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Molecular characterization of the red visual pigment gene of the American chameleon (*Anolis carolinensis*)

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The red sensitive visual pigment of the American chameleon, *Anolis carolinensis*, is unique in having absorption maximum some 50 nm further into the red than any other terrestrial vertebrate examined. We report here the isolation and sequence determination of the genomic DNA clone for the *Anolis* red visual pigment gene. Phylogenetic analysis shows that this gene is most closely related to the gecko green and chicken red visual pigment genes. We identified nine *Anolis*-specific amino acid replacements, seven of which reside in transmembrane domains and might contribute to the red-shift of the *Anolis* visual pigments.

Red visual pigment; Opsin; Color vision; Molecular evolution; *Anolis carolinensis*

1. INTRODUCTION

The lizard *Anolis carolinensis*, usually called green anole or American chameleon for its ability to change body color, is a diurnal and arboreal reptile and has been shown to be highly visual and possess color vision by behavioral experiments [1,2]. The *Anolis* retina is unique among terrestrial vertebrates, containing only cones [3–5], having deep central and shallow temporal foveas [6–8], and containing only 11-*cis*-3,4-dehydroretinal (vitamin A2) as a chromophore [9].

The *Anolis* cones have been classified into three morphologically distinct types: two types of single cones and double cones [10]. Although the correspondence to the morphological types has not been established, three spectral classes of photoreceptors have been identified by microspectrophotometry, having absorption maxima (λ_{max}) at about 462, 503, and 625 nm [9]. In particular, the λ_{max} of 625 nm is some 50 nm further into the red than any terrestrial vertebrates examined to date [9].

These prominent characters of the *Anolis* visual system prompted us to investigate their visual pigment genes. We report here the cloning and sequencing of the gene which most probably encodes the visual pigments having λ_{max} at about 625 nm. We have identified nine amino acid replacements specific to the *Anolis*. The dehydroretinal certainly contributes in absorbing light for higher wavelengths, but these amino acid changes might also be important in the red shift of the visual pigments.

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2. MATERIALS AND METHODS

2.1. *Southern blot analysis*

High molecular weight genomic DNAs were prepared from commercially supplied *Anolis carolinensis* by following a slightly modified procedure of Yokoyama and Yokoyama [11]. 10 μg per lane of genomic DNA were digested with restriction enzyme, electrophoresed on 0.5% agarose gel, and transferred to a Hybond-N nylon membrane (Amersham) by using the VacuGene vacuum blotting system (Pharmacia). The cDNA clone of human red visual pigment gene (hs7 in [12]; kindly provided by Dr. J. Nathans) was labeled with [α-^{32}P]dATP by the random-priming method and used as a hybridization probe. Hybridization was carried out at 55°C using the commercial protocol for Hybond-N membrane. Hybridized membrane was washed at 55°C four times (30 min each) in 1 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS.

2.2. *Genomic library screening and DNA sequencing*

A genomic library of the *Anolis* was constructed with *Bam*HI-digested lambda EMBL3 vector and *Sau*3AI partially digested *Anolis* genomic DNA (10–20 kb). The *Anolis carolinensis* has a diploid genome [13] and its DNA nuclear content is approximately 60% of the human genome [14]. We have screened about 1×10^6 recombinant plaques using hs7 as the probe. 11 lambda clones were obtained after three rounds of plaque hybridization, using the Southern hybridization conditions. One of these clones, λ24, was found to contain the entire coding region (designated R_{Ac}) and the hs7-hybridizing restriction fragments were subcloned into the Bluescript SK(−) vector. Nested deletions were introduced into the subclones using exonuclease III and mungbean nuclease. Then the nucleotide sequence of R_{Ac} was determined for both strands by the dideoxy-chain-termination method using double stranded templates [15].

