EYS is a major gene involved in retinitis pigmentosa in Japan: genetic landscapes revealed by stepwise genetic screening

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Next-generation sequencing (NGS) has greatly advanced the studies of causative genes and variants of inherited diseases. While it is sometimes challenging to determine the pathogenicity of identified variants in NGS, the American College of Medical Genetics and Genomics established the guidelines to help the interpretation. However, as to the genetic screenings for patients with retinitis pigmentosa (RP) in Japan, none of the previous studies utilized the guidelines. Considering that EYS is the major causative gene of RP in Japan, we conducted stepwise genetic screening of 220 Japanese patients with RP utilizing the guidelines. Step 1–4 comprised the following, in order: Sanger sequencing for two major EYS founder mutations; targeted sequencing of all coding regions of EYS; whole genome sequencing; Sanger sequencing for Alu element insertion in RP1, a recently determined founder mutation for RP. Among the detected variants, 2, 19, 173, and 1 variant(s) were considered pathogenic and 8, 41, 44, and 5 patients were genetically solved in step 1, 2, 3, and 4, respectively. Totally, 44.5% (98/220) of the patients were genetically solved, and 50 (51.0%) were EYS-associated and 5 (5.1%) were Alu element-associated. Among the unsolved 122 patients, 22 had at least one possible pathogenic variant.

The global estimate of retinitis pigmentosa (RP), the most prevalent form of the inherited retinal dystrophies (IRD) across all nations and ethnicities, is 1:4000, and it is a leading cause of severe visual disabilities and blindness in developed countries2–4. It is clinically and genetically heterogeneous. About 100 causative genes have been identified and novel causative genes and mutations are now being reported annually5–7.

Recent studies have revealed the spectrum of causative genes and steadily laid the groundwork for genetic approaches to treatments for IRD6–14. Many clinical trials of gene therapies are also ongoing. The approval of Voretigene neparvovec as the first gene therapy for Leber congenital amaurosis (LCA)15, signaled the dawn of gene therapy for IRD16–18. Given this background, identifying causative genes and their mutations for each IRD among various ethnicities will become more important.

Next-generation sequencing (NGS) can rapidly and accurately detect variants in DNA samples from large cohorts, and this has substantially contributed to genetic screening19–21. However, NGS screening has led to the novel difficulty of determining the pathogenicity of enormous numbers of detected variants. The American College of Medical Genetics and Genomics (ACMG) standards and guidelines have recently become the de-facto standard criteria to address this problem22.

The reported genetic diagnostic rates of retinitis pigmentosa in Japan have varied, being 36.6% in 2014 by Oishi M et al8 and 29.6% in 2018 by Koyanagi Y et al12. Those previous studies were performed without considering the ACMG classifications of variants, because the ACMG guidelines had not yet been launched in 2014, and because previously reported and null variants served as the criteria for pathogenic variants in the latter study.

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Therefore, it would be practical to investigate whether employing the ACMG methodology would improve genetically diagnostic yield.

Moreover, considering that EYS is the major causative gene of RP in Japan, we constructed the stepwise genetic screenings of patients with RP—starting with the direct Sanger sequencing for the two EYS founder mutations in Japan, followed by, targeted NGS for all exons of the EYS gene, and whole genome sequencing (WGS).

With the above research questions and the scientific backgrounds, the aim of our current study is to perform the stepwise genetic analyses for patients with RP and elucidate the landscapes of the causative genes and variants of RP in Japan using the ACMG guidelines.

**Methods**

All procedures used in the current study adhered to the tenets of the Declaration of Helsinki. The Institutional Review Board of Kyoto University Graduate School of Medicine approved the study protocols (G746). All patients and their relatives were fully informed of the purpose and procedures of the study, and written consent to participate was obtained from each patient and their family member, if available.

**Study participants.** The single-center study included 223 unrelated patients with RP who attended the Department of Ophthalmology at Kyoto University Hospital, Kyoto, Japan, between 2006 and 2016 and agreed to participate. An IRD specialist (O.A.) clinically diagnosed the subjects based on a history of visual symptoms and the findings of comprehensive ophthalmologic examinations. We excluded three patients with syndromic RP; two with cerebellar hypoplasia and one with mitochondrial disease (myoclonus epilepsy associated with ragged-red fibers). Finally, we analyzed data from 220 patients. Pedigrees were assembled based on detailed interviews of patients and their family members, from which inheritance patterns were inferred. With reference to a previous report, if at least one family member other than the proband was affected but the pedigree was not suggestive of an autosomal-recessive (AR), autosomal-dominant (AD), X-linked (XL), or sporadic trait, we regarded the result as “inconclusive”. Genomic DNA was extracted from venous blood using QuickGene-610L DNA extraction kits (Fujifilm, Minato, Tokyo, Japan).

