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# Sequence Divergence, Polymorphism and Evolution of the Middle-Wave and Long-Wave Visual Pigment Genes of Great Apes and Old World Monkeys

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**In man, the spectral shift between the middle-wave (MW) and long-wave (LW) visual pigments is largely achieved by amino acid substitution at two codons, both located in exon 5. A third amino acid site coded by exon 3 is polymorphic between pigments. We have studied the equivalent regions of the cone opsin genes in two members of the Hominidea (the gorilla, *Gorilla gorilla* and the chimpanzee, *Pan troglodytes*) and in three members of the Cercopithecoidea family of Old World primates (the diana monkey, *Cercopithecus diana*, the talapoin monkey, *Miopithecus talapoin*, and the crab-eating macaque, *Macaca fascicularis*). No variation in the codons that specify the amino acids involved in spectral tuning were found. We predict therefore that the MW and LW pigments of gorilla and chimpanzee have similar spectral characteristics to those of man. Multiple copies of the same opsin gene sequence were identified in the chimpanzee, talapoin and macaque and we also show that non-human Old World primates are similar to man in showing a bunching of polymorphic sites in exon 3. We discuss the ancestry of the separate MW and LW genes of Old World primates and the equivalent polymorphic gene of the marmoset, a New World primate.**

Visual pigments Molecular evolution Primates Colour vision

## INTRODUCTION

Vision depends on a group of light-sensitive pigments in the retina that are formed by the binding of a retinal, the aldehyde derivative of vitamin A, to an opsin protein. Opsins are members of the family of G protein-coupled receptors that share a common heptahelical structure of seven α-helical transmembrane regions linked by straight-chain extra-membrane loops (Schertler, Villa & Henderson, 1993). Most mammals enjoy a dichromatic system of colour vision, which depends on comparing signals of short-wave cones with those of a second class of cone maximally sensitive in the 500–570 nm range. Among the mammals, it is only in primates that a trichromatic system is seen (Bowmaker, 1991). In the case of man and the Old World or catarrhine monkeys, trichromacy is achieved by combining the short-wave cones with middle-wave (MW) cones having peak sensitivity ($\lambda_{max}$) near 530 nm and long-wave cones having $\lambda_{max}$ near 560 nm; the opsin component of the two latter pigments are coded by adjacent genes on the X chromosome (Nathans, Thomas & Hogness, 1986a; Vollrath, Nathans & Davies, 1988; Feil, Aubourg, Heilig & Mandel, 1990). This contrasts with the situation in New World or platyrrhine monkeys where there is only a single gene that codes for pigments with $\lambda_{max}$ in the red/green region of the spectrum. In these species, trichromatic colour vision is found only in females and arises when different polymorphic forms of this gene are present on the two X chromosomes; random X-inactivation will then ensure that only a single pigment is present in each cone photoreceptor (Mollon, Bowmaker & Jacobs, 1984; Jacobs & Neitz, 1987; Neitz, Neitz & Jacobs, 1991; Travis, Bowmaker & Mollon, 1988; Williams, Hunt, Bowmaker & Mollon, 1992; Bowmaker, Jacobs, Speigelhalter & Mollon, 1985; Bowmaker, Jacobs & Mollon, 1987; Tovée, Bowmaker & Mollon, 1992).

Only a small number of amino acid differences distinguish the MW and LW pigments of man (Nathans *et al.*, 1986a) and the same is true for the spectrally-distinct but allelic forms of the opsin gene in New World monkeys (Neitz *et al.*, 1991; Williams *et al.*, 1992). However, certain consistent differences are present and

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it has been possible to determine that the spectral shift between these pigments is largely achieved by amino acid substitution at only two sites, both in transmembrane region 6 (TM6) (Neitz *et al.*, 1991; Williams *et al.*, 1992; Winderickx, Lindsey, Sanocki, Teller, Motulsky & Deeb, 1992; Merbs & Nathans, 1993). In order to establish whether these substitutions are conserved over other primate species, we have looked at the MW and LW opsin gene sequences of two additional species of Great Ape, the gorilla, *Gorilla gorilla*, and chimpanzee, *Pan troglodytes*, and three species of Cercopithecoid monkeys. Except for the gorilla, all of these species are predominantly frugivorous in their diet; and it has been suggested that primate colour vision co-evolved with yellow and orange fruit, which only trichromatic dispersers can readily distinguish at a distance among dappled foliage (Mollon, 1991). There is therefore a special interest in the gorilla, a bulk eater, whose diet is largely composed of stalks, vines, leaves, bark, shoots and roots. Although the diet of the western gorilla includes some fruit, the gorilla is a slow-moving forager who does not seek out fruiting trees at a distance in the manner of many monkeys. Has the gorilla experienced a relaxation of the selection pressure for actue colour vision, analogous to the relaxation of selection pressure by which Post (1952) explained the high incidence of colour deficiences in modern human populations?

