Article

Long-Range Polymerase Chain Reaction Method for Detection of Human Red and Green Opsin Gene Polymorphisms

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Abstract: Human color-vision discriminates middle- from long-wavelength spectral light as a result of photopigment opsin genes found on the q-arm of the X-chromosome at location Xq28. These so called 'green' and 'red' opsin gene sequences are 98% homologous, producing middle-wavelength sensitive and long-wavelength sensitive photopigment opsin gene sequences which differ by only seven locations at which important amino-acid substitutions occur. Investigations of variations in green and red gene arrays, and the consequences on phenotype expression, are the basis for color vision anomalies, including those first described by Dalton in 1789 [1], and are important for understanding expression mechanisms contributing to evolution of photopigment opsin genotypes. Here we introduce a long-range polymerase chain reaction assay for specifying the presence of genetic dimorphisms in human red and green gene sequences, and examine the utility of the long-range PCR in predicting perceptual behaviors found in phenotypes arising from identified genotypes. Consistent with behavioral differences correlated with results from a short-range PCR genotyping method [2], the more specific long-range PCR analysis indicates that dimorphisms commonly found in green and red opsin genotypes are correlated with certain color perception behaviors. The long-range PCR presented provides an additional tool for use in evaluating hypotheses about photopigment opsin gene expression in color vision phenotypes.

Keywords: X-linked photopigment opsin genes, Amino acid substitution, Spectral tuning, Color vision, Color vision deficiencies, Daltonism.

1. Introduction

Research on the genetic basis of retinal photopigments has enabled an understanding of the biochemistry of photopigment response sensitivity as well as the genetic basis for individual differences in color perception, including some forms of color deficiency that are commonly referred to as *Daltonism* [1,3,4]. Such work has shown that normal color vision in humans and old-world primates is trichromatic, being based on three classes of photopigments that are maximally sensitive to reddish (560–565 *nm*), greenish (530–535 *nm*), and bluish (420– 430 *nm*) light [5-9]. The three photopigment opsins, as well as rhodopsin (the photopigment in retinal rods), are heptahelical proteins, composed of seven transmembrane α–helices that are linked by intra- and extracellular loops. Visual excitation following photon absorption occurs as the result of 11-*cis* to all-*trans* isomerization of the chromophore located at a binding site in helix 7. The genes encoding the opsins or apoproteins of the human red and green photopigments are each composed of six exons and are arranged in a head-to-tail tandem array located on the q-arm of the X-chromosome [3,4,10,11].

Individuals with normal color vision usually have one long-wavelenth sensitive "red" opsin gene in the proximal position of the gene array and one or more middle-wavelength sensitive "green" opsin genes. These X-linked opsin genes have 98% identity in nucleotide sequence (including introns and 3' flanking regions) [12]. The encoded ''red'' and ''green'' apoproteins differ by an estimated 15 residues, 7 of these known to occur at positionswhich influence photoreceptor responsivity in the expressed phenotype [13,3,14,15]. The photopigment gene-specific amino acids are at codons 116, 180, 230, 233, 277, 285 and 309.

Human genotype/perceptual-phenotype analyses show that genotypic variation corresponds to shifts in the absorption spectra of expressed retinal pigments [15-18], with concomitant shifts in perceptual spectral sensitivity, or "λ-max," in human observers [19-21].

More specifically, it has been show that single amino-acid substitutions at codons 180 in exon 3 and codons 277 and 285 in exon 5 produce large shifts in phenotypic spectral sensitivity whereas the amino acids at codons 230 and 233 in exon 4 produce smaller shifts [16,15]. The specific amino acids occurring at codons 180, 277 and 285 are highly conserved in vertebrates. The specific residues occurring at each position are associated with predictable shifts in λ-max for each species. Substitutions of amino acid residues involve the gain or loss of a hydroxyl-bearing group. Substitution of hydroxyl groups at key positions are associated with shifts in photopigment response sensitivity towards shorter wavelengths of the visible spectrum. Mammals whose middle-wavelength sensitive (*MWS*) and long-wavelength sensitive (*LWS*) genes demonstrate codon conservation at these key residues include cat, deer, guinea pig, horse, squirrel, goat, rabbit, dolphin, mouse, rat and several species of New World monkeys [22-24].

