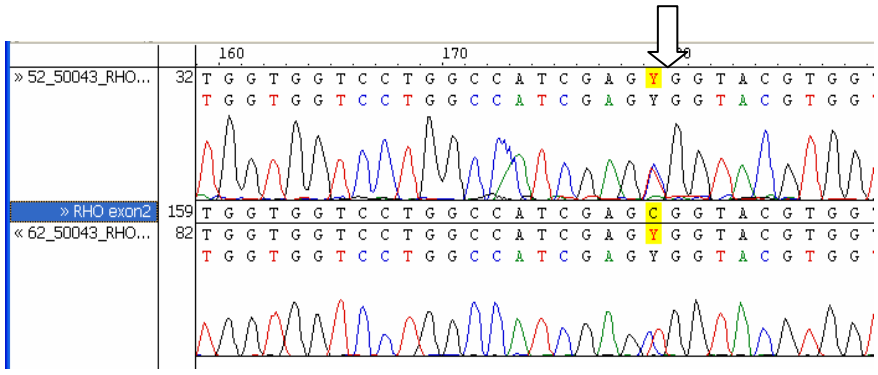
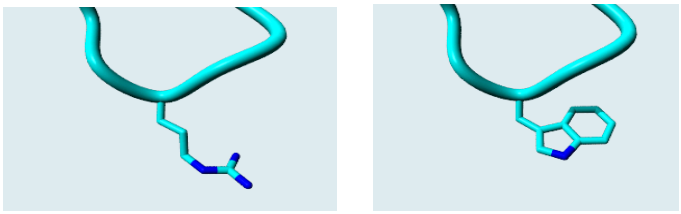


Figure23. Sequencing of RHO gene

An overview of sequencing DNA of RHO gene using the Align program on Vector NTI advance 11.0 software on patient 50043 which has mutation on p.R135W (arrow)

With certain program like Polymorphism Phenotyping (PolyPhen) prediction of possible impact of an amino acid substitution on the structure and function of a human protein can be done. Prediction is based on straightforward empirical rules which are applied to the sequence, phylogenetic and structural information characterizing the will show the differences between the wild type and the mutants and also may predict the consequences on the structure of the protein substitution. The table and figure below shows the changes of the amino acid may change the structures and eventually may cause severe phenotype.



Side chain of Wild type (R/Arginine) Side chain of Mutant type (W/Tryptophan)

Figure 24: Alteration of side chain

Prediction of amino acid structure changes caused the mutation by using polyphen. The wild type (R or Arginine) side chain and mutant type of the side chain (W or Tryptophan) because of the mutation on position 135 on RHO gene.

Patient 50043 start to felt quite difficult to see especially at a dim light at 14 years old. At that time his visual acuity still quite well because there was no other chief complain except the mild night blindness. As time goes by the complaint become worse and diagnosed with Retinitis pigmentosa. Lately the patient could not drive his own car because his visual acuity just only 0.5 and also has a tunnel vision using the confrontation test. For family W09-0043 the mutation could not be found, so we are going to examine other candidate gene that also causes autosomal dominant RP. There are several genes that might play a role in autosomal dominant RP such as retinal degeneration slow/Peripherin (*RDS*), fascin homolog, actin-bundling protein *(FSCN*), retinitis

pigmentosa 1 (RP1), semaphorin 4A (*SEMA4A*), Inosine monophosphate dehidrogenase 1 (*IMPDH1*), Precursor mRNA-processing factor 8 (*PRPF8*), Precursor mRNA-processing factor 3 (*PRPF3*).

Family W09-0038 and W09-0045

In family W09-0038 the largest homozygous region contained the *MERTK* gene. Exon 15 failed to amplify in the affected individuals so it presumed a genomic deletion (Figure 9) Detailed analysis proven the occurrence of the complex rearrangement that included a 1732-bp deletion containing exon 15 of MERTK. The absence of this exon is predicted to cause the frameshift and premature stop codon that may lead to truncation of the protein product of this gene or trigger nonsense-mediated mRNA decay.