**Stepwise sequence analyses.** Figure 1 shows the stepwise genetic analyses, starting with Sanger sequencing for two major EYS founder mutations (c.8805C>A; p.Y2935X and c.4957dupA; p.S1653fs) in Japanese patients with RP. Targeted NGS for all coding regions of EYS was conducted by NGS using an Illumina MiSeq.
| Step   | Description                                           | Detected variants | Variants evaluated as pathogenic by the criteria | Genetically solved patients |
|--------|-------------------------------------------------------|-------------------|-----------------------------------------------|-----------------------------|
| Step1  | Sanger sequence for two EYS founder mutations         | 2                 | (1) "DM" or "DM?" in HGMD professional          | 8                           |
| Step2  | Targeted sequencing for all EYS exons                 | 67                | (2) "pathogenic" or likely pathogenic by ACMG guidelines | 0                           |
| Step3  | Whole Genome Sequence                                 | 1730              | (3) Large deletions or insertions               | 41                          |
| Step4  | Sanger sequence for Alu element                       | 1                 |                                               | 44                          |

Total solved patients 98

Table 1. Number of detected variants, pathogenic variants, and genetically solved patients in each step. Totally 98 patients were genetically solved.

Results

We analyzed 220 patients (female, n = 102; male, n = 118) with RP (AD-RP, n = 4; AR-RP, n = 48; XL-RP, n = 5; sporadic RP, n = 137; inconclusive, n = 26).

We generated 5.7 Gb and 17.2 Tb DNA sequences for targeted sequencing and WGS analysis. On average, 98.7% and 99.1% of the reads were mapped to the reference sequence, which corresponded to 85.7% and 97.3%, respectively, of the target bases being covered with a depth of at least 10x.

The first step: Sanger sequencing of two major EYS founder mutations. In the first step, direct Sanger sequencing for two major EYS founder mutations in Japan, 3, 2, and 3 patients were genetically solved with homozygous c.4957dupA (p.S1653fs), homozygous c.8805C>A (p.Y2935X), and compound heterozygous c.4957dupA (p.S1653fs) and c.8805C>A (p.Y2935X), respectively.

Variant data analysis. A total of 10.7 kb DNA segments covering the exons of the EYS gene were sequenced using the Illumina MiSeq. The target regions were specifically amplified using 44 oligonucleotide pairs and the Tks Gflex enzyme (Takara Bio; Kyoto, Japan). Whole genome sequencing was conducted using an Illumina HiSeq X Ten system (Illumina Inc.). The sequence reads were aligned onto the reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner, then downstream analyses were conducted using Picard and GATK version 3.8, according to the GATK Best Practice recommendations included marking duplicates, base quality recalibration, haplotype calling, joint variant calling, and variant quality score recalibration (VQSR). Copy number variations were identified by the variance of read coverage in 5-kb bins along the reference genome sequence. Regions that were greater or less than 3/8 of the mode of the coverages were designated as alterations.

Definition of pathogenic variants and VUS. We analyzed all variants detected in the exons and their boundaries (± 2 bps) of the genes registered for IRD in RetNet (Retinal Information Network, https://sph.uth.edu/retnet/, Supplementary Table 1), and determined pathogenic variants and variants of uncertain significance (VUS) as follows (Table 1). In the current study, we presumed only the previously-reported inheritance pattern for each gene. For example, we evaluated SNRNP200 only for AD-trait inheritance because it is registered only for AD-trait in RetNet. Among the detected variants in the genes registered for IRD (or syndromic RP) in RetNet, we considered those satisfying at least one of the following criteria as pathogenic: 1) variants registered as "DM" or "DM?" for RP (or syndromic RP) in the Human Genome Mutation Database Professional (HGMD professional, 2019.2), but we filtered out variants with a MAF of >1% in gnomAD even if registered in HGMD professional because RP is a rare Mendelian disease; 2) variants evaluated as "likely pathogenic" or "pathogenic" by ACMG guidelines, and 3) large deletions or insertions including an Alu insertion in RP1.

Among the detected variants in the genes registered for IRD in RetNet, we determined VUS in accordance with the criteria of ACMG guidelines.