In their original report of the sequence of the human MW and LW opsin genes, Nathans *et al.* (1986a) reported the presence of multiple copies of the MW gene, and in a more extensive study (Drummond-Borg, Deeb & Motulsky, 1989), up to five such copies were identified, with a modal value of two per X chromosome. Polymorphic sites within the human opsin genes have also been identified (Nathans *et al.*, 1986a; Winderickx, Battisti, Hibiya, Motulsky & Deeb, 1993), together with hybrid MW/LW genes that are thought to underlie certain types of anomalous trichromacy in man (Nathans, Piatanida, Eddy, Shows & Hogness, 1986b; Neitz *et al.*, 1991; Deeb, Lindsey, Hibiya, Sanocki, Winderickx, Teller & Motulsky, 1992). We have previously shown that multiple opsin genes are also present in the talapoin monkey, *Miopithecus talapoin* (Ibbotson, Hunt, Bowmaker & Mollon, 1992). The sequences of the MW and LW genes reported in the present paper have enabled us to show that polymorphic sites are present in the opsin genes of non-human primates and that multiple copies of opsin genes are present in the Great Apes as well as in man.

Separate MW and LW genes are present only in Old World primates (Bowmaker, Astell, Hunt & Mollon, 1991; Ibbotson *et al.*, 1992), indicating that the duplication event that gave rise to this system of trichromacy arose after the separation of the Old and New World primate lineages. Using a molecular phylogenetic approach, we have examined the ancestry of these genes in the six species of Old World primates. The relationship between the MW and LW genes of Old World monkeys and the corresponding allelic variants found in New World primates is also discussed.

## MATERIALS AND METHODS

### *DNA samples*

DNA was prepared from frozen tissue stored from animals used in an earlier study (Ibbotson *et al.*, 1992) as follows: a female diana monkey (*Cercopithecus diana*), a male talapoin monkey (*M. talapoin*), and a male macaque (*Macaca fascicularis*). The DNA samples from a female gorilla, *G. gorilla*, and a male chimpanzee, *P. troglodytes*, were isolated from blood samples and were a kind gift of Dr Helen Stanley, Institute of Zoology, The Zoological Society of London.

### *Amplification and sequencing of opsin gene fragments*

The polymerase chain reaction (PCR) was used to amplify three regions of the MW and LW opsin genes, from base 479 to base 596 of exon 3 (using primer pair 5′-ATGACGGGTCTCTGGTCCCTG-3′ and 5′CTCCAACCAAAGATGGGCGG-3′), from base 650 to base 761 of exon 4 (using primer pair 5′-CACGGCCTGAAGACTTCATGC-3′ and 5′CGCTCGGATGGCCAGCCACAC-3′) and from base 830 to base 983 of exon 5 (using primer pair 5′-GAATTCCACCCAGAAGGCAGAG-3′ and 5′-GTCGACGGGGTTGTAGATAGTGGC-3′).

Each reaction contained approx. 200 ng of template DNA, 200 ng of each primer pair, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 unit of *Taq* polymerase and reaction buffer in a final volume of 50 $\mu$l. Either 30 or 35 cycles were used with an annealing temperature of 58°C, elongation temperature of 72°C, and denaturing temperature of 94°C. The products of the reaction were visualized by electrophoresis in a

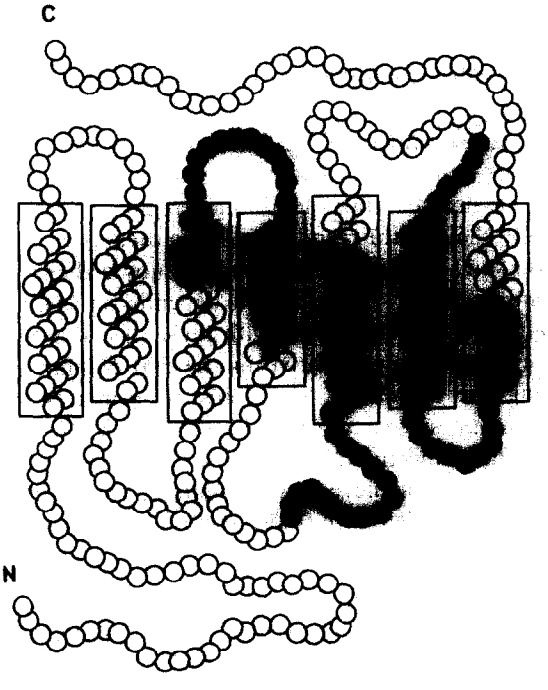


FIGURE 1. A two-dimensional model of the MW/LW visual pigment. The solid circles denote regions of the molecule that were sequenced in the present study.