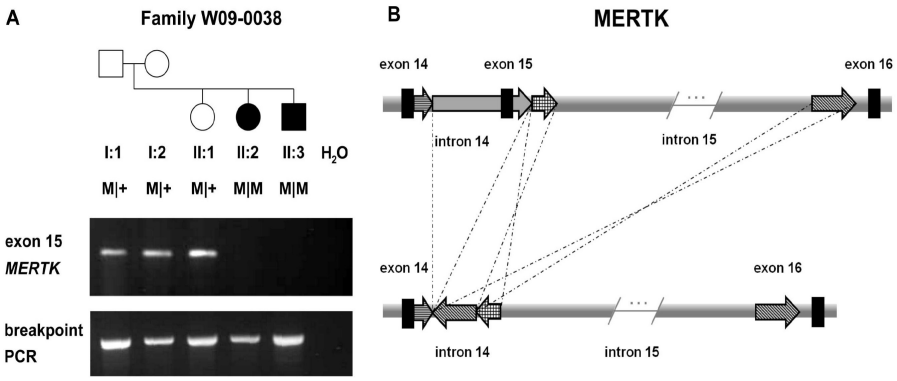


Figure 25:Molecular genetic analysis of *MERTK* in W09-0038

A) Upper panel: PCR analysis of exon 15 of *MERTK*. Exon 15 was not amplified in the two affected individuals of family W09-0038. All relatives and their position in the pedigree are indicated above the electropherogram. Lower panel: after identification of the breakpoints of the complex rearrangement, PCR primers were designed to amplify a product spanning the deletion. A PCR product indicating the presence of a rearrangement is observed in all individuals demonstrating that the unaffected family members are carriers.

B) Schematic representation of the complex rearrangement in *MERTK*. A deletion of a genomic region containing exon 15 is accompanied by duplication and an inversion event.

In family W09-0045 screened had been done to the candidate gene *MERTK* (C-mer proto oncogen tyrosinase kinase) for sequencing. This candidate gene *MERTK* was located in the second biggest homozygosity region with 5.1 MB on chromosome 2q13. The biggest homozygote region in this family was 7.4 MB with CORD9 locus on it. Splice site mutation has been on proband 50090 at position c.2487-2A>G (homozygous) at exon 19 *MERTK* gene. Two other affected individual 50093 and 50106 in this family also tested and have the same mutation as the proband.

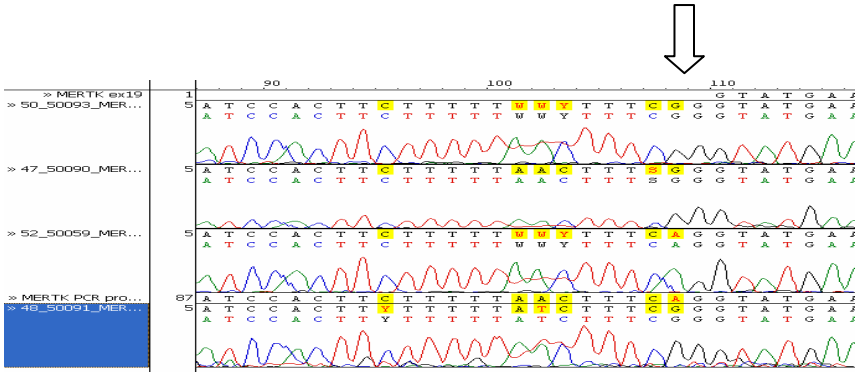


Figure26. ***Sequencing of MERTK gene***

Shown are the mutated on MERTK gene on DNA sequencing which occurred at c.2487-2A>G at exon 19 (arrow)

All affected individual in this family (W09-0045) had the same first symptoms like blur vision and night blindness at adulthood. Clinical features for affected person can be seen on the table 4 above. From the figure below the region of this mutation is quite conserve. Protein kinases play a role in a multitude of cellular processes from division, proliferation and differentiation. Mutation in this gene has been associated with disruption of the retinal pigment epithelium phagocytosis pathway and also the onset of autosomal recessive RP. The effect of the mutation in mRNA splicing was not confirmed by cDNA analysis yet. With cDNA analysis we could see if there was any alteration on

mRNA level and eventually might affect the tyrosine kinase domain. This situation could happen because there was a possibility of exonic skipping or intronic retaining.