2.3. *Sequence analysis*

The DNA sequence of R_{Ac} was compared to other red (long wavelength-sensitive; LWS) visual pigment genes from human (*Homo sapiens*) (R_{Hs}; [12]), chicken (*Gallus gallus* (R_{Gg}; [16]), cave fish (*Astyanax fasciatus*) ($R007_{Af}$; [17,18]) and gold fish (*Carassius auratus*) (R_{Ca}; [19]) as well as green (medium wavelength-sensitive; MWS) visual pigment genes from human (G_{Hs}; [12]), gecko (*Gekko gekko*) (G_{Gge}; P521 pigment in [20]), and the cave fish ($G101_{Af}$ and $G103_{Af}$; [11,17]). To

construct a rooted phylogenetic tree of the LWS and MWS (LWS/MWS) visual pigment genes, the rhodopsin genes from lamprey (Rh_{Lj}; [21]), sand goby (Rh_{Pm}; [22]), African water frog (Rh_{Xl}; Barry Knox, unpublished), chicken (Rh_{Gg}; [23]), human (Rh_{Hs}; [24]), bovine (Rh_{Bt}; [25]), and mouse (Rh_{Mm}; [26]) were used to represent the outgroup. All of these sequences were initially aligned by using a multiple alignment program in CLUSTAL V [27] and adjusted further visually to maximize similarity.

The phylogenetic tree was constructed by using the maximum likelihood (ML) method (DNAML program version 3.41) in PHILIP 3.4 [28].

3. RESULTS

3.1. *Presence of a single copy gene of the* Anolis *red visual pigment*

To estimate the copy number of the LWS/MWS visual pigment genes in the *Anolis* genome, the human red cDNA (hs7) was hybridized to the *Anolis* genomic DNA. The Southern hybridization revealed only one band for each of three different restriction digests (Fig. 1). The sizes of these bands were consistent with those expected from the restriction map of λ24 (data not shown). Furthermore, when other restriction enzymes, which are known to digest λ24 internally, were used, the number and sizes of bands detected in the genomic Southern again corresponded exactly to those expected from the restriction map of λ24 (data not shown). These observations strongly suggest that the *Anolis* genome contains only a single copy of the LWS/MWS visual pigment gene and that λ24 is an unrearranged copy of it.

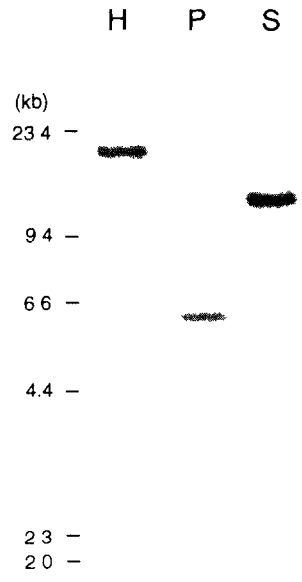

Fig. 1. Southern hybridization of *Hin*dIII (H), *Pst*I (P), and *Sst*I (S) digested genomic *Anolis carolinensis* DNA with human red cDNA clone (hs7, [12]) λ*Hin*dIII size standards are indicated in kb at the left margin.

3.2. *Structure of the* Anolis *red visual pigment gene*

R_{Ac} contains six exons and five introns spanning 3.7 kb from start to stop codons (Fig. 2). Introns interrupt the coding sequence at exactly the same positions as those of R_{Hs} and G_{Hs} in human and $R007_{Af}$, $G101_{Af}$, and $G103_{Af}$ in fish. A consensus TATA box sequence (TATAAA) was found 194 bp upstream from the start codon and two putative polyadenylation signals (AATAAA) were located 403 bp and 940 bp downstream from the stop codon. Splice junction signals (GT/AG) are conserved in all introns and there is no nonsense mutation in the coding region.

From the deduced amino acid sequence (369 residues long), potentially important amino acids can be identified: Lys-315, the site of Schiff base linkage to the retinal chromophore [29,30]; Glu-132, the Schiff base counterion [31–33]; Cys-129 and Cys-206, the sites for an essential disulfide bond [34]; Glu-153, Arg-154 and Lys-257, thought to interact with transducin [35,36]; Asn-37, a possible N-glycosylation site common in LWS/MWS visual pigments [11,12,16–20]; Thr-106, Ser-110 and Thr-135, candidates to form a counterion complex with Glu-132 in LWS/MWS pigments [37]; Trp-145, Trp-284 and Tyr-287, involved in conformational change during retinal isomerization and formation of retinal binding pocket [38]; Cys-159, involved in phosphorylation through interacting with C-terminal tail [39]; and multiple serines and threonines in the C-terminal region, potential phosphorylation sites for opsin kinase [40].

Importantly, the deduced amino acid sequence also possesses three critical amino acids (Ser-183, Tyr-280 and Thr-288) which distinguish red visual pigments from green visual pigments in primates and fish [17,41–45].