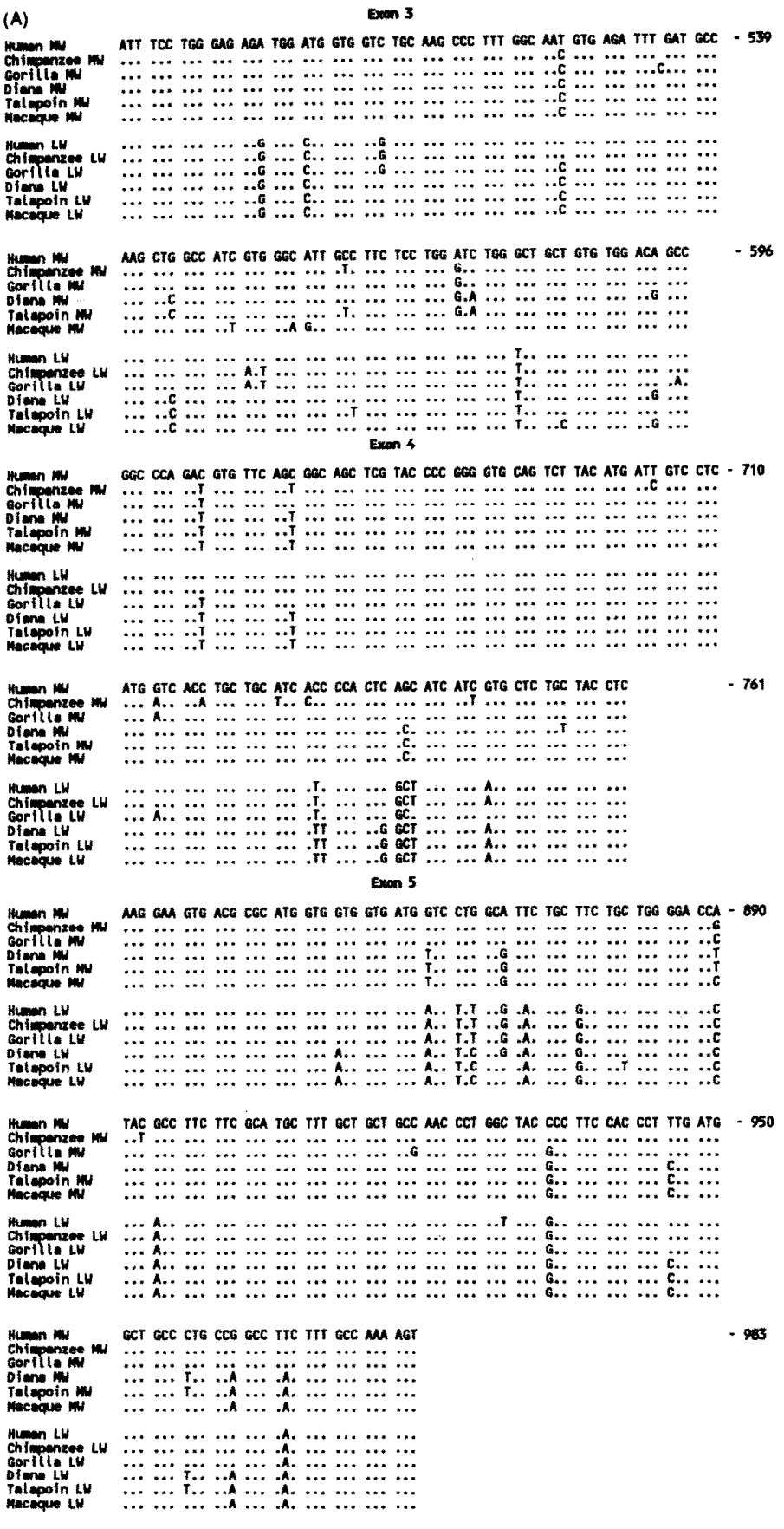


FIGURE 2. *Caption overleaf.*

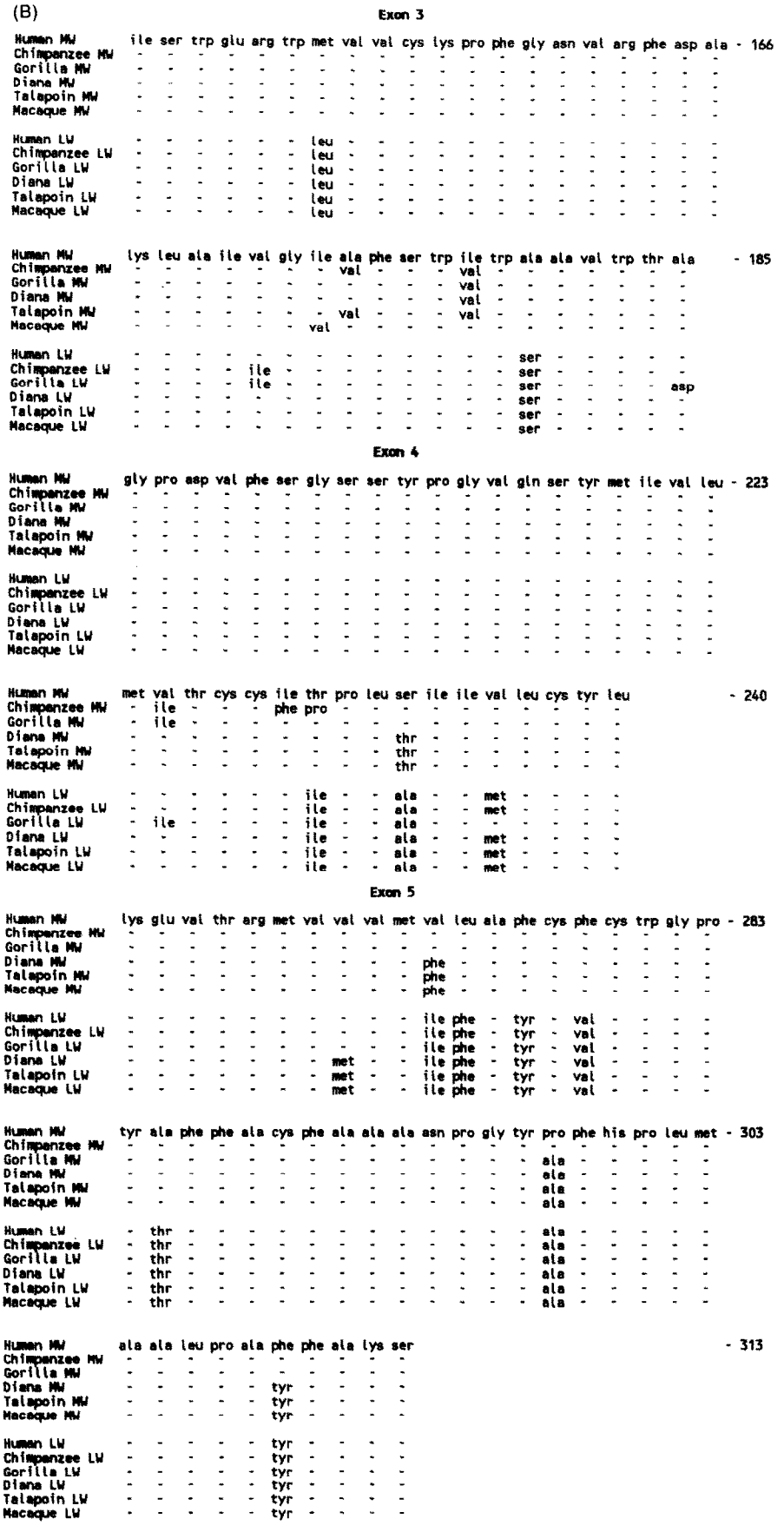


FIGURE 2(B)

FIGURE 2. Sequence of opsin genes from six species of Old World primates. Nucleotide (A) and deduced amino acid sequence (B). The human sequences are from Nathans *et al.* (1986a). Exons 4 and 5 of the Cercopithecoid monkeys were originally reported by Ibbotson *et al.* (1992).

1.5% low-melting-point agarose gel using a 0.09 M Tris–borate, 0.002 M EDTA buffer pH 8.0.

Amplified fragments were TA cloned (Invitrogen) into either the pCR100 or pCRII plasmids and sequenced by the dideoxy method using T7 polymerase and $^{35}$S-labelled dATP. The products of the sequencing reaction were loaded on to a 6.0% polyacrylamide gel, separated at 1500 V for about 3 hr, and autoradiographed overnight.

All clones were sequenced in both directions and in order to allow for *Taq* polymerase incorporation errors, the sequence was confirmed by cloning from at least two separate PCRs.

*Sequence analysis*

Phylogenetic trees were generated by the neighbour-joining method of Saitou and Nei (1987) using a computer program kindly supplied by Dr M. Nei of the Center for Demographic and Population Genetics, The University of Texas Health Center at Houston, Tex. The number of nucleotide substitutions for each pair of genes ($n$) was determined and the total number of nucleotide substitutions per site ($d$) was corrected for multiple substitutions by the method of Jukes and Cantor (1969), where the corrected value of $d = -(\frac{3}{4})\ln[1 - (\frac{4}{3})n]$.