Family W09-0041

There were four affected siblings that shared only one large homozygous region on chromosome 15 which is harboring the *NR2E3* gene. Mutation analysis of *NR2E3* identified a homozygous missense mutation that substituting a glycine for a valine residue (c.1025T>G;p.Val342Gly). The mutation was homozygously present in five affected family members and was absent of heterozygously present in three unaffected siblings. By using the 374 ethnically matched normal control panel DNA from the same population of this family, this variant was not detected. *NR2E3* encoded a transcription factor belongs to the family of nuclear hormone receptors. RP patients with mutated *NR2E3* usually present with clumped pigment deposits that are distinct from typical bone spicules observed in RP. All affected individuals have the age onset at teenager as shown at table 4 above.

Family W09-0042

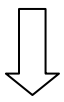
Screening *ABCA4* gene has been performed in this family on the biggest region at chromosome 1 which has 24.8 MB. Mutation in photoreceptor-specific ATP binding cassette transporter (*ABCA4*) gene are responsible for several disease such Stargardt disease (*STGD1*), autosomal recessive cone rod dystrophy, age related macular degeneration and also autosomal recessive retinitis pigmentosa.[84-86] The contribution of *ABCA4* to the development of arRP is about less than 5 %, and it has been account that this gene is associated in 60 % of the arCRD. The *ABCA4* gene which located 1p22.1 encodes an ATP-binding cassette (ABC) transport protein that located at the rim of the

photoreceptor discs. ABC protein transports various molecules across extra and intracellular membranes. The mutation on this gene may cause the accumulation of the all Trans retinal in the photoreceptor discs due to dysfunction of the ABCR protein.[40,41]

The intronic mutation found on patient 50085 at position c.302+4A>C (homozygous) shown at figure 9. To ensure the effect of this mutation several techniques can be used like functional study (mRNA) segregation on other family. In this case segregation analysis will be performed on other family member. In addition, the variant was also not detected in 326 ethnically matched control chromosomes. *In silico* prediction of the strength of the splice donor site showed a small decrease due to the alteration, indicating that this variant might alter *ABCA4* splicing. Hence the pathogenicity of this variant remains unclear.

Affected on this family has the early onset on childhood with blurred vision and night blindness. Most *ABCA4* associated RP patients with early loss of visual function and severe atrophy will carry the homozygous null whereas in patients with classic RP mostly heterozygous *ABCA4.*

The rate of photoreceptor cell death was related to the severity of mutation on *ABCA4* gene. A combination of mutations that results in a moderate dysfunction of the ABCR protein leads to STGD1. A more severe impairment of ABCR result in a CRD phenotype, whereas complete absence of function causes RP.[88]



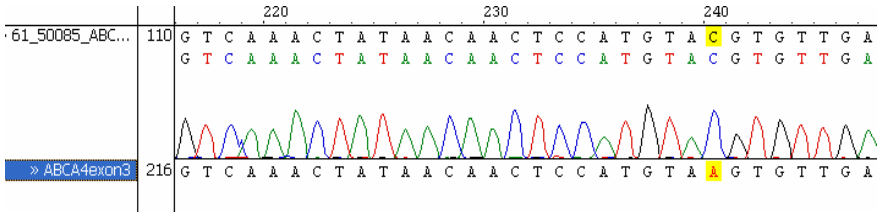


Figure 27. ***Sequencing of ABCA4 gene***

Sequencing DNA of ABCA4 gene using the Align program on vector NTI advance 11.0 software on patient 50085 which has mutation on c.302+4A>C (arrow)

Family W09-0046

In this family, screened has been performed on the biggest homozygosity region (69.5 MB) which is consisting the *EYS* gene. *EYS* gene located on chromosome 6q12 and consists of 44 exons with 3165 amino acids. This gene contains multiple epidermal growth factor (EGF) like and Laminin G (lamG) domain. Protein product from this gene is expressed in the photoreceptor of the human retina. *EYS* gene also interact with other gene like *PROM1,CRB1* that may play a role in rod outer segment disc morphogenesis and form a critical component of Muller cell and photoreceptor, photoreceptor cell and photoreceptor cell interaction.[77]

Missense mutation in c.9082G>T; p.D3028Y has been found on patient 50084. This mutation takes place in laminin AG domain. Actually laminins are the major protein in the lamina basalis which is the protein network foundation for the most cells and organs.