Photopigments in invertebrates also consist of seven transmembrane α-helices. Although amino acid sequences in invertebrates are not conserved with respect to vertebrates, the specific amino acid residues at codons 180, 277 and 285 in long- and middle-wavelength-sensitive photopigment genes are invariant in some insect species, *e.g. Papilio* [25], suggesting some evolutionary conservation of tertiary protein structure and biochemical mechanisms for photosignal transduction.

In contrast to the highly conserved relationship between the amino acids at codons 230, 233, 277 and 285 and perception of light as red or green, the specific amino acid occurring at codon 180 in each

photopigment opsin gene is variable or polymorphic in *Homo sapiens* [15,26]. In the Caucasian population, substitution of the amino acid serine for alanine in the MWS, or "green", gene occurs in an estimated 6-9% of males. The amino acid alanine is substituted for serine in the LWS, or "red", gene in an estimated 38% of individuals [26]. Substitution of a hydrophobic residue for a hydroxl-bearing amino acid at codon 180 produces a relatively large shift in λ-max in the LWS photopigment, but a lesser shift in λ-max in the MWS photopigment. Individuals with the more common serine at codon 180 in their LWS opsin gene will demonstrate an average spectral response, or λ-max, of 557 *nm* for red light. Individuals inheriting alanine at codon 180 in their LWS gene demonstrate a 5 *nm* shift in their average λ-max for red light to 552 *nm*, moving their spectral sensitivity for red light towards the λ-max for green light, which is 532 nm [13,26].

Complicating further the analysis of the relationship between genotype and perceptual behavior is the chromosomal location of the MWS and LWS genes. The MWS and LWS photopigment opsin genes occur in a tandem array on the X chromosome. Females have two X chromosomes while males have a single X chromosome. Hence, females have two sets of such genes, one on each X chromosome, whereas males, who have only one X chromosome, have only one set of genes. As a result, females have potentially greater genetic variability in their MWS and LWS photopigment gene combination than is possible for males. Whereas there are four possible MWS/LWS genotype combinations at codon 180 for males, there are nine possible MWS/LWS genotype combinations for females. Thus, based on the reasoning that greater genotype diversity increases the diversity of expressed phenotypes, it might be reasonable to expect color perception variation in females not seen in male photopigment opsin genotypes.

In this article we present a new long-range PCR method (hereafter abbreviate LR PCR) for specifiying amino-acid substitutions at exon 3, codon-180 of the MWS and LWS photopigment opsin genes. As described below, the method is a modification of existing methods using a short-range PCR technique that simply detects the presence of codon-180 polymorphisms [2]. The method described here extends earlier analyses of Jameson et al. by permitting greater specificity of the identified polymorphisms, and permits a more informative analysis of genotype-correlated behaviors previously reported by Jameson and colleagues [2]. In addition to predicting more accurately our measures of color perception behavior, the LR PCR is offered as a new tool to refine the correlation between perceptual behavior measures and photopigment opsin genotypes.

2. LR PCR Rationale and Method

Investigating the relationships between opsin genotype/phenotype and color vision behaviors can be approached through analyses of postmortem retinal mRNA [27,28]. The present procedures use only venous blood specimens and perceptual data, and aim to further complement existing methods by expediting investigation of the relationships among opsin genotype, inferred phenotype and color perception in live observers.

Because of the extensive DNA sequence homology between the green and red opsin genes, conventional PCR amplification of DNA through exon 3 has found it difficult to distinguish between MWS and LWS gene sequences at codon 180. The method to be described here uses a combination of molecular methods to enable accurate genotyping at exon 3, codon 180 for both the MWS and LWS

genes. The method draws from various procedures and theories of Winderickx et al., Neitz and Neitz and colleagues, Asenjo et al. and Sharpe et al. In particular, the method is similar to one described by Winderickx et al. [20] but differs because it incorporates an additional conformation of exon 4 and 5 gene-specific amino acids sequences. This additional confirmation is based on descriptions given by Asenjo and colleagues [15] and Sharpe and colleagues [26] combined with other results [19,21,20] to distinguish the genomic regions of DNA sequence variation between MWS and LWS genes. These studies provided the empirical justification for the method described here. The method uses a combination of three molecular approaches in order first to create MWS and LWS gene-specific DNA templates and then to use those templates to distinguish between their respective codon 180 sequences. A long-range polymerase chain reaction technique (LR PCR) generates gene-specific PCR products. DNA sequencing of each PCR template confirms this gene specificity, and PCR and a restriction digest determines MWS and LWS codon 180 genotypes.