## RESULTS AND DISCUSSION

The regions of exons 3, 4 and 5 that were sequenced code respectively for about half of TM3 and most of TM4, most of TM5, and all of TM6 and half of TM7, as shown in Fig. 1. The nucleotide and deduced amino acid sequences are shown in Fig. 2, together with the corresponding regions of the human genes (Nathans *et al.*, 1986a).

*Spectral tuning sequence variation of the MW and LW visual pigments*

The sequences are described as MW or LW on the basis of homology with the corresponding human sequences as originally reported by Nathans *et al.* (1986a). A complication with this assignment is that the human gene is known to be highly polymorphic for exon 3 (Nathans *et al.*, 1986a) and for codon 180 in particular, such that two different MW and two different LW pigments are found in the human population (Winderickx *et al.*, 1992; Neitz, Neitz & Jacobs, 1993). The association of a particular exon 3 with either a MW or LW exon 4 was not established in this study so the classification of exon 3 sequences as either MW or LW is based solely on homology with the original sequences reported by Nathans *et al.* (1986a). What is clear however is that the two types of exon 3, which code for either serine or alanine at position 180, are present throughout the Old World primates and are not just a feature of the human visual pigment genes.

Some substitutions are restricted to either the Cercopithecoid or the Hominoid lineages (Fig. 2). Serine-233, valine-274 and phenylalanine-309 are found only in the Hominoid MW gene, whereas methionine-271 is present only in the LW gene of the Cercopithecoids. Of these sites, only 233 has been implicated in spectral tuning (Williams *et al.*, 1992); the replacement of threonine by serine at this site in Hominoids is considered to be a conservative change (Lehninger, 1982) and recent work by Merbs and Nathans (1993) indicates that substitution at this site produces less than a 1 nm spectral shift. The Old World species exhibit no variation in the remaining three amino acids (specified by codons 180, 277 and 285) thought to be critical for spectral tuning (Neitz *et al.*, 1991; Williams *et al.*, 1992; Merbs & Nathans, 1992). The $\lambda_{max}$ of the MW (533 nm) and LW (563 nm) pigments of the three species of Cercopithecoid monkeys (Bowmaker, Dartnall & Lythgoe, 1980; Bowmaker *et al.*, 1991) are known to be very similar to those of man (Bowmaker & Dartnall, 1980) and, given the conserved amino acids at positions 180, 277 and 285 in the Great Apes, we would predict that the MW and LW pigments of gorilla and chimpanzee would have similar spectral characteristics to these Old World monkeys and to man. It would appear unlikely that any change in the spectral characteristics of the MW and LW pigments has arisen from the folivorous life style of the gorilla.

Although nothing is known behaviourally about the colour vision of gorillas, our results for the chimpanzee are concordant with the carefully conducted experiments of Grether (1940a, b, c, 1941), who found that chimpanzees behaviourally resembled human subjects in their Rayleigh matches, in their spectral limits, in their wavelength discrimination and in their saturation discrimination. The main differences that Grether observed between human and chimpanzee subjects were small: relative to human subjects tested in the same apparatus, chimpanzees had somewhat elevated hue-discrimination thresholds at long wavelengths, and there was a shift from 575 to 570 nm in the least saturated wavelength of the spectrum.

*Sequence polymorphism in Old World primates*

A number of polymorphisms in human MW and LW opsin gene sequences have been reported (Nathans *et al.*, 1986a; Winderickx *et al.*, 1993), and we have found a similar pattern of polymorphisms in non-human primates. The haplotypes of the polymorphic exons are shown in Table 1. The polymorphisms in the talapoin monkey and macaque are restricted to exon 3, whereas the chimpanzee and gorilla are polymorphic for both exons 3 and 4, although for both species, the polymorphism in exon 4 is limited to a single site. No polymorphic variation was found in exon 5 of any species examined. This bunching of polymorphic sites in exon 3 (Fig. 3) is also seen in humans (Nathans *et al.*, 1986a; Winderickx *et al.*, 1993), with eight sites at positions 494, 498, 500, 506, 552, 554, 562 and 573 common to humans and Old World primates. Since the alternative nucleotide present is in all cases identical to that present in the other type of opsin gene, the production of these polymorphisms by inter-genic recombination would appear to be

