

ARTICLE

Comprehensive genotyping reveals *RPE65* as the most frequently mutated gene in Leber congenital amaurosis in Denmark

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Leber congenital amaurosis (LCA) represents the most severe form of inherited retinal dystrophies with an onset during the first year of life. Currently, 21 genes are known to be associated with LCA and recurrent mutations have been observed in *AIPL1*, *CEP290*, *CRB1* and *GUCY2D*. In addition, sequence analysis of *LRAT* and *RPE65* may be important in view of treatments that are emerging for patients carrying variants in these genes. Screening of the aforementioned variants and genes was performed in 64 Danish LCA probands. Upon the identification of heterozygous variants, Sanger sequencing was performed of the relevant genes to identify the second allele. In combination with prior arrayed primer extension analysis, this led to the identification of two variants in 42 of 86 cases (49%). Remarkably, biallelic *RPE65* variants were identified in 16% of the cases, and one novel variant, p.(D110G), was found in seven *RPE65* alleles. We also collected all previously published *RPE65* variants, identified in 914 alleles of 539 patients with LCA or early-onset retinitis pigmentosa, and deposited them in the *RPE65* Leiden Open Variation Database (LOVD). The *in silico* pathogenicity assessment of the missense and noncanonical splice site variants, as well as an analysis of their frequency in ~60 000 control individuals, rendered 864 of the alleles to affect function or probably affect function. This comprehensive database can now be used to select patients eligible for gene augmentation or retinoid supplementation therapies.

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INTRODUCTION

Leber congenital amaurosis (LCA; OMIM 204000) is a group of rare inherited retinal dystrophies that affects ~1 in 50 000 individuals worldwide.^{1,2} LCA is characterized by a severe visual impairment in the first year of life, sensory nystagmus, amaurotic pupils and markedly diminished or extinguished rod and cone electroretinograms (ERG).^{3,4} LCA is predominantly inherited in an autosomal recessive manner, although autosomal dominant inheritance has also been reported a few times.⁴ To date, mutations in 21 genes underlie at least 70% of LCA cases.^{5,6} These genes encode proteins that exert their functions in diverse processes and pathways in the retina, such as photoreceptor morphogenesis (*CRB1*, *CRX*, *GDF6*), retinal differentiation (*OTX2*), phototransduction (*AIPL1*, *GUCY2D*, *RD3*), retinoid cycle (*LRAT*, *RDH12*, *RPE65*), guanine synthesis (*IMPDH1*), signal transduction (*CABP4*, *KCNJ13*), outer segment phagocytosis (*MERTK*), coenzyme NAD biosynthesis (*NMNAT1*) or intra-photoreceptor ciliary transport processes (*CEP290*, *IQCB1*, *LCA5*, *RPGRIP1*, *SPATA7*, *TULP1*).^{4,6} Mutations in many of these genes can also give rise to early-onset retinal dystrophies.

The *RPE65* and *CHM* gene augmentation trials in patients with LCA^{7–9} and choroideremia,¹⁰ respectively, have been milestones in the

retinal therapeutics field. An oral retinoid-based treatment was also reported to be beneficial for individuals with *RPE65* or *LRAT* mutations.¹¹ Preclinical gene augmentation studies in animal models due to mutations in several other genes have also demonstrated safety and efficacy.¹² However, the identification of molecular genetic causes is a prerequisite to assess the patients' eligibility in receiving suitable treatments. Various techniques, including classical genetic linkage analysis, identity-by-descent (IBD) mapping, Sanger sequencing of candidate genes or next-generation sequencing, have been used for gene discovery and diagnostic methods in LCA. Previous studies in LCA revealed the intronic *CEP290* variant c.2991+1655A>G as the most frequent in the Caucasian population, explaining ~12% of cases in Caucasian populations originating from North-West Europe.^{13–15} In addition, the *AIPL1* variant p.(W278*) in exon 6, a conspicuous cluster of *CRB1* variants in exons 7 and 9, and the *GUCY2D* exon 12 variant p.(R768W) together may explain ~14% of the cases.^{16,17} Therefore, prescreening of these 5 exons potentially identifies 26% of the mutations, serving as a cost- and time-effective genotyping procedure for LCA. Finally, screening of *RPE65* and *LRAT* should uncover the cause of disease in 7 and 1% of LCA cases, respectively.⁴ The identification of individuals with mutations in

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these genes is very relevant in view of the above-mentioned treatment options.

By using a combination of different genotyping techniques, we identified the underlying mutations in approximately half of the Danish LCA probands, and most significantly found *RPE65* variants to be the most prevalent causes of LCA and early-onset RP. In addition, we collected all published *RPE65* variants and completed the *RPE65* Leiden Open Variation Database. These results are essential for genetic counseling and for the selection of individuals who are eligible for therapeutic interventions.

MATERIALS AND METHODS

Subjects and clinical evaluation

A total of 86 unrelated Danish families comprising 80 isolated and 6 multiplex families diagnosed with LCA were ascertained in this study. The diagnosis of LCA was based on clinical history, a full eye exam including slit-lamp and retinal examination, full-field electroretinography (ffERG), visual acuity, refraction, visual field and optical coherence tomography (OCT) in some cases. The diagnostic criteria were based on a congenital or early infantile onset of a generalized retinal dystrophy with substantially reduced cone and rod ffERG responses. Many patients showed nystagmus and/or hypermetropia, but these clinical signs were not compulsory for the diagnosis.^{18,19} In this study, we consider LCA and early-onset retinitis pigmentosa as a continuum of phenotypes, as they are clinically and genetically overlapping and the true age at disease onset can be difficult to establish. Research procedures were in accordance with institutional guidelines and adhered to the tenets of the Declaration of Helsinki. An ethics committee approval was obtained and all samples were acquired after written informed consent was obtained from the patient or, in the case of children, their legal guardians.