In the current study, we considered AIPL1, CRB1, and RCBTB1 to be causative only for the AR trait, and did not consider FSCN2, GUCA1B, OR2W3, PITPNM3, RIMS1, ROM1, SEMA4A, UNC119, and CA4 to be causative for IRD, because their pathogenicity for the AD trait was in doubt as a result of an allele frequency analysis in a recent study by Hanany et al.26 (The latter group of genes; FSCN2, GUCA1B, OR2W3, PITPNM3, RIMS1, ROM1, SEMA4A, UNC119, and CA4 were registered only in AD traits in RetNet).

Supplementary Table 1 shows the reference numbers of the genes analyzed herein.

References

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The first step: Sanger sequencing of two major EYS founder mutations. In the first step, direct Sanger sequencing for two major EYS founder mutations in Japan, 3, 2, and 3 patients were genetically solved with homozygous c.4957dupA (p.S1653fs), homozygous c.8805C>A (p.Y2935X), and compound heterozygous c.4957dupA (p.S1653fs) and c.8805C>A (p.Y2935X), respectively.
Totally, 8 patients were genetically solved in this step, and the 212 unsolved patients proceeded to the next step. Among the 212 patients, heterozygous c.4957dupA (p.S1653fs) and heterozygous c.8805C>A (p.Y2935X) were detected in 25 and 8 patients, respectively.

The second step: targeted sequencing for all coding regions of EYS. In the second step, targeted sequencing for all coding regions of EYS, 67 variants were detected, and 14 and 5 variants were evaluated as pathogenic satisfying the criteria of 1) registered as causative for RP in HGMD professional 2019.2 and 2) evaluated as likely pathogenic in ACMG guidelines, respectively.

Among the 212 unsolved patients in the first step, 41 patients were genetically solved in the second step. Focusing on the 33 carriers of two founder mutations analyzed in the first step, 14 patients had compound heterozygous variants c.4957dupA (p.S1653fs) and other pathogenic variants, and 5 patients had compound heterozygous variants c.8805C>A (p.Y2935X) and other pathogenic variants.

The 171 unsolved patients proceeded to the third step of WGS.

The third step: WGS. In the third step, WGS, 1730 variants were detected in the IRD genes shown in Supplementary Table 1, and among them, 1142 variants belonged to the genes registered as causative for RP or syndromic RP in RetNet. After filtering out by the criteria in Method section, 86, 81, and 6 out of the 1142 variants were evaluated as pathogenic satisfying the criteria of 1) registered as causative for RP in HGMD professional 2019.2, 2) evaluated as likely pathogenic in ACMG guidelines, and 3) large deletions and insertions, respectively. As to large deletions and insertions, read-depth analysis revealed unreported 5 large deletions and 1 large insertion in the genes registered for IRD in RetNet; 3 large deletions and 1 large insertion in EYS, 1 large deletion in IQCB1, and 1 complex rearrangement in PRPF31.

Among the 171 unsolved patients in the second step, 44 patients were genetically solved in the third step. Among the 6 gross changes detected by read-depth method, only two contributed to the genetic diagnoses; a heterozygous deletion that spanned exon 42 of the EYS and complex rearrangements including the PRPF31.

The additional fourth step: Alu element. As the fourth step, we performed Sanger sequencing to detect the Alu element insertion in exon 4 of RP1 in all 220 included patients. The results of our analysis showed that three patients each had homozygous and heterozygous Alu element insertions. Among the three patients with a heterozygous Alu element insertion, two had another pathogenic RP1 variant and were genetically solved for AR-trait RP; one had c.4196del and the other had c.5797C>T, both of which have recently been reported27. Therefore, 5 patients were genetically solved in this additional step.

The allele frequency of the Alu element in this RP cohort was 2.0% (9 of 440 alleles), and it was ranked fifth among the most frequent variants that contributed to the genetic diagnoses for at least one patient (All the top five most frequent variants belonged to EYS, and the Alu element allele frequency was the same as the fifth most frequent EYS variant).

Summaries of genetic diagnoses. Summarizing all the genetic diagnostic steps, we genetically diagnosed 98 (44.5%) of the 220 analyzed patients (Table 1 and Supplementary Table 2).

Eight patients were genetically solved just by two founder mutations in EYS, 41 were solved by targeted NGS of all coding regions in EYS, 44 were solved by WGS, and 5 were solved by additional PCR and Sanger sequencing for the Alu insertion in exon 4 of RP1. Thus, 49 (50%) of the patients were solved by the first and second steps, which searched only variants of EYS.