TABLE 1. Haplotypes of polymorphic exons

| | Exon 3 | | | | | | | | | | | | | | Exon 4 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chimpanzee | | | Gorilla | | | Talapoin | | | | Macaque | | | | Chimpanzee | | | Gorilla | | |
| Site | MW | LW1 | LW2 | MW1 | MW2 | LW | MW1 | MW2 | MW3 | LW | MW1 | MW2 | MW3 | LW | MW1 | MW2 | LW | MW | LW1 | LW2 |
| 494* | | | | A — | G — | G — | | | | | | | | | | | | | | |
| 498 | | | | A met | C leu | C leu | | | | | | | | | | | | | | |
| 500 | | | | | | | | | | | G ile | G ile | A met | G ile | | | | | | |
| 506* | | | | C — | G — | G — | | | | | | | | | | | | | | |
| 524 | | | | | | | T — | T — | C — | C — | C — | T — | C — | C — | | | | | | |
| 528 | | | | G gly | A arg | A arg | | | | | | | | | | | | | | |
| 545 | | | | | | | G — | C — | C — | C — | | | | | | | | | | |
| 551 | C — | C — | T — | | | | | | | | T — | C — | T — | C — | | | | | | |
| 552* | G val | A ile | G val | | | | | | | | | | | | | | | | | |
| 554* | G — | T — | G — | | | | | | | | | | | | | | | | | |
| 557 | C — | C — | A — | | | | | | | | A — | C — | A — | C — | | | | | | |
| 558 | A ile | A val | G val | | | | | | | | G val | A ile | G val | A ile | | | | | | |
| 562* | | | | | | | C ala | C ala | T val | C ala | | | | | | | | | | |
| 563 | | | | | | | C — | T — | C — | T — | | | | | | | | | | |
| 566 | C — | C — | T — | | | | | | | | | | | | | | | | | |
| 573* | | | | | | | A — | A ile | G val | A ile | | | | | | | | | | |
| 575 | | | | | | | C — | C — | A — | C — | | | | | | | | | | |
| 587 | G — | G — | T — | | | | | | | | | | | | | | | | | |
| 593 | A — | A — | G — | | | | | | | | | | | | | | | | | |
| 659 | | | | | | | | | | | | | | | | | | T — | T — | C — |
| 758 | | | | | | | | | | | | | | | C — | T — | C — | | | |

Genomic DNA was obtained from a male chimpanzee, a female gorilla, a male talapoin monkey and a male macaque. Only sites that are polymorphic in at least one species are shown. Where a polymorphic site produces an amino acid change, the amino acid coded by the haplotype is indicated. Sites that are also polymorphic in man (Nathans *et al.*, 1986a; Winderickx *et al.* 1993) are indicated with an asterisk.

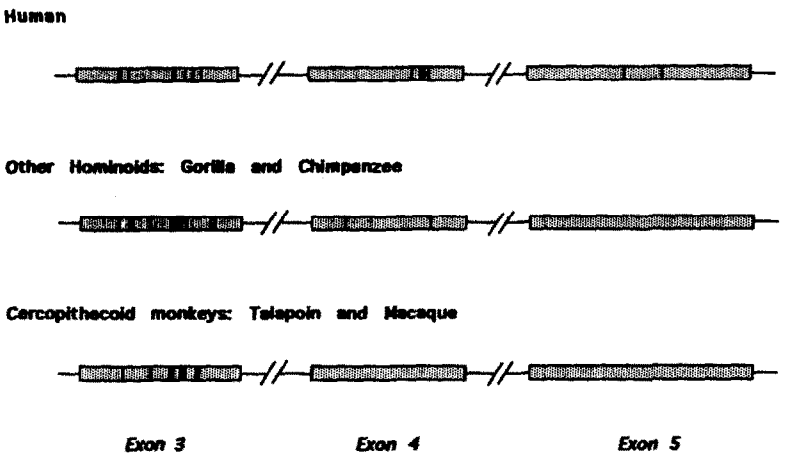


FIGURE 3. Position of polymorphic sites in the MW and LW genes of Old World primates. The human polymorphic sites are taken from previously published data (Nathans *et al.*, 1986a; Winderickx *et al.*, 1993).

the most likely mechanism. Such exchanges must occur therefore much more frequently in exon 3 than in either exon 4 or 5, and since only in a few cases are the positions of these polymorphic sites the same in the different species, the production of such exchanges must be an ongoing process. Winderickx *et al.* (1993) have identified a sequence (from 497 to 504) that resembles the hypervariable minisatellite sequence. This sequence has been shown to promote recombination (Wahls, Wallace & Moore, 1990) and has been found at certain chromosomal breakpoints (Kenter & Birshtein, 1981; Krowczynska, Rudders & Krontix, 1990). This region is polymorphic at site 498 but is otherwise conserved across the five species of Old World monkeys examined here. The high rate of intergenic exchanges seen in this region may be the consequence therefore of the presence of this sequence (Winderickx *et al.*, 1993).