The method enables accurate genotyping of codon 180 polymorphisms on each photopigment opsin gene. Thus fine comparisons can be made between genotype and color matching behavior. Analyses using this method demonstrate a close correlation with perceptual behavior and provide significant insight into mechanisms contributing to the variability in perceptual behavior.

Subjects. With permission of the UCSD Human Subjects Committee, informed consent was obtained from 38 female and 26 male UCSD undergraduates for participation in this study. For simplicity of presentation, analyses in this article consider only data from female subjects. Three milliliters of venous blood from each student was collected into EDTA vacutainer tubes by a trained phlebotomist. Subjects were solicited through either the Psychology Department Human Subjects pool, or by posted solicitations for experimental participation for either cash payment for course extra-credit. To address specific empirical hypotheses subject solicitations were designed to maximize the yield of participants that were carriers of genes underlying color vision deficiencies or anomalies. Thus, genotype frequencies of the present study in no way represent population frequency estimates.

DNA Extraction Method. DNA was isolated from peripheral blood leukocytes using the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN).

Long Range PCR Rationale. As mentioned above, the genetic polymorphism of interest at codon 180 in the LWS and MWS genes occurs in exon 3 of each gene. Conventional PCR methods used to amplify DNA through this region of the gene cannot distinguish between DNA sequences within the LWS from DNA sequences within the MWS genes because the sequences of the two genes are identical except for the presence or absence of this polymorphism. The only significant region of sequence divergence between the two genes occurs in exon 5 which is more than 3000 bases away from the polymorphic region in exon 3 and beyond the limits of standard PCR methods. The LR PCR method described here makes use of PCR reagents specifically designed to extend the length of PCR-amplified DNA to several thousand base pairs. In addition, the method makes use of findings reported by Sharpe et al. of invariant amino acid sequences in exon 4 of each gene which distinguish LWS from MWS genomic DNA sequences [26]. The method relies on these invariant sequences to confirm the gene specificity of each LR PCR product.

The LR PCR method consists of three steps:

(*i.*) Gene-specific antisense primers through exon 5 and a common sense primer recognizing sequences within intron 2 are used to amplify 4000bp (with13646) base pair DNA products specific to each gene.

(*ii.*) A portion of each LR PCR product is amplified using primers through exon 4 to amplify and then DNA sequence PCR products which include codons 230 and 233 of each gene to confirm the gene specificity of each LR PCR product.

(*iii.*) Following confirmation of the gene-specificity of each LR PCR by DNA sequencing of exon 4, the remainder of each LR PCR product is amplified with primers from exon 3, followed by restriction digest, to determine the presence or absence of the codon 180 polymorphism.

Long Range PCR Technique. Following the manufacturer's recommended protocol, 100 ng DNA is added to a PCR mixture containing 1X Taq Extender buffer (Stratagene, La Jolla, CA), 350 uM each of dATP, dCTP, dGTP, and dTTP, 300 nM each primer, 5 U Taq Extender and 5 U Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA) into a total volume of 28.6 ul. (Primer sequences are shown in Table 1 below.) Following denaturation at 94°C for 3 minute, each reaction undergoes 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 4 minutes, ending with a final 5 minutes of extension at 72°C. Below are the gene-specific sequences within exon 5 which were used to generate gene-specific exon 5 antisense primers. Unique amino acids are in italics and DNA bases unique to each gene are underlined and bolded.

EXON 5

LWS Gene Codons:

	274	275	276	277	278	279
	Ile	*Phe*	Ala	*Tyr*	Cys	*Val*
DNA:	**A**TC	**TT**T	GC**G**	T**A**C	TGC	**G**TC

MWS Gene Codons:

	274	275	276	277	278	279
	Val	*Leu*	Ala	*Phe*	Cys	*Phe*
DNA:	**G**TC	**C**T**G**	GC**A**	T**T**C	TGC	**T**TC

Exon 4 Sequence Confirmation. 5 ul of LR PCR product is added to a PCR mixture containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS HCl, pH 8.3, 0.001% gelatin, 200 uM each of dATP, dCTP, dGTP, and dTTP, 500 nM each primer (see Table 1 for primer sequences) and 1 U Taq DNA polymerase (Applied Biosystems) to a final volume of 50 ul. Following 3 minutes of denaturation at 94°C, each reaction undergoes 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. PCR products then undergo column purification (QIAGEN, La Valencia, CA) and are submitted to the UCSD Cancer Center DNA Sequencing Shared Resource for sequencing. Figure 1 below provides the

gene-specific sequences within exon 4 used to confirm the specificity of each LR PCR product. Unique amino acids are in italics and DNA bases unique to each gene are bolded.