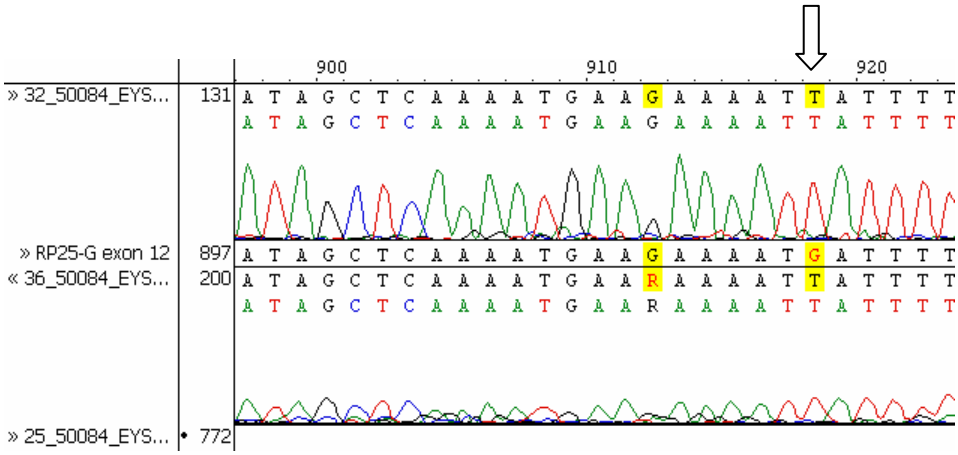
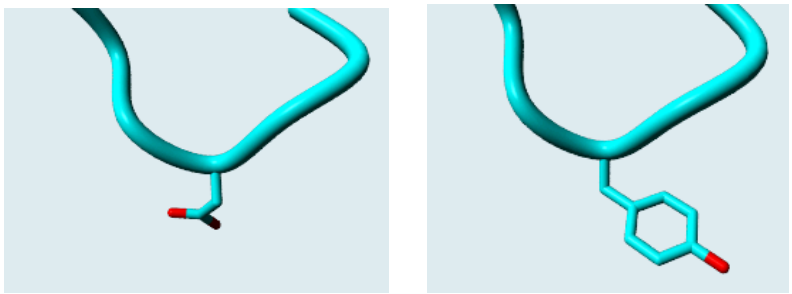


Figure 28.*Sequencing of EYS gene*

Sequencing DNA of EYS gene using the Align program on Vector NTI advance 11.0 software on patient 50084 which has mutation on c.9082G>T (arrow)

Using the polyphen we can predict the possibility that might happen if there is a change in the amino acid. According to this prediction, the mutation causes a change a negative residue into neutral residues. This may also affect the ionic interaction and eventually destabilize the structure. The D residue also more hydrophilic than the new (Y) one, it means that it can make hydrogen bonds with either other residues when located in the core or with other molecules when located on the surface. The new residues are also bigger than the old one and it can be detrimental for the structure because the new residue simply does not fit at that position (figure 13).



Side chain D/Aspartic(wild type) Side chain Y/Tyrosine(mutant)

Figure29. Alteration of side chain

Missense mutation c.9082G>T may cause alteration on side chain and finally also affect the protein product itself.

When this happens on the surface interaction with other molecules can be disturbed. The protein motifs of EYS gene could be signal peptide, EGF-domain, Calcium binding EGF-domain, EGF-like domains, o-linked glycosylation, laminin A G-like domains.

The clinical feature of affected patients can be seen on table 4. Restriction fragment length polymorphism (RFLP) or ARMS (Amplification Refractory Mutation System) could be used to check whether this variant also occur at the control panel to find the prevalence and the pathogenicity.

Family W09-0047

Mutation in the *PDE6A* that encoding the alpha subunit of rod cyclic guanosin monophospate (cGMP) phospodiesterase is responsible for autosomal recessive retinitis pigmentosa. cGMP is an important regulator of rod cell membrane current and its dynamic concentration is establish by phosphodiesterase 6A cGMP hydrolysis and guanylate cyclase cGMP synthesis. This protein is a subunit of a key photo transduction enzyme and participates in processes of transmission and amplification of the visual signals.