Genetic analysis

Genomic DNA of participating individuals was isolated from peripheral leukocytes using a standard salting out procedure.²⁰ A candidate gene approach and an arrayed primer-extension (APEX) analysis were performed in several probands before recurrent mutation screening. Complete genotyping methods for the affected individuals are listed in Supplementary Table S1.

Sanger sequencing in selected LCA-associated genes

Sanger sequencing based on a candidate gene approach was performed in the LCA-associated genes *AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *LRAT*, *RDH12*, *RPE65*, *RPGRIP1* and *TULP1* by a commercial supplier (Seqlab, Göttingen, Germany) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a ABI 3730XL platform (Applied Biosystems).

SSCP and APEX analysis

Single-strand conformation polymorphism (SSCP) analysis was applied to 52 index patients as described previously²¹ followed by subsequent Sanger sequencing of exons with aberrant bands in *AIPL1*, *CEP290*, *CRX*, *CRB1*, *GUCY2D*, *LRAT*, *RDH12*, *RPE65*, *RPGRIP1* and *TULP1*. Thereafter, APEX LCA microarray analysis, versions 2007–2012 (Asper Ophthalmics, Tartu, Estonia) was performed in the 15 remaining unsolved cases.²² This microarray-based genotyping method allowed the simultaneous analysis of previously identified disease-associated sequence variants in the LCA-associated genes *AIPL1*, *CEP290*, *CRB1*, *CRX*, *GUCY2D*, *IQCB1*, *LCA5*, *LRAT*, *MERTK*, *RDH12*, *RD3*, *RPE65*, *RPGRIP1*, *SPATA7* and *TULP1*. The methods employed in this analysis have been described elsewhere (www.asperbio.com; Asper Biotech, Ltd).²³

LCA recurrent mutation screening

The screening was performed in cases carrying one or no variant identified using Sanger sequencing of selected LCA-associated genes or APEX analysis, and 19 additional cases that had not been previously analyzed. Recurrent mutation screening was performed in 13 amplicons of four LCA-associated genes. The *CEP290* c.2991+1655A>G variant was assessed by utilizing the amplification-refractory mutation system (ARMS) as described by den

Hollander *et al.*¹³ In addition, *AIPL1* exon 6, *GUCY2D* exon 12 and *CRB1* exons 7 and 9 were amplified and sequenced by the Sanger method. Primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and are listed in Supplementary Table S2. Purified PCR products were analyzed in either sense or antisense direction using dye termination chemistry (BigDye Terminator, version 1.1, on a 3730 DNA analyzer; Applied Biosystems, Inc.). All identified sequence variants were validated with independent PCR reactions.

Sequence analysis of *RPE65* and *LRAT*

All coding exons and flanking intronic sequences of *RPE65* and *LRAT* were amplified by PCR, purified and analyzed using Sanger sequencing as described above. PCR primers and conditions are available upon request.

Ethnically matched control frequencies

The allele frequency of each identified variant in the Danish population was assessed using whole-exome data of 2000 ethnically matched individuals from the Danish exome study.²⁴

Pathogenicity interpretation of sequence variants

To determine the pathogenicity of missense variants, we used the following *in silico* prediction methods: Sorting Tolerant from Intolerant (SIFT, <http://sift.jcvi.org/>), Polymorphism Phenotyping v2 (PolyPhen-2; <http://genetics.bwh.harvard.edu/pph2/>) and PhyloP (<http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=cons46-way>) the latter of which assesses the evolutionary conservation at the nucleotide level. We also assessed the allele frequency data of identified variants derived from an available 'control' exome data set provided by NHLBI Exome Variant Server (EVS, <http://evs.gs.washington.edu/EVS/>) and the Danish exome study.²⁴ For the *RPE65* variants deposited in the *RPE65* LOVD, we also assessed their prevalence using the Exome Aggregate Consortium (ExAC) database (<http://exac.broadinstitute.org>). Five computational prediction programs, SpliceSite finder-like,²⁵ MaxEntScan,²⁶ NNSPLICE,²⁷ Gene Splicer²⁸ and Human Splicing Finder²⁹ were used to determine the pathogenic effect of noncanonical splice variants.

Sequence variants nomenclature

All identified variants are described as recommended by the Human Genome Variation Society (HGVS) nomenclature guidelines, considering nucleotide +1 as the A of start codon (ATG).³⁰ When the nucleotide position is indicated, the GenBank cDNA reference sequences were used as follows: *AIPL1* (NG_008474.1); *CEP290* (NG_008417.1); *CRB1* (NG_008483.1); *GUCY2D* (NG_009092.1); *LRAT* (NG_009110.1); *RDH12* (NG_008321.1); *RPE65* (NG_008472.1); *RPGRIP1* (NG_008933.1); and *TULP1* (NG_009077.1).³¹

RPE65 Leiden open variation database variant depositions

RPE65 variants were collected from all articles published until 2014 (Supplementary Tables S5–S7). To assess the pathogenicity of missense variants, we combined three prediction programs, that is, PhyloP, SIFT and PolyPhen-2, as well as the allele count in the ExAC database that contains exome data of ~60 000 individuals (<http://exac.broadinstitute.org>). A PhyloP score of ≥ 2.5 was employed as one of the criteria to consider a variant to be potentially disease causative. Subsequently, we classified missense variants as 'probably no functional effect', 'variants of unknown significance' or 'affects function'. They were considered to have 'probably no functional effect' when none of the prediction programs described the variant as damaging and/or if the ExAC allele count was ≥ 18 . The latter cutoff number was chosen as the three most frequent pathologic *RPE65* variants, p.(R91W), p.(Y368H) and c.11+5G>A, representing 11.6, 8.0 and 7.2% of the published 864 autosomal recessive *RPE65* function-affecting variants, respectively, were found in 10, 8 and 11 alleles in the ExAC database. Statistical analysis of the most frequent *RPE65* variant c.11+5G>A, with a total allele count of 120 956 in the ExAC database for this position, results in a 95% confidence interval between 0.3×10^{-4} and 1.4×10^{-4} . From this we can infer that any variant with an allele frequency $> 1.4 \times 10^{-4}$ can be considered to be benign with 95% certainty. Assuming the maximum number of alleles present in the ExAC database (122 972 alleles),

benign variants are expected to be present in ≥ 18 alleles. The pathogenicity of a variant was considered as 'variants of unknown significance' if one of three prediction programs described the variant as damaging and the allele count in ExAC was < 18 . Finally, a variant was considered to 'affect function' when at least two prediction programs described the variant as damaging and the ExAC allele count was < 18 .