Exon 3 Codon-180 Serine to Alanine Polymorphism Detection. 5 ul LR PCR product is added to a PCR mixture containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS HCl, pH 8.3, 0.001% gelatin, 200 uM each of dATP, dCTP, dGTP, and dTTP, 500 nM each primer and 1 U Taq DNA polymerase (Applied biosystems(as above)) to a final volume of 50 ul. Following 3 minutes of denaturation at 94°C, each reaction undergoes 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. Digestion of 15 ul of exon 3 PCR product with 3 U of the restriction enzyme Fnu 4HI at 37°C for 1 hour determines the presence or absence of the codon 180 serine (abbreviated "Ser") to alanine (abbreviated "Ala") DNA polymorphism. When the digested PCR products are size-separated on a 3% agarose gel run in 1X TAE buffer, the presence of the DNA sequence coding for alanine is detected as a 152 bp band whereas the DNA sequence coding for serine is detected as a 193 bp band.

Figure 1. Gene-specific sequences within exon 4 of the red (LWS) and green (MWS) opsin genes. DNA sequence codings were used to confirm specificity of each long-range PCR product. Amino acids and DNA bases unique to each gene are shown in bold and italics.

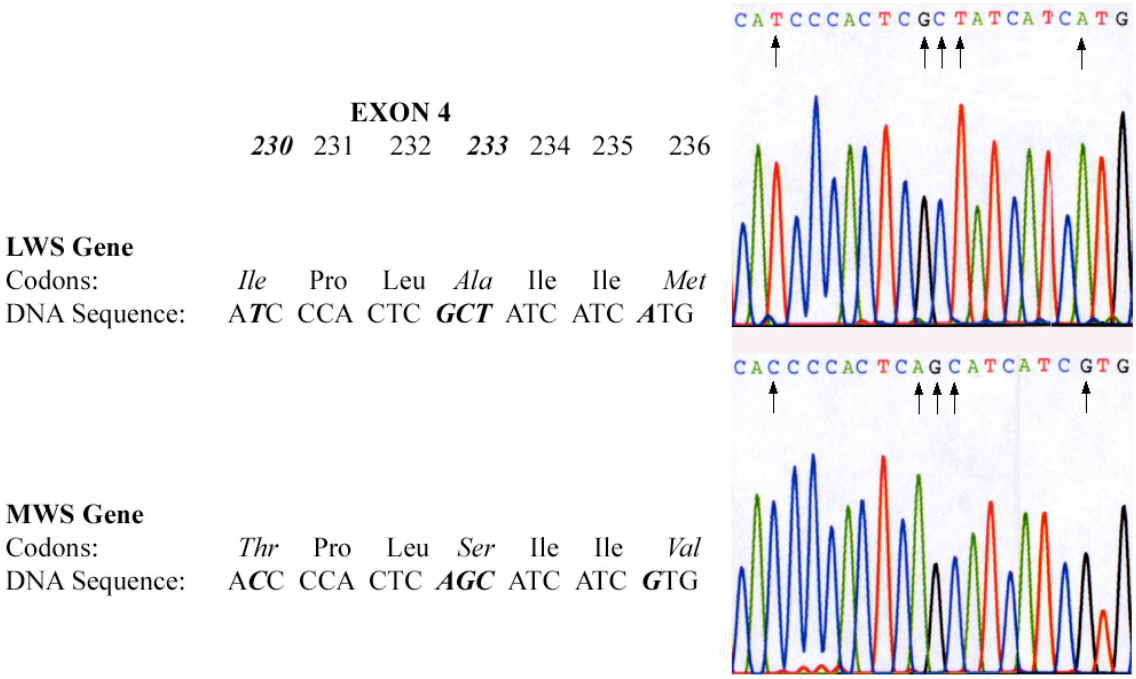

Table 1. Oligonucleotide primers used in amplification or sequencing reactions.