*Multiple opsin genes*

For the reasons discussed above, either of the exon 3 haplotypes identified as LW in the male chimpanzee could be part of a MW gene. These results are consistent therefore with the original suggestion (Nathans *et al.*, 1986a) that only the MW gene can be present in multiple copies. The sex of the animals studied is critical to the understanding of these multiple sequences. In the case of a female, the simplest explanation for the presence of more than two sequences is that there is a different version of the MW or the LW gene on her two X chromosomes; this would account then for the multiple MW exon 3 and LW exon 4 sequences found in the gorilla. In males however, the multiple sequences identify the presence of more than two opsin genes on a single X chromsome. The multiple exon 3 sequences that were found in the male chimpanzee, the male talapoin monkey and the male macaque indicate therefore that these animals carry three, four and four opsin genes respectively on the X chromosome.

These observations are consistent with the previous identification of multiple opsin genes in humans (Nathans *et al.*, 1986a; Drummond-Borg *et al.*, 1989), and in talapoin monkeys (Ibbotson *et al.*, 1992). In both

TABLE 2. Average number of substitutions per site

| | C MW | G MW | D MW | T MW | M MW | H LW | C LW | G LW | D LW | T LW | M LW | 563 | 556 | 543 | CH-I |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H MW | 0.036 | 0.028 | 0.048 | 0.045 | 0.039 | 0.042 | 0.042 | 0.048 | 0.066 | 0.066 | 0.063 | 0.081 | 0.066 | 0.066 | 0.255 |
| C MW | | 0.039 | 0.060 | 0.051 | 0.057 | 0.078 | 0.078 | 0.066 | 0.084 | 0.084 | 0.081 | 0.063 | 0.048 | 0.054 | 0.255 |
| G MW | | | 0.045 | 0.042 | 0.039 | 0.054 | 0.060 | 0.048 | 0.072 | 0.072 | 0.069 | 0.081 | 0.066 | 0.072 | 0.251 |
| D MW | | | | 0.008 | 0.028 | 0.063 | 0.069 | 0.063 | 0.045 | 0.057 | 0.054 | 0.072 | 0.063 | 0.069 | 0.255 |
| T MW | | | | | 0.025 | 0.060 | 0.066 | 0.060 | 0.048 | 0.054 | 0.057 | 0.075 | 0.060 | 0.066 | 0.247 |
| M MW | | | | | | 0.051 | 0.057 | 0.051 | 0.051 | 0.057 | 0.054 | 0.054 | 0.051 | 0.051 | 0.239 |
| H LW | | | | | | | 0.011 | 0.022 | 0.042 | 0.048 | 0.045 | 0.069 | 0.054 | 0.054 | 0.239 |
| C LW | | | | | | | | 0.011 | 0.042 | 0.048 | 0.045 | 0.069 | 0.054 | 0.054 | 0.239 |
| G LW | | | | | | | | | 0.042 | 0.048 | 0.045 | 0.057 | 0.042 | 0.048 | 0.239 |
| D LW | | | | | | | | | | 0.011 | 0.008 | 0.054 | 0.048 | 0.054 | 0.247 |
| T LW | | | | | | | | | | | 0.014 | 0.066 | 0.054 | 0.060 | 0.255 |
| M LW | | | | | | | | | | | | 0.057 | 0.051 | 0.057 | 0.239 |
| 563 | | | | | | | | | | | | | 0.014 | 0.019 | 0.243 |
| 556 | | | | | | | | | | | | | | 0.006 | 0.236 |
| 543 | | | | | | | | | | | | | | | 0.239 |

Values have been corrected for multiple substitutions by the method of Jukes and Cantor (1969).

H, human; C, chimpanzee; G, gorilla; D, diana monkey; T, talapoin monkey; M, macaque; 563, 556, 543, marmoset alleles; CH-I, chicken iodopsin.

Where polymorphism is present, the sequence shown in Fig. 2 has been used in the analysis.