Figure 2 shows the list of causative genes in 98 genetically solved patients. The most frequent causative gene was EYS, and 50 (51.0%) of the genetically solved patients were EYS-associated. Variants in only six genes, EYS, USH2A, RPGR, RP1, PDE6B, and CNGA1, led to genetic diagnoses in 82 (83.6%) of the 98 solved patients. Supplementary Table 3 shows details of the identified causative variants.

Details of EYS mutations contributing to genetic diagnoses. Adding the 67 variants detected in the second step of targeted sequencing for all coding regions of EYS, we detected 8 other variants in the third step of WGS; 4 variants in the exons and their boundaries, 3 large deletions and 1 large insertion. Among the total of 75 variants detected in the exons and their boundaries of EYS in 220 patients, 26 of them were considered pathogenic. Furthermore, 20 EYS variants including a single 6 kb gross deletion throughout exon 42 were causative for at least one patient (Supplementary Table 3). From the perspective of variant types, the 20 causative variants comprised five stop-gain single-nucleotide variations (SNV), six frameshift deletions, eight missense SNV, and one gross deletion. The most frequent variants in EYS were c.4957dupA(p.S1653fs), c.8805C>A(p.Y2935X), and c.2528G>A(p.G843E), all of which are understood to be founder mutations in Japanese populations26.

Fourteen causative variants of EYS have been registered in HGMD Professional 2019.2 as “DM” or “DM?” with published evidence, and among the other six causative variants, two (c.8268_8272del and c.8868delT) had referenced reports in which their pathogenicity was inferred. Therefore, we describe the other four variants (c.5847C>A (p.Y1949X, stopgain), c.7613delC (p.P2538fs, frameshift deletion), the 6 kb gross deletion including all of exon 42, and c.8268delC (p.S2756fs, frameshift deletion) herein as novel causative variants.

Mild or late-onset phenotypes of patients with homozygous c.2528G>A (p.G843E) in EYS. Among the six patients with homozygous c.2528G>A (p.G843E) in EYS, four retained visual acuity ≥ 20/20 and the ratio of the patients with visual acuity ≥ 20/20 was higher than that of the 186 patients with
other variants or who were genetically undiagnosed (4:2 vs. 39:147 patients with best corrected visual acuity of ≥20/20:<20/20 in both eyes; P = 0.0235, Fisher exact tests). One patient who harbored homozygous c.2528G > A (p.G843E) had a wholly devastated pigmented retina and could perceive only light with both eyes at the age of 77 years. His visual acuity had been sufficient to renew his driver’s license at the age of 43 years, when a first visit to an ophthalmologist identified slight subjective symptoms. The experience of this patient indicates a late onset phenotype.

Analysis to interpret VUS. After the genetic diagnoses (Fig. 3), we analyzed the 122 genetically unsolved patients and their variants and found that 90 were carriers of at least one pathogenic variant in the genes registered for IRD in RetNet. Among them, seven had other variants evaluated as VUS in the same gene as the pathogenic variant. Furthermore, three of the 122 unsolved patients had homozygous VUS, eight had compound heterozygous VUS on the genes of AR-trait IRD, and four had heterozygous VUS on genes of AD-trait IRD. Overall, with respect to VUS, 22 patients were possibly genetically solved with candidate variants that need to be replicated in further study for confirmation of pathogenicity before any genetic counseling and any available clinical trials of gene therapies. The related genes were **USH2A** (n = 4), **EYS** and **SNRNP200** (n = 2 each), and **CACNA1F, CLN3, CNGA1, CRB1, FLVCR1, GPR98, GUCY2D, IFT140, MAK, PDE6B, PRPF8, RHO, TOPORS, and ZNF408** (n = 1 each). Among these 17 genes, 14 were registered as causative for RP or syndromic RP in RetNet, and **CLN3, GUCY2D, and CACNA1F** were registered as causative for other IRD. Supplementary Tables 4 and 5 show details of the patients and the involved VUS.