RESULTS

Molecular genetic analysis

Genotyping analysis before recurrent mutation screening.

For diagnostic purposes, genotyping analysis using Sanger sequencing of selected LCA-associated genes and/or APEX analyses were performed in 27 and 40 LCA cases, respectively (Figure 1). Using the selected Sanger sequencing approach, two function-affecting variants were identified in eight cases and one variant in three cases. In addition, APEX analysis detected two variants in 14 cases and one variant in one case. Subsequently, 41 cases without any identified variants were included in our recurrent mutation screening.

LCA recurrent mutation screening and second allele sequencing.

Sixty-four individuals carrying no or one variant were included in the screening for the presence of frequent LCA mutations. Two function-affecting variants were identified in 11 cases and 15 carried only one variant. Additional Sanger sequencing to find a second allele identified additional variants in nine of the latter individuals. Taken together, the LCA recurrent mutation screening

followed by a search for a second allele by Sanger sequencing identified two variants in 20 cases (23%) and one variant in six cases (7%), as shown in Figure 1.

Interestingly, based on the number of cases with two causal variants, this study revealed the *RPE65* gene as the most frequently mutated gene in this Danish LCA cohort (16%). Out of 46 identified variants, 17 are novel variants (Table 1 and Supplementary Table S4) with the 3 most frequent alleles being c.2991+1655A>G in *CEP290* (8 alleles), p.(W278*) in *AIPL1* (7 alleles) and, surprisingly, a novel p.(D110G) variant in *RPE65* (7 alleles). Segregation analysis in 11 families in whom parents and/or siblings were available showed that the unaffected parents and/or siblings each carried one of the two variants (Figure 2).

Three variants, each identified in a heterozygous state in one LCA proband, *AIPL1* c.286G>A; p.(V96I), *AIPL1* c.286G>A; p.(Y134F) and *GUCY2D* c.2101C>T; p.(P701S), were identified in 2.8, 1.0 and 2.6% of 2000 ethnically matched Danish control individuals, respectively, ruling them out as highly penetrant LCA-causing alleles. In four probands with one or two causal variants in a given gene, we identified an additional variant in a different gene.

The benign *GUCY2D* p.(P701S) variant was identified in one proband, together with a likely pathogenic variant, that is, p.(A950V). However, as p.(P701S) is deemed benign and sequencing was not completely performed for all exons of *GUCY2D*, this case remains unsolved but a second mutation can be located in another exon.

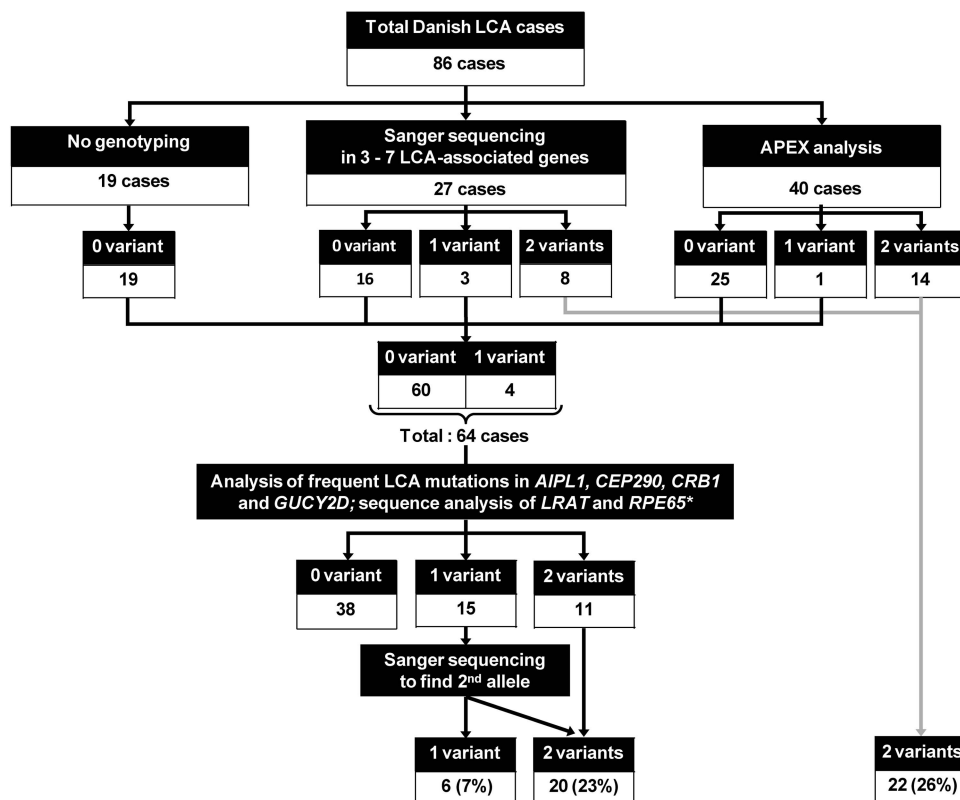


Figure 1 Genotyping workflow in this study. Eighty-six individuals with LCA were included in this study. Twenty-seven cases were sequenced for variants in several LCA-associated genes, 40 cases were analyzed using APEX analysis and 19 cases had not been analyzed. Thereafter, 60 patients carrying no variants and 4 patients with one variant were included in the screening of common LCA mutations in *AIPL1* exon 6, *CEP290* c.2991+1655A>G variant, *CRB1* exons 7 and 9, *GUCY2D* exon 12 and all coding exons of *RPE65* and *LRAT*. Together with the previous genotyping analysis, this screening yielded one variant in 6 cases and two variants in 42 cases.