On the biggest homozygosity region (24.3 MB) which is harbor the *PDE6A* on chromosome 13 mutations has been found. Sequencing of *PDE6A* showed a homozygous single base pair change c.1675C>A (homozygous) which lead to stop mutation p.Y558X. Stop mutation on exon 13 might cause the truncation of the protein product and finally might deteriorate the function of this gene because of mediated decay of *PDE6A* mRNA. This mutation occur in the HDc domain (556 -734) that is found in the superfamily of enzymes with a predicted or known phosphohydrolase activity. These enzymes appear to be involved in the nucleic acid metabolism, signal transduction. This domain also found in

3',5' cGMP phospodiesterase which is located in photoreceptor outer segments and it light activated and playing a pivotal role in signal photo transduction.

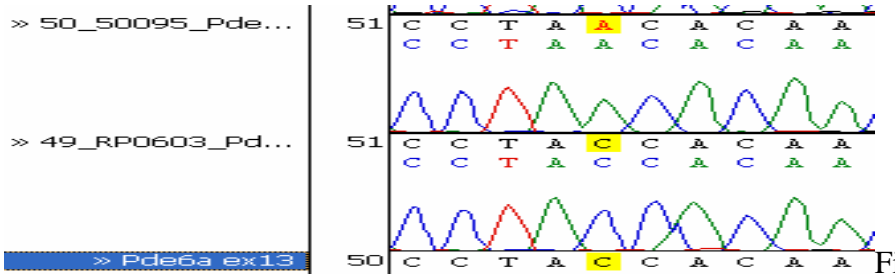


Figure 30:

Sequencing of PDE6A gene

Sequencing DNA of PDE6A gene using the Align program on Vector NTI advance 11.0 software on patient 50095 which has mutation on c.1675C>A (arrow)

Family W09-0048.

The largest homozygous region detected in the affected person 50068 from family W09-0048 harbored the *CRB1* gene, and sequence analysis found a transition of cytosine to thymine and resulting the substitution of a leucine for a proline residue (c.3914C>T; p.Pro1305Leu). This alteration did not detected in 298 ethnically matched normal control panel. The fact that the proline residue is highly conserved throughout vertebrae evolution and of course it may suggests that this mutation is causative for arRP in this family.

IV.2.DISCUSSION

This is the first report about molecular genetic analysis of RP in the Indonesian population. Ophthalmologic examination by using the simple techniques such as visual acuity with Snellen chart, slit lamp biomicroscopy and direct funduscopy may useful to screen the RP patient. Visual field analysis and photo fundus color examination also beneficial. All the RP patients were taken their vein blood and extracted their DNA by using salting out procedure.

Pedigree analysis / reconstruction were performed to classify the type of inheritance among those families. Affected family members have been identified by pedigree reconstruction. By doing the pedigree analysis the type of inheritance of the family could be made. Two individuals are identical-by-descent for a particular allele if they each have a copy of the same ancestral allele.

There were several consideration that should taken into account to classified to adRP or arRP such as consanguineous, affected individual in every level of the family. By combining the type of inheritance, homozygosity mapping (Illumina 6k or Affymetrix 5.0

SNP array) and mutation analysis has revealed eight different probable pathogenic mutations in eight out of sixteen families that were analyzed.

This method is quite accurate to harbor the mutation on the RP gene. SNP array is also a useful tool to study the whole genome. The most important application of SNP array is in determining disease susceptibility and consequently, in pharmaco-genomics by measuring the efficacy of drug therapies specifically for the individual.