Long Range PCR Primers:	
Common Intron 2 Sense Primer	5'-GGC AAC ATA GTG AGA CCT CTT CTC-3'
LWS Exon 5 Antisense Primer	5'-CCAGCAGACGCAGTACGCAAAGAT-3'
MWS Exon 5 Antisense Primer	5'-CCAGCAGAAGCAGAATGCCAGGAC-3'

Exon 4 Primers:	
Exon 4 Sense Primer	5'-ACAAACCCCACCCGAGTTAG-3'
Exon 4 Antisense Primer	5'-GACTCATTTGAGGGCAGAGC-3'
Exon 3 Primers:	
Exon 3 Sense Primer	5'-TCATCTGTCTGCTCTCCCCAT-3'
Exon 3 Antisense Primer	5'-ACCCTTACCTGCTCCAACCA-3'

3. Long Range PCR Results

Before describing results for this LR PCR genotyping method, a previously published genotyping method is summarized for comparison. Jameson, Highnote & Wasserman [2] previously published a genotyping method for codon 180 dimorphisms which generates three possible genotypes in female subjects: Ser/Ser, Ser/Ala and Ala/Ala. Females who are Ser/Ser or Ala/Ala from this method have the same amino acid at codon 180 in both their LWS and MWS genes. For females who are Ser/Ala, it is not possible to determine which amino-acid is specific to LWS and MWS genotypes. Nevertheless, even with this restricted classification system, Jameson et al. reasonably surmised that the "heterozygous" genotype individuals exhibiting *both* serine and alanine (i.e., Ser/Ala) necessarily possess a more *diverse* genotype than either homozygous genotype exhibiting a single amino-acid residue (i.e., Ser/Ser or Ala/Ala).

In light of the above mentioned fact that shifts in spectral sensitivity follow from codon-180 opsin gene substitutions, Jameson and colleagues tested the following hypothesis: *individuals with the genetic potential to express more diverse phenotypes (i.e., ser/ala) are likely to exhibit color perception behaviors which differ more than those in comparisons of homozygous genotypes (i.e., ser/ser or ala/ala)*. Using this simple genotyping method they showed that individuals of the Ser/Ala classification demonstrated robust differences in color perception behaviors when compared with their homozygous female counterparts. Results of the classification given by the Jameson et al.'s [2] PCR method are summarized in Table 2, column 2.

Compared with the Jameson et al. method, under a new genotyping system that uses the LR PCR method described here, there are nine possible classifications of LWS-, MWS-genotypes at codon 180. As illustrated by Figure 2's restriction gel digest and the LR PCR classification given in Table 2, column 1, accurate genotyping of codon 180 for both the LWS and MWS genes is achieved. Thus the LR PCR method successfully specifies the L- and M-cone genotypes of 37 female undergraduates. (Note hereafter N=37 due to insufficient DNA specimen for one participant).

Based on the results of the LR PCR, 37 female subjects can be classified into six of the nine possible genotype categories (Table 2, column 1). A comparison of columns 1 and 2 of Table 2 show that the LR PCR permits a more specific classification of subjects' opsin genotypes. The utility of the LR PCR's additional specificity can also be evaluated using the perceptual data of Jameson et al. [2]. For this we examine the degree of association between subject's perceptual data and the new LR PCR classification method, and compare that association with analogous measures under the original genotype classification method published [2].

Figure 2. Fnu 4HI restriction digestion assay of genomic DNA from exon 3 codon-180 of the Green opsin (M-cone) gene of seven female donors (Lanes 1-7). Presence of the DNA sequence coding for alanine is detected as a 160 bp band whereas the DNA sequence coding for serine is detected as a 190 bp band. Lanes 1, 2 and 5, genomic DNA from human females with alanine at exon 3, codon-180 of the green gene. Lane 7, one female with serine at exon 3, codon-180. Lanes 3, 4, and 6, females with a serine-alanine dimorphism at exon 3, codon-180 of the green gene. Lane 8, DNA Ladder. Restriction gel digest products depicting analogous dimorphisms occurring at codon-180, exon 3 of the Red opsin (L-cone) gene are not depicted here, but are similar to those presented for the Green gene above.

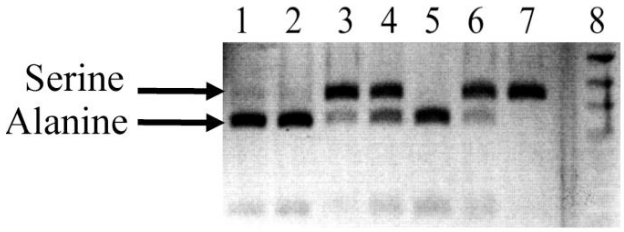

Table 2. Frequencies of genotypes from a group of 37 participants evaluated using the new LR PCR method (column 1) compared with the original short-range PCR genotyping (column 2) from Jameson et al. [2].