Table 1 Overview of function-affecting variants identified in Danish LCA patients

Fam. ID	Gene	Allele 1		Allele 2		Gene	Allele 3		Segregation analysis
		Nucleotide change	Amino acid change	Nucleotide change	Amino acid change		Nucleotide change	Amino acid change	
1	<i>GUCY2D</i>	c.2513G>C	p.(R838P)	+	+				NA
2	<i>CRB1</i>	c.2549G>T	p.(G850V)	c.4006G>A	p.(V1340I)				ND
4	<i>CEP290</i>	c.2991+1655A>G	p.(C998*)/p.= ^a	c.2991+1655A>G	p.(C998*)/p.= ^a				ND
5	<i>CEP290</i>	c.2991+1655A>G	p.(C998*)/p.= ^a	+	+				NA
14	<i>RPE65</i>	c.329A>G	p.(D110G)	+	+	<i>GUCY2D</i>	c.1093C>T	p.(R365W)	NA
17	<i>CEP290</i>	c.2991+1655A>G	p.(C998*)/p.= ^a	c.5364+2T>G	p.(?)				ND
20	<i>CRB1</i>	c.2290C>T	p.(R764C)	c.2843G>A	p.(C948Y)				ND
21	<i>RPE65</i>	c.495+1dup	p.(?)	c.130C>T	p.(R44*)				Yes
23	<i>CEP290</i>	c.2991+1655A>G	p.(C998*)/p.= ^a	c.2119_2123dupCAGCT	p.(Q709Sfs*11)				ND
26	<i>GUCY2D</i>	c.2302C>T	p.(R768W)	c.1877T>C	p.(S626F)				ND
27	<i>CEP290</i>	c.2991+1655A>G	p.(C998*)/p.= ^a	c.2119_2123dupCAGCT	p.(Q709Sfs*11)				ND
28	<i>AIPL1</i>	c.834G>A	p.(W278*)	+	+				NA
30	<i>CEP290</i>	c.2991+1655A>G	p.(C998*)/p.= ^a	c.5364+2T>G	p.(?)	<i>AIPL1</i>	c.244C>T	p.(H82Y)	ND
33	<i>RPE65</i>	c.1543C>T	p.(R515W)	c.130C>T	p.(R44*)				ND
38	<i>RPE65</i>	c.329A>G	p.(D110G)	c.11+5G>A	p.(?)				ND
42	<i>RPE65</i>	c.329A>G	p.(D110G)	c.11+5G>A	p.(?)				ND
43	<i>AIPL1</i>	c.834G>A	p.(W278*)	c.834G>A	p.(W278*)				ND
46	<i>RPE65</i>	c.94G>T	p.(G32C)	c.130C>T	p.(R44*)				Yes
49	<i>GUCY2D</i>	c.2302C>T	p.(R768W)	c.1694T>C	p.(F565S)				ND
51	<i>RPE65</i>	c.1328T>C	p.(V443A)	c.1102T>C	p.(Y368H)				ND
52	<i>RPE65</i>	c.11+5G>A	p.(?)	c.1102T>C	p.(Y368H)				ND
55	<i>RPE65</i>	c.11+5G>A	p.(?)	c.1088C>G	p.(P363R)				ND
58	<i>GUCY2D</i>	c.2135_2136delinsTC	p.(E712V)	c.3043+5G>A	p.(?)				ND
59	<i>AIPL1</i>	c.815G>C	p.(R272P)	+	+				NA
62	<i>AIPL1</i>	c.834G>A	p.(W278*)	c.834G>A	p.(W278*)				ND
63	<i>AIPL1</i>	c.834G>A	p.(W278*)	c.834G>A	p.(W278*)				ND
64	<i>CRB1</i>	c.4121_4130del10	p.(A1374Nfs*20)	c.4121_4130del10	p.(A1374Nfs*20)				ND
65	<i>CRB1</i>	c.613_619delATAGGAA	p.(I205Dfs*13)	c.2401A>T	p.(K801*)				ND
66	<i>CRB1</i>	c.584G>T	p.(C195F)	c.613_619delATAGGAA	p.(I205Dfs*13)	<i>RPGRIP1</i>	c.2869G>A	p.(E957K)	ND
67	<i>CEP290</i>	c.1219_1220delAT	p.(M407Efs*14)	c.2991+1655A>G	p.(C998*)/p.= ^a				ND
68	<i>GUCY2D</i>	c.1561C>T	p.(R521*)	c.1561C>T	p.(R521*)				ND
69	<i>RDH12</i>	c.379G>T	p.(G127*)	c.379G>T	p.(G127*)				ND
70	<i>RDH12</i>	c.2T>C	p.(M1T)	c.582C>G	p.(Y194*)				ND
71	<i>GUCY2D</i>	c.2302C>T	p.(R768W)	c.1877T>C	p.(S626F)	<i>RPGRIP1</i>	c.2555G>A	p.(R852Q)	Yes
72	<i>GUCY2D</i>	c.1957G>A	p.(G653R)	c.2234delC	p.(P745Lfs*39)				Yes
73	<i>GUCY2D</i>	c.2302C>T	p.(R768W)	c.2302C>T	p.(R768W)				Yes
74	<i>GUCY2D</i>	c.2849C>T	p.(A950V)	+	+				NA
75	<i>GUCY2D</i>	c.779T>C	p.(L260P)	c.779T>C	p.(L260P)				Yes
76	<i>GUCY2D</i>	c.2849C>T	p.(A950V)	c.380C>T	p.(P127L)				NA
77	<i>RPE65</i>	c.495+1dup	p.(?)	c.1374G>A	p.(W458*)				Yes
78	<i>RPE65</i>	c.271C>T	p.(R91W)	c.271C>T	p.(R91W)				Yes
79	<i>RPE65</i>	c.329A>G	p.(D110G)	c.329A>G	p.(D110G)				Yes
80	<i>RPE65</i>	c.769T>G	p.(Y239D)	c.1102T>C	p.(Y368H)				Yes
81	<i>RPGRIP1</i>	c.930+3A>G	p.(?)	c.3618-5del	p.(?)				ND
82	<i>TULP1</i>	c.1495_1496dup	p.(D500Vfs*4)	c.1495_1496dup	p.(D500Vfs*4)				ND
83	<i>CRB1</i>	c.613_619delATAGGAA	p.(I205Dfs*13)	c.2401A>T	p.(K801*)				ND
85	<i>RPE65</i>	c.1102T>C	p.(Y368H)	c.130C>T	p.(R44*)				Yes
86	<i>RPE65</i>	c.329A>G	p.(D110G)	c.329A>G	p.(D110G)				ND