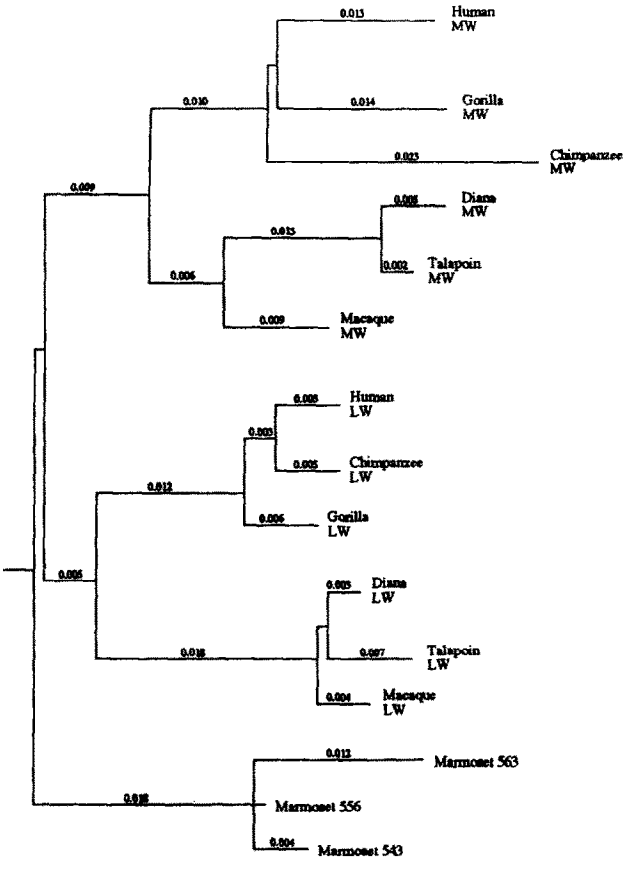


FIGURE 4. Phylogenetic tree generated from sequence divergence of the MW and LW opsin genes of Old World primates, the three allelic variants of the marmoset gene, and the chicken iodopsin gene (not shown). The tree was generated by the neighbour-joining method of Saitou and Nei (1987). The average number of substitutions per site for each branch of the tree is shown.

cases, it was thought that only MW sequences are present in multiple copies, although an alternative hypothesis that multiple LW genes can also be present has now been proposed by Neitz and Neitz (1992). In either case, the conclusion that multiple opsin genes are present in Cercopithecoid and Hominoid primates is not affected.

*Origin of separate MW and LW genes in Old World primates*

The neighbour-joining method of Saitou and Nei (1967) was used to generate the molecular phylogeny of the MW and LW opsin genes. The equivalent gene sequences from the marmoset (Hunt, Williams, Bowmaker & Mollon, 1993) were included in this analysis, and the chicken iodopsin (Kuwata, Imamoto, Okano, Kokame, Kojima, Matsumoto, Morodome, Fukada, Shichida, Yasuda, Shimura & Yoshizawa, 1990) was used as an outgroup. The average number of base pair substitutions (silent and coding) in pairwise comparisons of the sequences were calculated and corrected for multiple substitutions (Table 2) by the method of Jukes and Cantor (1969). To make maximal use of the sequence data, all substitutions were included except those at codons 180, 277 and 285 that are known to affect spectral tuning (Neitz *et al.*, 1991; Williams *et al.*, 1992; Winderickx *et al.*, 1992).

As shown in Fig. 4, the separate MW and LW sequences of Old World primates appear only after the divergence of New and Old World primates. Each gene then shows a separate lineage into the Cercoipithecoid and Hominoid branches. This is consistent with the notion that the duplication event that gave rise to these separate genes occurred early in the evolution of Old World primates, before the separation of the Cercopithecoid and Hominoid lineages.

The marmoset (New World) gene is represented by three different allelic variants that specify pigments with $\lambda_{max}$ of 563, 556 and 543 nm respectively, and the phylogenetic analysis indicates that these variants arose some time after the establishment of the New World lineage. Since Old and New World primates depend on a common set of amino acid substitutions to achieve the spectral shifts of the MW (543 nm pigment in marmoset) and LW (563 nm pigment in marmoset) visual pigments (Neitz *et al.*, 1991; Ibbotson *et al.*, 1992; Williams *et al.*, 1992; Winderickx *et al.*, 1992), this would imply that the mechanism of spectral tuning in Old and New World primates arose separately in the two lineages; the use of a common set of amino acid sites in the spectral tuning process must be the result therefore of convergent evolution. The phylogenetic analysis may underestimate however the antiquity of the three marmoset sequences. Since these are allelic variants of a single polymorphic gene, the process of recombination will tend to limit sequence divergence, whereas the separate MW and LW genes of Old World primates will undergo exchange only by the considerably less frequent process of gene conversion (Balding, Nichols & Hunt, 1992; Winderickx *et al.*, 1993). It is possible therefore that the appearance of this allelic variation pre-dates the separation of Old and New World lineages. In this case, different spectral forms of the opsin gene would have been present in the ancestral primate, and an unequal exchange that placed two different allelic forms of this gene on to a single X chromosome would have resulted in the retention of the same key amino acid substitutions in both lineages.

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