Long Range PCR Genotypes:	Original Genotyping from Jameson et al. [2]:
1. L-180-Ser/Ser & M-180-Ser/Ser : 10	Ser/Ser: 10
2. L-180-Ser/Ser & M-180-Ser/Ala : 7	Ser/Ala
3. L-180-Ser/Ala & M-180-Ser/Ala : 11	Ser/Ala 20
4. L-180-Ser/Ser & M-180-Ala/Ala : 1	Ser/Ala
5. L-180-Ser/Ala & M-180-Ala/Ala : 1	Ser/Ala
6. L-180-Ala/Ala & M-180-Ala/Ala : 7	Ala/Ala: 7

4. Relationships between LR PCR Genotype Classifications and Perceptual Behaviors

Through an additional step involving exon 4 sequence confirmation the LR PCR provides an accurate method for specifying L- and M-cone photopigment opsin genotypes. But the greater specificity illustrated in Table 2 is only of value in such studies if it reveals meaningful relationships in the genotype-phenotype linkage. Thus, the question is whether the greater specificity provided by the LR PCR genotyping furthers our understanding of the genotype-phenotype linkage beyond the findings of our earlier classification system [2] which showed that *a more diverse opsin genotype is associated with more complex (or richer) color experience behaviors*.

Note that although the present LR PCR genotyping provides greater specificity compared to the original genotyping method, it does not automatically follow that such specificity will necessarily be a better predictor of perceptual phenomena. With these issues in mind, we examine the perceptual results

of the individuals classified by the two methods in Table 2 and ask two specific questions (see 4.1 and 4.2 below) of the relationships in the data.

Table 3. Question 1 analyses using Kendall's *Tau* nonparametric measures of association between two genotyping classifications, O-PCR and LR PCR, and the Median Number of Chromatic Percepts, MNCP, experienced by 37 genotypically classified observers.

Variables correlated	Kendall's *Tau*	Significance level
O-PCR classification & MNCP	0.363	P < .002
LR PCR classification & MNCP	0.361	P < .0001

4.1. Which genotyping method is more informative regarding color-perception behaviors?

Question 4.1 can be addressed using measures of association between (*i.*) the two methods' resulting genotype classifications [i.e., the *original PCR method* (abbreviated "O-PCR"), and the "LR PCR" method introduced above], and (*ii.*) the measures of perceptual behavior used in the Jameson et al. [2] investigation. The measure of perceptual behavior is summarized here as "median number of perceived colors," and is tantamount to *the median number of different chromatic percepts a given observer detects in a series of judgments for diffracted spectrum stimulus* (see [2] for details of the stimulus and empirical task).

For the purpose of addressing the above question using correlational analyses*, Appendix A* provides the numeric code assigned to the O-PCR and LR PCR genotype classifications listed in Table 2. The coding system is an ordinal code following the hypothesis that greater genotype diversity predicts greater diversity in an observer's percept. For example, individuals genotyped as double-heterozygous for green and red gene dimorphisms (i.e., genotype in row *3*, column 1, Table 2) are deemed the most genotypically diverse in our sample, and are assigned a ordinal code value of '4,' whereas genotypes with a lesser degree of genetic variation (i.e., homozygous genotype in row *1*, column 1, Table 2) are assigned a lower ordinal value reflecting the likelihood of a *less-diverse* expressed phenotype. (See *Appendix A* for the O-PCR and LR PCR numeric codes.) Table 3 provides the Kendall's *Tau* statistics for the Question 1 analysis. Kendall's *Tau* is a nonparametric symmetric measure of ordinal association that ranges from '–1' (indicating a total negative association between variables) to '1' (indicating total positive association).

Table 3's measures of association demonstrate that the different genotype classifications given by the original genotyping method (O-PCR) and the long-range genotyping method introduced here (LR PCR) are similarly correlated with the color perception behaviors observed using the Jameson et al. (2001) task. The measures indicate that in addition to giving a more specific genotyping, the LR PCR is at least as predictive of color perception behaviors as the originally used genotyping method.