NA, not applicable; ND, not done.

Novel variants are marked in bold.

^aThe deep-intronic *CEP290* mutation c.2991+1655A>G in ~50% of the RNA results in the insertion of an intronic sequence in the mRNA that carries a stop mutation, and in ~50% of the RNA has no effect on splicing.**Pathogenicity prediction of novel variants**

Of the 46 identified variants in this study, 17 are novel, consisting of 8 missense, 5 splice site, 2 frameshift and 2 nonsense variants. None of the missense variants was present in 6503 individuals in the EVS

database or in 2000 Danish control individuals. Based on *in silico* predictions, all missense variants except p.(V1340I) in *CRB1* (found in F2) are deemed pathogenic (Table 2). The *AIPL1* missense variant p.(R272P) affects a highly conserved amino acid

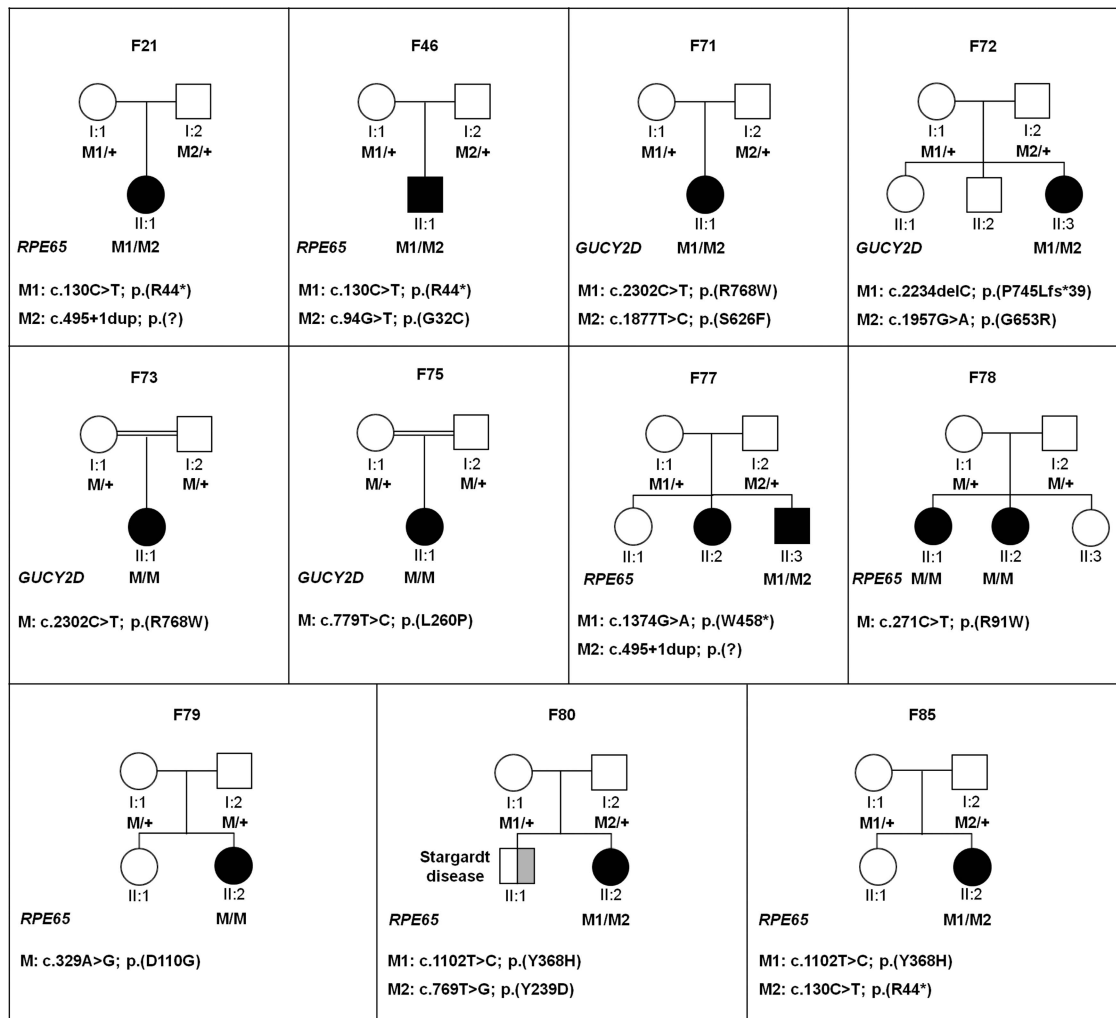


Figure 2 Family pedigrees and segregation analysis. Pedigrees of patients for whom parent samples were available. All variants segregate as expected in the families.