3.3. *Phylogenetic analysis*

Using the neighbor-joining (NJ) method [46], we have shown that the LWS/MWS visual pigment genes can be distinguished into two groups: (1) R_{Hs} and G_{Hs} (Group I) and (2) G_{Gge}, R_{Gg}, $R007_{AF}$, $G101_{Af}$ and $G103_{Af}$ (Group II) [18]. Within Group II, $R007_{AF}$, $G101_{Af}$ and $G103_{Af}$ are most closely related, their common ancestor diverged from the ancestor of G_{Gg}, and their common ancestor diverged from the ancestor of G_{Gge} [18]. However, the better tree topology can be found by using the ML method, where Group II genes are further subdivided into two separate groups: (1) $R007_{Af}$, $G101_{Af}$ and $G103_{Af}$ and (2) G_{Gge} and R_{Gg} [18].

Using the rhodopsins as the outgroup and applying the NJ method on the amino acid sequences of the visual pigments encoded by the seven LWS/MWS visual pigment genes as well as R_{Ac} and R_{Ca}, it can be shown that the latter two genes also belong to Group II. The phylogenetic analysis based on the NJ method shows that, within Group II, R_{Ca}, $R007_{Af}$, $G101_{Af}$ and $G103_{Af}$ form one subgroup (Group IIa), their common ancestor

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                                ctgggctaaa aggtccctta gcctatttag aaaaatgggc cattaggaaa ttgcaaggaa gaaccattcg tgagagggat
tagctgagct cttttgactc tctaatcacc cctccgtgct catccctcac ctgaagtgtc cagcaaatac accaagggtg acgcaggaca agcatgagcc attcatactg
ctgcaaccag agagagggag caggaaaatg agacagggag ggggccaaat cacagcccaa ttaagagata aaacgtttaa gggctttgag gatcttgttc tccaagtcag
gggcttaatc cgcctggggg ggtgggtata aaaatgggca gtgctctggg ccctgtcact gacgtttgga tgggagagac tgagcggtag agagggaaag aagagaagaa
gagagagagg agagaaaagg ggggagagag cgaaactagg aagagaagga aagtgagggg ataggaaagg ggcaaaaaac aacaagcgct ggtagtaggc gggaaagaag