As each individual has many SNP together create a unique DNA sequence. SNP-based genetic linkage analysis could be performed to map disease loci, and hence determine disease susceptibility genes for an individual especially in RP patients. The combination of SNP maps and high density SNP array allows the use of SNPs as the markers for Mendelian diseases with complex traits efficiently. [1,14,17] The more markers that been used for the array the more sensitive that array as well, and of course the possibility to harbor the mutations on the candidate gene was higher.[26,27]

Despite a tremendous gain in knowledge about the genetic causes of RP over the last decade, nothing was known about the genetic causes of RP in the Indonesian population. The identification of several novel mutations in this study indicates that the same genes compared to other populations are involved in the etiology of arRP and adRP in Indonesia.[16,17,32]

There were three of these mutations are missense changes that have not been previously described.[16,48,88] .The pathogenic effect of missense alteration may be difficult to interpret in the absence of suitable functional assay or animal models. However, due to the high degree of conservation of the mutated amino acid residues their location within predicted function domains of the encoded protein and the absence of these alleles in

ethnically matched control individuals, the missense variants in *NR2E3, EYS* and *CRB1* are considered to be the likely pathogenic mutation in the respective families. Mutations in NR2E3 actually might cause dominant or recessive retinal degeneration. Thirty two different mutations in NR2E3 have been identified in either homozygous or compound heterozygous state in the recessively retinal degenerations.[87,88] The high variability of clinical phenotypes observed in patients affected by NR2E3-linked retinal degenerations may be caused by different disease mechanisms, including absence of DNA-binding, altered interactions with transcriptional co-regulators, and differential activity of modifier genes.[88] In family W09-0041 fortunately was the autosomal recessive RP. As seen on the high LOD score 3.0 on chromosome 15, its mutation (p.Val342Gly) on *NR2E3* also takes place. Such as *NR2E3* the other missense mutation (*CRB1* and *EYS*) also the novel mutation whereas there were no report with the same site with these mutations.[48,52,87]

On family W09-0035 the mutation was on p.R135W which is one of the causative gene of the adRP that already published on several journal. In this family the proband (50043) and the mother has the same phenotype. Mutations in the rhodopsin gene (*RHO*; OMIM ID: +180380) account for about 25% of the dominantly inherited RP cases and less than a few percent of recessively inherited cases The Pro23His mutation is the most frequently reported rhodopsin mutation in the United States, accounting for about 8.5% of all dominant RP cases or about 1/3 of those with a dominant rhodopsin mutation.[90,91]

MERTK mutation on family W09-0045 and W09-0045 were also the novel ones because have not published yet in other journal. On the journal the mutation were took place in , c.718G→T in exon 4, which results in a premature termination of p.E240X and also the Long-range PCR identified a ~9 kb deletion within *MERTK* that removes exon 8

deletion.[92,93]. In our study the 1732 bp deletion containing exon 15 also happen accompanied by a inversion and duplication event and also splicing site alteration on c.2487-2A>G at exon 19

Since the identification of the *ABCA4* gene as the cause of autosomal recessive (ar) Stargardt disease/fundus flavimaculatus (STGD/FF) much has been draw up of the phenotypic variability in *ABCA4* retinopathy, from age-related macular degeneration (ARMD) in heterozygous carriers to bull's eye maculopathy (BEM), ar-cone rod dystrophy (CRD) and ar-retinitis pigmentosa (RP). That is why the spectrum of disease seen in patients with *ABCA4* retinopathy is very broad. In order to fully characterize a patient's phenotype, examination using multiple modalities of investigation, as well as careful history and clinical examination is necessary.[94,95] With the several high linkage plot it was really tricky to choose the right location whereas the mutation takes place. By using the information at genome browser screened the ABCA4 in chromosome 1 reveal the mutation. The intronic mutation gene in consanguineous family W09-0042 at c.302+4A>C (*ABCA4)* was also the novel one .[40,41,94]

Phosphodiesterase 6A, cGMP-specific rod alfa mutation (*PDE6A*) on family W09-0047 at p.Y558X (homozygous) also the novel mutation based on the several articles.[33,34,87] Truncation of the protein product because of the stop mutation may affect the severity of phenotype on the RP patients on this family. Eventhough their age is quite young at 33 and39 years old but their visual acuity is very poor just light perception.

The *EYS* gene until now was the biggest gene that may affect the RP if there was the mutation on it.[17] The mutations p.D3028 described an affect highly conserved residues at homologous positions in laminin A G-like domains and support the notion that missense

mutations in *EYS* can cause arRP.[96] Two affected individuals in the family W09-0046 has this mutation and suffer the impaired vision with light perception (46 years old) and counting finger (40 years old). As seen on the pedigree that the mutation must be homozygous.