Table 2 The *in silico* predictions of the functional effects of the identified novel missense variants

Gene	cDNA	Amino acid	SIFT	PPH2	PhyloP value	Final classification
<i>AIP1L1</i>	c.815G>C	p.(R272P)	Deleterious	Probably damaging	1.09	Affects function
<i>CRB1</i>	c.2549G>T	p.(G850V)	Deleterious	Probably damaging	5.37	Affects function
<i>CRB1</i>	c.4018G>A	p.(V1340I)	Tolerated	Benign	-0.04	No functional effect
<i>GUCY2D</i>	c.1877C>T	p.(S626F)	Deleterious	Probably damaging	3.03	Affects function
<i>GUCY2D</i>	c.2135_2136delinsTC	p.(E712V)	Deleterious	Probably damaging	4.56/0.45	Affects function
<i>GUCY2D</i>	c.380C>T	p.(P127L)	Deleterious	Probably damaging	2.87	Affects function
<i>RPE65</i>	c.94G>T	p.(G32C)	Deleterious	Probably damaging	5.53	Affects function
<i>RPE65</i>	c.329A>G	p.(D110G)	Deleterious	Probably damaging	4.73	Affects function
<i>RPE65</i>	c.1088C>G	p.(P363R)	Deleterious	Probably damaging	5.77	Affects function

SIFT, <http://sift.jcvi.org/>.

Polymorphism Phenotyping v2 (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>).

PhyloP, <http://genome.ucsc.edu/cgi-bin/hgTrackUI?g=cons46way>.

in the tetratricopeptide (TPR) domain and may disrupt the interaction with the Hsp70 and Hsp90 molecular chaperones, crucial for facilitating retinal protein maturation.³² The *CRB1* missense variant p.(G850V) affects a conserved glycine in the second laminin G domain, encoded by one of the most frequently

mutated regions in *CRB1*. Three missense variants in *RPE65* affect conserved amino acid residues located in the carotenoid oxygenase that is essential for its isomerohydrolase activity. By using several *in silico* tools, three out of five novel splice site variants in *CEP290*, *GUCY2D*, *RPE65* and *RPGRIP1* are predicted to affect normal

Table 3 The *in silico* predictions of the functional effects of the novel splice site variants

	Splice Site prediction tool	Splice Site				EVS	Final classification	
		Finder-like ²⁵	MaxEntScan ²⁶	NNSPLICE ²⁷	GeneSplicer ²⁸			HSF ²⁹
	Score range	(0–100)	(0–12)	(0–1)	(0–15)	(0–100)		
	Threshold	≥ 70	≥ 0	> 0.4	≥ 0	≥ 60		
CEP290	c.5364+2T>G	77.40 ⇒ —	5.54 ⇒ —	0.68 ⇒ —		83.55 ⇒ —	T = 0/G = 13006	Affects function
GUCY2D	c.3043+5G>A		7.55 ⇒ —	0.53 ⇒ —	9.83 ⇒ 2.60 (–73.5%)	80.99 ⇒ 68.82 (–15.0%)	T = 0/A = 13006	Affects function
RPE65	c.495+1dup	80.23 ⇒ —	6.71 ⇒ —	0.74 ⇒ —		92.09 ⇒ —	A1 = 0/R = 13006	Affects function
RPGRIP1	c.3618–5del	81.46 ⇒ 75.54 (–7.3%)	5.98 ⇒ 6.71 (+12.2%)	0.97 ⇒ 0.79 (–19.3%)		82.94 ⇒ 78.83 (–5.0%)	A1 = 0/R = 13006	No functional effect
RPGRIP1	c.930+3A>G	76.52 ⇒ —	8.40 ⇒ 0.05 (–99.4%)	0.85 ⇒ —		96.23 ⇒ 80.67 (–16.2%)	G = 33/A = 12973	No functional effect

splicing (Table 3). None of these splice site variants were found in 2000 Danish control individuals.

Clinical findings

Clinical characteristics of the patients with novel variants are presented in Table 4 and Figure 3. Patients carrying *CRB1* variants display an early-onset retinal degeneration with maculopathy and nummular pigmentations in the retina that are typical features for individuals with variants in *CRB1*.¹⁷ As reported for other cases with *RPGRIP1* variants, patient 81 had poor vision in the first year of life with nystagmus and hypermetropic eyes (+5.5/+6.0).³³ Early-onset visual impairment and nystagmus were also observed in patient 71 carrying *GUCY2D* variants who showed no ERG responses at the age of 1 year.^{34,35}

Patients carrying two *RPE65* variants presented with symptoms of nystagmus, lack of fixation, night blindness and/or orientation difficulties within the first year of age, except one patient who according to the anamnesis presented with visual impairment at 2 years of age. All patients showed extinct ERG responses and suffered from nystagmus and severe night blindness. The fundi were generally characterized by retinal degeneration and attenuated vessels with later signs of optic nerve atrophy and in some patients mottled or bone spicule pigmentations. Mean visual acuity at the age of 7 years for all *RPE65*-associated cases was 0.18 (SD 0.17). Most patients had severely constricted visual fields at the age of 10 years; however, a few patients had preserved visual fields at later stages. Similar phenotype data were described for individuals with *RPE65* variants in several previous studies.^{36,37}

RPE65 LOVD variant depositions

Novel and previously described variants identified in this study have been listed in the respective LOVD for the mutated genes.^{38–43} In addition, we collected all *RPE65* variants that were published up to 2014. In total, we identified 914 variant alleles in 539 affected individuals that were described in 79 papers. In Supplementary Table S5, we list the 152 different variants according to their position in the *RPE65* gene. Almost all variants were inherited in an autosomal recessive manner. Exceptions are p.(D477G), which was reported in a large autosomal dominant RP family,⁴⁴ and one case showing uniparental isodisomy of chromosome 1 carrying c.11+5G>A.⁴⁵ For the missense and noncanonical splice site variants, we show the *in silico* predictions of their pathogenic nature in Supplementary Tables S6 and S7, respectively. Based on the pathogenicity criteria described in the Materials and methods, 128 variants were designated

‘affects function’, 10 with no functional effect and 12 as variants of unknown significance.