One potential advantage of the LR PCR's genotyping specificity is that the additional information given by the classification permits evaluation of specific hypotheses related to the phenotypic expression of identified genes. For example, competing hypotheses from the literature propose selective expression for M- versus L-genes that are distal from their locus of control region on the array [29-31]. One such hypothesis suggests that, despite the occasional presence of multiple red gene variants in the genetic array, expression mechanisms allow only a single red gene – that most proximal

to the locus of control region – to be expressed phenotypically. Thus, it is believed that distal red gene variants do not phenotypically manifest. An alternative viewpoint suggests that multiple red genes can be phenotypically expressed [32]. Using the present LR PCR it is possible to examine plausible behavioral consequences of such hypotheses, as expressed in Question 4.2 below.

4.2. Under the assumption that multiple red gene variants are not expressed in the phenotype, which genotyping method is more informative regarding color-perception behaviors?

In essence, the assumption in Question 4.2 permits further assessment of Table 3's correlations between genotype classification and perceptual behavior. According to one theory at least, distal variants of red genes are not expressed. It follows from this idea that the perceptual consequences arising from the expression of green gene dimorphisms may be unchanged by the presence of more than one red gene variant (i.e., a L-opsin dimorphism) in the gene array.

As with Question 4.1, Question 4.2 is examined using Kendall's *Tau* measures. *Appendix A, section 2* describes a revised ordinal code which effectively lowers the rank assignment used to encode Table 2's, column 1 genotypes possessing more than one red gene variant. The basic relationship between the numeric values of the reassigned ordinal code and the genotype classifications remains preserved such that increasingly *diverse* photopigment opsin genotypes are encoded by monotonically increasing ordinal values.

Table 4's measures of association demonstrate that the genotype classification schemes given by the less-specific O-PCR method and the improved LR PCR method are also found to be well-correlated with differences in color perception behaviors similar to that seen in Table 3.

For Question 4.2's analysis the O-PCR numeric code remained unchanged from that described under the Question 4.1 analysis because the genotype classification of the O-PCR does not permit further refinement. However, the LR PCR's specificity permits updating and, thus, Table 4's correlations are based on the recoded values of the LR PCR classifications that reflect increased association consistent with the hypothesis of increased diversity of the genotype, presumably because only one red gene is phenotypically expressed (i.e., Table 3, $\tau = 0.361$ versus Table 4, $\tau = 0.412$). This additional information permits analyses which reveal that the differences in color perception behaviors observed by Jameson et al. [2] are likely attributable to increases in genotype dimorphisms. While this new information is consistent with some existing theories of opsin gene phenotype expression [33-35], direct tests are needed to determine whether the differences in perceptual behavior found by Jameson et al. (2001) for heterozygous females might be attributable to expressed variants of L-cone photoreceptor classes.

On this issue, however, it is informative that in the present analyses in which the ordinal numeric code gives greater significance to green gene dimorphisms, the statistical trends show additional increases in the Kendall's *Tau* measures of association. These analyses suggest the LR PCR classification and the behavioral data are even more highly correlated. Although physiological confirmation is needed, such analyses underscore the suggestion that the LR PCR's specificity is informative concerning assessment of expressed phenotypes.

These correlational measures between the LR PCR and perceptual behaviors are a good indication that in addition to giving more specific genotyping classifications, the LR PCR is as useful a predictor

of color perception behaviors as the short-range genotyping method used by Jameson et al. [2]. Moreover the LR PCR's specificity permits the kind of hypothesis testing illustrated here in the straightforward assessment of the hypothesis of single red gene expression.

Table 4. Question 4.2 analyses using Kendall's *Tau* nonparametric measures of association between two genotyping classifications, O-PCR and LR PCR, and the Median Number of Chromatic Percepts, MNCP, experienced by 37 genotypically classified observers.

Variables correlated	Kendall's *Tau*	Significance level
O-PCR classification & MNCP	0.363	P < .002
LR PCR classification & MNCP	0.412	P < .0001

5. Conclusion

A long-range polymerase chain reaction technique for genotyping codon 180 polymorphisms in the MWS and LWS genes has been described. The method extends previous work using a short-range PCR technique [2] and provides a way of classifying red and green photopigment opsin genotypes that permits detailed analysis of the perceptual consequences of the opsin gene polymorphisms, despite uncertainties regarding individually expressed phenotypes. The utility of the technique was demonstrated through comparative analyses with a previously used genotyping method. In all analyses, the LR PCR method proved to be more accurate, more specific. Here the method served as a more sophisticated test of the hypothesis that more diverse opsin genotypes yield phenotypes producing more complex color perception experience. As a classification tool the method has also contributed to clarifying complex color perception behaviors of putative phenotype groups [36,37] and been used as a basis for defining mathematical models of color vision observer variants in evolutionary game theoretic simulations of color categorization [38,39].