Atypical phenotypes of possibly solved patients with candidate variants in RP genes that need to be replicated in further study for confirmation of pathogenicity. Among the 19 possibly solved patients with VUS, candidate variants that need to be replicated in further study, in the genes registered as causative for RP or syndromic RP, three had atypical phenotypes. Supplementary Fig. 3 shows the phenotype of a female patient with a homozygous VUS of **CNGA1** (c.41A>T). Dystrophy was limited to the nasal area, and her macula region and temporal retina were almost normal in both eyes. Full-field electroretinography (ffERG) showed essentially undetectable rod and subnormal cone responses despite having temporal retina in almost normal state. Supplementary Fig. 4 shows the phenotype of a male patient with a compound heterozygous VUS of **GPR98** (c.940G>A and c.3046G>A), who lacked characteristic peripheral bone-spicule-like pig-mentary changes (retinitis pigmentosa sine pigmento). Supplementary Fig. 5 shows the atypical phenotype of a female patient with a heterozygous VUS of **CRB1** (c.3131C>T) for the AD trait. She had bilaterally symmetrical paravenous dystrophy, which is obvious in autofluorescence images of the fundus, and essentially undetectable rod and cone responses in ffERG.

![Figure 2. List of causative genes identified. About half of the solved patients were EYS-associated.](https://doi.org/10.1038/s41598-020-77558-1)
Phenotypes of possibly solved patients with candidate variants in non-RP IRD genes that need to be replicated in further study for confirmation of pathogenicity.

Supplementary Fig. 6 shows the phenotype of a male patient with a homozygous VUS of CLN3 (c.1007C > A). He had a dystrophic retina without pigmentation (retinitis pigmentosa sine pigmento), and his rod and cone responses in ffERG were non-recordable at the age of 26 years. Supplementary Fig. 7 shows the phenotype of a male patient with a heterozygous VUS of GUCY2D (c.162C>G). He had a typically dystrophic retina with pigment near the arcade vessel, and his rod and cone responses were subnormal at the age of 64 years. A female patient with a compound heterozygous VUS of CACNA1F (c.3334G>T and c.3346 T>G) had the typical phenotype of diffuse pigment and narrowed vessels in the fundus, non-recordable rod and essentially undetectable cone responses in ffERG. However, her medical records contained only poor-quality images.

Discussion
We conducted stepwise genetic screening for Japanese patients with RP using direct Sanger sequencing for the two EYS founder mutations in Japan, targeted NGS of all coding regions of EYS, WGS, and additional Sanger sequencing for the Alu element insertion in RP1, and determined genetic diagnoses in 98 (44.5%) of 220 analyzed patients. The six major causative genes, EYS, USH2A, RPGR, RP1, PDE6B, and CNGA1, were responsible for 83.6% of the diagnosed patients.

Since the first description of the pathogenic effect of EYS variants on RP in 2008, many studies have revealed the prevalence of EYS-associated RP in patients with various ethnicities: 15.9% AR traits among those in Spain, 12% sporadic or AR traits in those from France, 11% in UK and China, 10% in Germany, 9.1% in Korea, 7% in Israel, 5% in The Netherlands, and 0% in Northern Ireland. The present findings reconfirmed the significant contribution of EYS to genetic diagnoses for Japanese patients with RP. Among our 98 genetically solved patients, EYS accounted for 50 (51.0%) of them, and only five EYS variants (c.2528G>A, c.4957dupA, c.6557G>A, c.7919G>A, and c.8805C>A) accounted for 38 (38.8%). The results of our analysis showed that including the three EYS mutations; c.2528G>A, c.6557G>A, and c.7919G>A, into the two well-known EYS founder mutations would improve the rate of genetic diagnoses by Sanger sequence in Japanese patients with RP.
The most frequent EYS variants in our cohort were c.2528G>A (EYS v1), c.4957dupA (EYS v2), and c.8805C>A (EYS v3) with allele frequencies of 10.4%, 7.7%, and 3.4%, respectively. The founder mutations, EYS v2 and EYS v3, are frequently reported in Japan23–26. Even though the pathogenicity of EYS v1 has remained controversial due to its high prevalence in Japanese public databases (2.2% in HGVD and 1.7% in ToMMo 4.7KIPN), we considered it pathogenic, as reported by Iwanami et al.26. They found the missense variant segregated with RP in 10 families and included five patients who harbored the homozygous variant. In agreement with these findings, the allele frequency of the variant in patients with RP was significantly and statistically higher than that in controls (P < 0.0001 chi-squared tests). Iwanami et al. also referred to the possibility of a mild or late-onset phenotype in patients with EYS v1, and our findings mentioned in Results section supported that notion.