Missense variants that are present in ≥ 18 of ~ 60 000 individuals in ExAC (p.(A132T); p.(K294T); p.(N321K); p.(A434V); each of which are present in > 300 alleles in ExAC) are considered as variants with no functional effect. Altogether, 231 and 144 individuals carried homozygous and compound heterozygous variants, respectively. The number of individuals with heterozygous variants is more difficult to assess as the *RPE65* gene was not fully sequenced in many studies. For 6 individuals with one protein-truncating variant and 10 individuals with one probably function-affecting missense variant, the entire gene was analyzed by sequence analysis.

The three most frequent variants are c.271C>T; p.(R91W), c.1102T>C; (p.Y368H) and c.11+5G>A, identified in 100/864 (11.6%), 69/864 (8%) and 62/864 (7.2%) of the disease-causing alleles, respectively.

DISCUSSION

In this study, disease-causing variants were identified in 49% (42/86) of the Danish LCA probands using different genotyping techniques. The recurrent mutation screening identified causal variants in 23% of the cases. The full potential of this approach was not used as APEX analysis and selective Sanger sequencing had previously been performed for diagnostic purposes in some cases. Assuming that these cases also had undergone the recurrent mutation analysis, the potential yield would have been 36% (Table 1), rendering it an inexpensive prescreening method for Danish LCA cases, and potentially also for other populations.

Sequence analysis of *LRAT* yielded no results but, remarkably, *RPE65* was found to be the most frequently mutated gene (16%) in the Danish LCA cohort, followed by *GUCY2D* (10%), *CEP290* (7%), *CRB1* (7%), *AIPL1* (4%), *RDH12* (3%), *TULP1* (1%), and *RPGRIP1* (1%). We cannot rule out that as yet undiscovered LCA-associated genes are among frequently mutated genes in Danish LCA cases. Importantly, the most frequent *RPE65* variant identified in this study was p.(D110G) (seven alleles), which hitherto was not described. It thereby is almost as frequent as the *CEP290* variant c.2991+1655A>G, found in eight alleles, and equally frequent as the *AIPL1* variant p.(W278*) in seven alleles. The p.(D110G) variant was found in individuals from Denmark and Jordan, and hence it remains to be investigated whether this is a founder mutation.

This study revealed the thus far highest prevalence for *RPE65* variants in the Caucasian population. An equally high frequency of

Table 4 Clinical phenotypes of affected individuals carrying two novel causal variants

Fam. ID	Gene	Origin	Gender	Age at disease presentation	Refraction		Nystagmus	ERG (age)	Fundus	Other pathology
					LE/RE (Age)	(Age)				
2	<i>CRB1</i>	Denmark	F	3 Years	+6.0/+5.5 (6)		No	Nearly extinct (6)	Early bull's eye maculopathy and later nummulate pigmentations and attenuated vessels	None
21	<i>RPE65</i>	Denmark	F	Congenital	+7 (22)		Yes	Extinct (1)	Attenuated vessels, no hyperpigmentation	Epilepsia
46	<i>RPE65</i>	Denmark	M	Congenital	-4.0/-4.0 (20)		Yes	Extinct (1)	Attenuated vessels, no hyperpigmentation	None
55	<i>RPE65</i>	Denmark	F	Congenital	-1/+2 (1)		Yes	Extinct (1)	Attenuated vessels and muddy retinal pigmentation	None
71	<i>GUCY2D</i>	Denmark	F	Congenital	+6/+6 (1)		Yes	Extinct (1)	Attenuated vessels and muddy retinal pigmentation	None
79	<i>RPE65</i>	Denmark	F	Congenital	-2/-3.25 (1)		Yes	Extinct cone response and reduced rod response (1)	Attenuated vessels and pale optic nerve	None
81	<i>RPGRIP1</i>	Pakistan	F	1 Year	+5.5/+6.0 (1)		Yes	Extinct (3)	Attenuated vessels and no hyperpigmentation	None
86	<i>RPE65</i>	Jordan	M	2 Years	-1.5/-2.0 (15)		Yes	Extinct (17)	Attenuated vessels, atrophic optic nerve and bone spicule pigmentations	None