The LR PCR method also permitted evaluation of a hypothesis from the existing literature regarding the expression of gene variants located on the distal end of the genetic array. Although the ultimate demonstration of opsin gene expression mechanisms, and their resulting individual retinal phenotypes, will be provided by physiologists and geneticsts, this LR PCR method provides an alternative tool which might be used with existing methods for assessing relationships between the kinds of opsin gene expression mechanisms, retinal photopigment phenotypes, and color perception behaviors examined here.

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Appendix

Ordinal codes assigned to genotype classifications used in Kendall's *Tau* correlational analyses of two empirical questions.

QUESTION 4.1: Which genotyping method is more informative regarding color-perception behaviors, the short-range PCR partitions from Jameson et al. [2] or the new Long-Range PCR partitions?

Original-PCR Partition (or "O-PCR") Code from Jameson et al. [2]:

S/A: 2 (possibly heterozygous on one gene, possibly on both Red and Green genes)
S: 1 (presumably homozygous-wildtype S & S on Red and green Genes)
A: 1 (presumably homozygous-polymorphA & A on Red and Green genes)

(In this Original-PCR coding, 'S' denotes serine, and 'A' denotes alanine, as amino acids detected at codon 180 in **either**, or **both**, red and green genes.)

By comparison the LR PCR coding below allows for greater specificity in identifying allelic variations in red and green genes.

Long-Range PCR (or "LR PCR") Partition Code:

S/A & S/A: 4 (double-heterozygous)
S & S/A: 3 (homozygous-wild on Red, heterozygous on Green)
S/A & A: 3 (heterozygous on Red, homozygous-wild on Green)
S/A & S: 3 (heterozygous on Red, homozygous-polymorph on Green)
A & S/A: 3 (homozygous-polymorph on Red, heterozygous on Green)

S/S & A/A: 2 (multiple wildtype copies on Red, multiple wildtype copies on Green)
S/S & S/S: 2 (multiple wildtype copies on Red, multiple polymorphic copies on Green)
A/A & A/A: 2 (multiple polymorphic copies on Red, multiple wildtype copies on Green)
S & A: 1 (homozygous-wild on Red, homozygous-wild on Green)
A & S: 1 (homozygous-poly on Red, homozygous-poly on Green)
S & S: 1 (homozygous-wild on Red, homozygous-poly on Green)
A & A: 1 (homozygous-poly on Red, homozygous-wild on Green)

QUESTION 4.2: Under the assumption that multiple red gene variants are not expressed in the phenotype, which genotyping method is more informative regarding color-perception behaviors, the 0-PCR partitions from Jameson et al. [2] or the recoded LR PCR partitions?

Ordinal codes reassigned to LR PCR classification to reflect the theory that only one, proximal, red gene variant is expressed in the phenotype. In the New Long-Range PCR Partition code below, bolded code values reflect recoding based on this single red expression hypothesis. In the Question 2 analysis the O-PCR code remains as given above:

S/A & S/A: **3** (double-heterozygous)
S & S/A: 3 (homozygous-wild on Red, heterozygous on Green)
S/A & A: **2** (heterozygous on Red, homozygous-wild on Green)
S/A & S: **2** (heterozygous on Red, homozygous-polymorph on Green)
A & S/A: 3 (homozygous-polymorph on Red, heterozygous on Green)
S/S & A/A: 2 (multiple wild copies on Red, multiple wild copies on Green)
S/S & S/S: 2 (multiple wild copies on Red, multiple polymorphic copies on Green)
A/A & A/A: 2 (multiple polymorphic copies on Red, multiple wild copies on Green)
S & A: 1 (homozygous-wild on Red, homozygous-wild on Green)
A & S: 1 (homozygous-poly on Red, homozygous-poly on Green)
S & S: 1 (homozygous-wild on Red, homozygous-poly on Green)
A & A: 1 (homozygous-poly on Red, homozygous-wild on Green).