We detected the Alu element insertion including a premature termination codon in the coding region (p.Y1352A fs*) in six patients and genetically solved five (2.3% of the analyzed patients) as having the RPI Alu-associated RP for the AR trait. Considering its high allelic frequency in Japanese patients with RP (2.0%) and its significant contribution to genetic screening, it might be a reasonable strategy to screen this large insertion in addition to the five frequent EYS variants determined by direct Sanger sequencing before whole exome sequencing (WES) or WGS. Nikopoulos et al. reported that the Alu element insertion, RPI frameshift variant c.4196del (p.C1399Lfs*5) and the RPI stop-gain variant c.5797C>T (p.R1933*) were not pathogenic for AD-RP when heterozygous. Furthermore, the RPI stop-gain variant c.5797C>T (p.R1933*) was benign in the homozygous form but pathogenic with Alu element insertion in the compound heterozygous form27. This non-Mendelian pattern of inheritance might explain the genetically undiagnosed patients to some extent as well as novel causative genes, deep-intronic variants37–39, and medium-to-large deletions and insertions as well as structural variants, that are difficult to detect by WES or WGS using short-read sequencers34–42.

Figure 3 shows that 22 out of the 122 genetically unsolved patients were possibly genetically solved with the candidate causative variants. We must add prudent and cautious comments on these candidate variants shown in Supplementary Table 5. These variants satisfied the criteria of ACMG guidelines not for pathogenic variants but for VUS, therefore they need to be replicated in further study for confirmation of pathogenicity before the patients of RP with the candidate variants are recruited to any available clinical trials of gene therapies.

Of note, the atypical phenotypes of the patients who were possibly genetically solved with the candidate variants that need to be replicated in further study resembled those that were previously reported. For example, the phenotype of our patient with a homozygous VUS of CLN3 (Supplementary Fig. 6), was similar to that of five patients with unpigmented atypical RP described by Ku C A et al. (2017)43. Another example is the atypical paravenous pigmented phenotype of our patient having heterozygous CRB1 (Supplementary Fig. 5). CRB1 is known to be causative for pigmentary retinochoroidal atrophy, which is similar to our case. Of course, the pathogenicity of these VUS is very difficult to verify because most of the variants have not been functionally studied, animal models have yet to supply confirmatory data and in silico prediction programs are just supportive but not decisive. More controls, patients with RP, and accumulated illustrative familial data are essential to determine the pathogenicity of the VUS listed in Supplementary Table 5 using the evidence framework of the ACMG guidelines.

The rate of genetic diagnosis herein was 44.5%, which was an increase of 7.9% compared with the previous reports from our institute in 20144. The following factors might explain the difference. Although previously regarded as non-pathogenic, we considered the most frequently causative variant, EYS v1, as pathogenic based on more evidence24–26; the criteria of the ACMG guidelines22 that were launched in 2015, and new causative variants of RP, including Alu element insertion in RPI that have recently been identified27.

This study has several limitations that might have affected the rate of the genetic diagnoses. Short-read sequencing could not detect some types of variants, such as gross structural variants and variants in long repeating regions. Novel third generation, long-read sequencing would contribute to the genetic diagnoses of patients who were hitherto genetically undiagnosed, especially carriers of pathogenic AR-RP variants40–42. We assessed previous findings considering the deleterious effects of the detected variants, but we did not fully analyze conflicting reports suggesting that the variants might be benign. However, the various methods applied in previous studies were not standardized. Therefore, we did not include evidence for benignancy in the current study. We did not conduct segregation analyses due to the lack of familial samples and data. Considering that segregation data are highly valued in ACMG guidelines and are regarded as a “Strong” level evidence when increased, the accumulation of not only samples from patients but also their families will be very important for further determinations of variant pathogenicity.

In conclusion, we identified causative variants in 98 (44.5%) of 220 Japanese patients with RP by stepwise genetic screening. Over half of the solved patients were EYS-associated, and our stepwise screening protocol functioned well for this cohort. We detected Alu element insertions in the RPI genes of six patients, and the allelic frequency was 2.0%. Applying the ACMG guidelines to evaluate unreported variants and the steady accumulation of IRD and familial samples will contribute to more accurate genetic diagnoses and be of considerable importance to patients with inherited visual defects in the era of gene therapy.

Data availability
All data generated/analyzed in this study are included in this article or in the Supplementary Information files and can be provided upon request.

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Author contributions
S.N. and A.O. designed the study. S.N., A.O., K.H., M.O., M.M., T.H., H.I., Y.O., F.M., and A.T. acquired the data. S.N., A.O., K.H. analyzed and interpreted data. A.O., F.M., and A.T. supervised the study. S.N., A.O., and K.H. wrote the manuscript. M.O., M.M., T.H., I.H., Y.O., F.M., and A.T. revised the manuscript. All authors agreed with the final version of manuscript.

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Competing interests
The authors declare no competing interests.

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