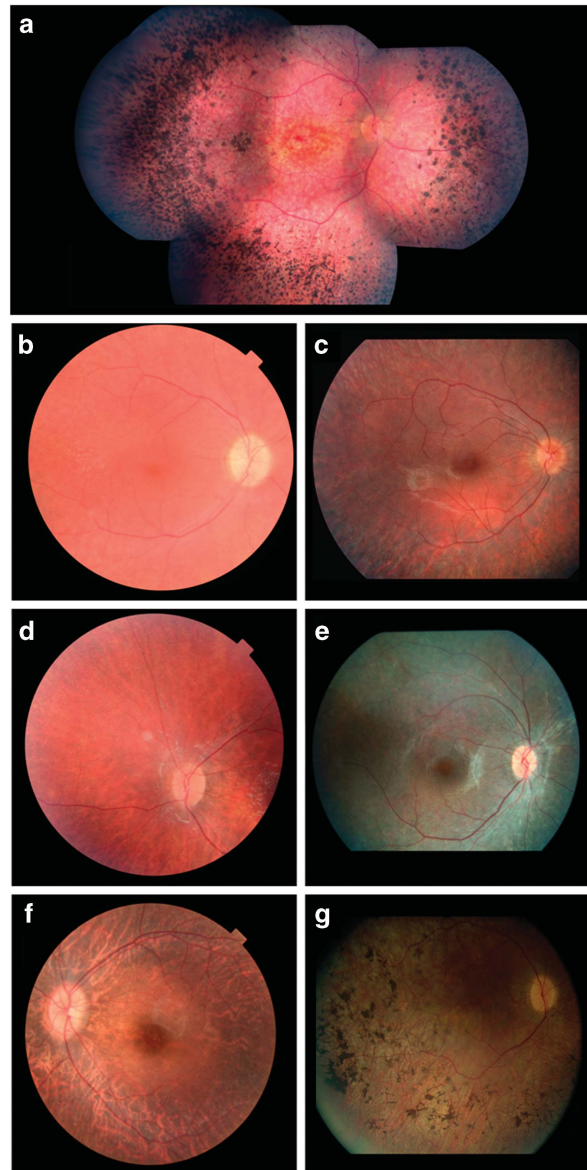


Figure 3 Fundus pictures of LCA cases with novel variants. (a) The fundus of proband F2 with variants p.(G850V) and p.(V1340I) in *CRB1*, at the age of 28 years, showing central maculopathy, peripheral nummular pigmentations and attenuated vessels. F2 had a refraction of +6.0/+5.5 at the age of 6 years. (b) Fundus of patient F21-II:1 with variants c.495+1dup and p.(R44*) in *RPE65*, at the age of 17 years, showing attenuated vessels and no hyperpigmentation. At 22 years, the refraction was +7/+7. (c) Fundus of patient F46-II:1 with variants p.(G32C) and p.(R44*) in *RPE65*, at the age of 17 years, showing attenuated vessels and no hyperpigmentation. Refraction was -4.0/-4.0 at the age of 20 years. (d) Fundus picture of proband F55 with variants c.11+5G>A and p.(P363R) in *RPE65*, at the age of 6 years, showing attenuated vessels and slight muddy retinal pigmentation in the periphery. Refraction was -1/+2 at the age of 1 year. (e) Fundus picture of proband F81 with variants c.930+3A>G and c.3618-5del in *RPGRIP1*, at 9 years of age, showing attenuated vessels and no hyperpigmentation. Refraction was +5.5/+6.0 at the age of 1 year. (f) Fundus of patient F79-II:2 with a homozygous *RPE65* p.(D110G) variant, at the age of 9 years, showing attenuated vessels and a pale optic nerve. Refraction was -2/-3.25 at the age of 1 year. (g) Fundus of proband F86 with a homozygous p.(D110G) variant in *RPE65*, at the age of 40 years. The fundus shows attenuated vessels, atrophic optic nerve and pigment aggregations. Refraction was -1.5/-2.0 at the age of 1 year.

RPE65 variants (16.5%) was reported recently in the Indian population.⁴⁶ Our findings facilitate the identification of Danish patients who are eligible for the AAV-*RPE65* gene augmentation and/or 9-*cis*-retinoid supplementation therapies. By including the sequence analysis of *RPE65* exons 1, 3, 4 and 10, carrying 19 variants, to the recurrent mutation screening (8 exons) for Danish LCA cases, the potential yield would increase to 45%. The clinical phenotype of patients with two *RPE65* variants was relatively mild compared with several other more severe genetic types of LCA and in general similar to what was previously reported.³⁶

In five individuals with LCA (F1, F5, F14, F28, F59), one heterozygous variant was identified upon sequence analysis of the entire coding regions of the corresponding genes. The *GUCY2D* variant p.(R838P) has been reported in previous LCA studies as an autosomal dominant mutation. In our study, this variant occurs in a sporadic case. In the absence of parental DNAs, we cannot test the possibility that it is a *de novo* variant. In the remaining cases, the identification of heterozygous variants could be attributed to one of the following possibilities. A deletion or duplication in the other allele may not be detected as copy number variant analysis was not performed. Our screening also does not cover deep-intronic regions, and thus variants in this part of the genome will not be identified.^{15,47} Moreover, variants in regulatory elements located far away from the respective genes cannot be ruled out. Finally, the aggregate carrier frequency of variants in the most intensively studied genes in this study (*AIPL1*, *CEP290*, *CRB1*, *GUCY2D*, *LRAT* and *RPE65*) is estimated 1 in 25 individuals (Supplementary Table S3), meaning that three or four probands could carry a variant in one of these genes in this cohort of 86 families, without pathologic consequences. The latter may also explain the identification of more than two variants in different genes in four probands in this study. The identification of the remaining causal variants requires a more comprehensive analysis of all genes associated with LCA and early-onset RP, for example, using next-generation sequencing-based gene-panel sequencing, whole-exome or whole-genome sequencing.

By collecting all *RPE65* variants reported in literature, we found *RPE65* variants in 539 individuals, of whom 375 carried two variants. Among all reported variants, several variants tend to occur exclusively or in high frequency in certain ethnicities suggesting possible founder effects, for example, variant c.304G>T; p.(E102*) in the Greek population, c.1102T>C; p.(Y368H) in the Dutch population and c.271C>T; p.(R91W) in Saudi Arabia and Tunisia. Three variants (p.(R91W), p.(Y368H) and c.11+5G>A) together comprise 26.7% of all *RPE65* variants identified to date. One splice site variant (c.94+10A>G) and 10 missense variants were designated benign. However, the latter missense variants are rare in LCA patients and in ExAC and therefore in the absence of functional assay data cannot completely be excluded to have functional effects.

Sixteen individuals carried only one pathogenic *RPE65* variant, despite the fact that all exons and exon-intron boundaries were analyzed by Sanger sequencing. As described above and in Supplementary Table S3, we estimate the carrier frequency for *RPE65* variants in the general population to be 1/158 individuals. Among the 516 individuals with one or two recessive *RPE65* variants, three or four would be expected to carry such a variant in a heterozygous state by chance. The fact that 16 individuals were found to be heterozygous carriers of variants may point to the existence of 'missed mutations'. These could be heterozygous deletions, deep-intronic variants or other variants affecting the expression of *RPE65*

mRNA. Individuals carrying two causal *RPE65* variants are eligible for the above-mentioned novel therapies.

In summary, we identified the underlying mutations in a significant group of cases providing the most comprehensive genetic data of LCA in the Danish population. Most significantly, we identified *RPE65* as the most frequently mutated LCA gene in Denmark, and this is of particular interest in view of the ongoing therapeutic trials using gene augmentation or 9-*cis* retinoid supplementation. Finally, we collected all published variants of the *RPE65* gene and performed *in silico* assessment of the missense and noncanonical splice variants to predict their functional effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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