With this kind of pedigree it is quite difficult to predict whether this is dominant or recessive RP. By using the SNP array the biggest homozygous region with 19.3 Mb there was a missense mutation (p.Pro1305Leu) in *CRB1*. The visual acuity on the affected individual (50068) was no light perception. This mutation also the novel one according to the human genome variation society with databases of mutation on this gene.

All those mutations that have been found also already checked by using at least 200 control DNA panel, and there was no polymorphism or mutation on the control. In seven arRP families no causative mutation was detected in one of the known RP genes. Hopefully DNA sequencing on the other region especially the regions that are homozygously shared by two or more affected sibs from one family might aid the identification of novel genes that are causative for arRP.

Mostly of the mutated gene in this study occurred at the first up to third biggest of the homozygous region, this fact similar with the several articles that have been published. In addition, next generation sequencing technologies, either using predefined linkage intervals or homozygous regions will be instrumental in identifying novel genetic causes of RP in Indonesian patients.

Association of a specific genetic defect (genotype) with a specific clinical feature (phenotype) is called genotype-phenotype correlation. In general genotype-phenotype correlation refers to the association of alteration of a specific gene with specific clinical

feature such as the association of the mutations in this study with retinitis pigmentosa. In a specific gene is invariably association with specific phenotype. Some genes and their defects such as in ABCA4 gene and its associated phenotype show a considerable degree of genotype-phenotype correlation.

Clinical feature is also strongly influenced by additional modifying factors such as nutritional factors, light exposure and smoking. Retinal disorders like RP may also show considerable genetic heterogeneity, meaning that mutations in different genes result in clinical similar phenotype. [97,98]

Parents who have a child with a recessive disease who are normal themselves can expect that 25 % of their future children will be affected. Unaffected parents who have a child with an X-linked disease have a 50 % risk of having affected male children. Females only carry the X-linked gene, while males suffer from the disease. A better known example of this pattern of inheritance is color blindness and RP. Dominantly inherited disease is manifested in all individuals who carry the gene. Thus one parent is usually affected as are 50 % of the children. Sporadic diseases are new mutations and the particular inheritance is unclear until further generations exhibit the trait. Genetic counselling depends on an accurate diagnosis, determination of the mode of inheritance in each family, and results of molecular genetic testing, thus by doing the DNA sequencing, the prediction of the phenotype to the siblings could be established.

Counselling about prognosis should include information regarding the great variation among and within inheritance groups, families, and individuals with respect to age of onset and natural history of the disorder. Because the study of the treatment is

currently available but not for all RP patients, genetic counselling and supportive follow-up should be viewed as an essential service for this common group of genetic disorders, and cooperation with the ophthalmologic diagnostician should be actively sought.

Genetic testing and determining a molecular diagnosis of diseases such as the RP might provide the ophthalmologist and involved families an estimate of the probable clinical course of the disease and also the chances/possibilities to affect for their offspring. There are several purposes for doing genetic testing as it aids ophthalmologists in the clinical management of RP patients and facilitates vision scientists in their quest to identify novel disease genes.

1. Improve diagnostic accuracy.
2. Provide prognostic information.
3. Establish a genotype–phenotype correlation system, in order to suggest the causal gene from the retinal phenotype.
4. Identify new retinal pathways.
5. Provide prenatal screening.
6. Identify new genes.
7. Guide therapy.

The limitation of this study is because the current technology is not fully 100% sensitive because of false positive results such as polymorphic variants. To determine whether the mutation might cause the disease of polymorphism it ought to be performed the functional assays in vitro cell culture. In our study this kind of assay could not be performed because the mRNA was not ready yet. The other limitation also related to the

high cost of performing the high resolution homozygosity mapping whereas it is possible to perform this array in the developing country like Indonesia without any contribution/charity from other institution. If there were a very big region of homozygosity in the families this kind of method is not effective also because still the priority should me made among genes that consists of these region. To tackle this limitation fortunately there was the next generation of sequencing analysis which could sequence the whole genome.