                                    10                                  20                                  30
Met Ala Gly Thr Val Thr Glu Ala Trp Asp Val Ala Val Phe Ala Ala Arg Arg Arg Asn Asp Glu Asp Asp Thr Thr Arg Asp Ser Leu
ATG GCA GGG ACC GTG ACG GAA GCC TGG GAT GTG GCA GTA TTT GCT GCC CGA CGG CGC AAT GAT GAA GAC GAC ACC ACA AGG GAT AGC TTG
                                    40                                                                      50
Phe Thr Tyr Thr Asn Ser Asn Asn Thr Arg G                                    ly Pro Phe Glu Gly Pro Asn Tyr His Ile Ala
TTC ACT TAT ACC AAC AGC AAC AAT ACC CGG G gtgagcccag...Intron 1...tctctggcag GC CCC TTT GAA GGT CCA AAC TAT CAC ATT GCG
                                    60                                  70                                  80
Pro Arg Trp Val Tyr Asn Ile Thr Ser Val Trp Met Ile Phe Val Val Ile Ala Ser Ile Phe Thr Asn Gly Leu Val Leu Val Ala Thr
CCA CGC TGG GTC TAC AAT ATC ACT TCT GTC TGG ATG ATT TTT GTG GTC ATC GCT TCA ATC TTC ACC AAT GGT TTG GTA TTG GTG GCC ACT
                                    90                                  100                                 110
Ala Lys Phe Lys Lys Leu Arg His Pro Leu Asn Trp Ile Leu Val Asn Leu Ala Ile Ala Asp Leu Gly Glu Thr Val Ile Ala Ser Thr
GCC AAA TTC AAG AAG CTA CGG CAT CCT CTG AAC TGG ATT CTG GTA AAC TTG GCG ATA GCT GAT CTG GGT GAG ACG GTT ATT GCC AGT ACC
                                    120                                 130                                 140
Ile Ser Val Ile Asn Gln Ile Ser Gly Tyr Phe Ile Leu Gly His Pro Met Cys Val Leu Glu Gly Tyr Thr Val Ser Thr Cys G
ATC AGT GTC ATC AAC CAG ATC TCT GGC TAC TTC ATC CTT GGC CAC CCT ATG TGT GTG CTG GAG GGA TAC ACT GTT TCA ACT TGT G gtaaga
                                                        150                                                     160
                              ly Ile Ser Ala Leu Trp Ser Leu Ala Val Ile Ser Trp Glu Arg Trp Val Val Val Cys Lys Pro Phe
gaca...Intron 2...ttctccatag GT ATC TCA GCT CTC TGG TCC TTG GCC GTT ATA TCT TGG GAA CGC TGG GTC GTT GTC TGC AAG CCC TTT
                            170                                 180                                 190
Gly Asn Val Lys Phe Asp Ala Lys Leu Ala Val Ala Gly Ile Val Phe Ser Trp Val Trp Ser Ala Val Trp Thr Ala Pro Pro Val Phe
GGA AAT GTC AAG TTC GAT GCC AAA CTG GCC GTG GCT GGC ATT GTC TTC TCC TGG GTG TGG TCT GCT GTA TGG ACA GCA CCA CCC GTC TTT
                                                        200                                 210
Gly Trp Ser Ar                                    g Tyr Trp Pro His Gly Leu Lys Thr Ser Cys Gly Pro Asp Val Phe Ser Gly
GGC TGG AGT AG gtgagtgtga...Intron 3...ttttctctag G TAC TGG CCC CAT GGC TTG AAG ACC TCA TGC GGT CCA GAT GTA TTC AGT GGC
                            220                                 230                                 240
Ser Asp Asp Pro Gly Val Leu Ser Tyr Met Ile Val Leu Met Ile Thr Cys Cys Phe Ile Pro Leu Ala Val Ile Leu Leu Cys Tyr Leu
AGT GAT GAC CCC GGT GTT CTG TCT TAC ATG ATT GTC CTC ATG ATC ACC TGT TGC TTC ATT CCC CTG GCT GTC ATC CTT CTC TGC TAC CTG
                            250                                                             260
Gln Val Trp Leu Ala Ile Arg Ala                                         Val Ala Ala Gln Gln Lys Glu Ser Glu Ser Thr Gln Lys
CAA GTG TGG TTG GCT ATC CGT GCG gtaggtactc...Intron 4...ctcatacacag GTT GCT GCC CAG CAG AAA GAG TCT GAA TCT ACA CAG AAG
                        270                                 280                                 290
Ala Glu Lys Glu Val Ser Arg Met Val Val Val Met Ile Ile Ala Tyr Cys Phe Cys Trp Gly Pro Tyr Thr Val Phe Ala Cys Phe Ala
GCT GAG AAG GAA GTG TCG AGG ATG GTG GTA GTC ATG ATA ATT GCC TAT TGC TTC TGC TGG GGG CCA TAT ACG GTC TTT GCC TGT TTT GCT
                        300                                 310                                 320
Ala Ala Asn Pro Gly Tyr Ala Phe His Pro Leu Ala Ala Ala Leu Pro Ala Tyr Phe Ala Lys Ser Ala Thr Ile Tyr Asn Pro Ile Ile
GCT GCC AAC CCA GGC TAT GCC TTC CAC CCC CTT GCA GCT GCT CTG CCT GCT TAC TTT GCA AAG AGC GCA ACT ATC TAC AAC CCA ATT ATC
                        330                                                             340
Tyr Val Phe Met Asn Arg Gln                                             Phe Arg Asn Cys Ile Met Gln Leu Phe Gly Lys Lys Val Asp
TAC GTC TTC ATG AAC AGA CAG gtaattttct...Intron 5...tcttctggcag TTC CGT AAC TGC ATA ATG CAG CTC TTT GGC AAG AAG GTG GAT
                    350                                 360
Asp Gly Ser Glu Leu Ser Ser Thr Ser Arg Thr Glu Val Ser Ser Val Ser Asn Ser Ser Val Ser Pro Ala
GAT GGC TCT GAA TTA TCC TCC ACC TCC CGC ACT GAG GTC TCT TCT GTC TCT AAT TCC TCT GTA TCG CCG GCA TAA gagccccctg cctgaatagc

cacaaagact ggagattctg cctcccagga gagccccagt gagaacttcc tctttcctcc tggactgatt gctgctgaac agattcccct tgagaatatg aaatactggc
ctgccatctc tacagatcct cttctttgaa cttatactgc accctcagct gggagtgctg ctgtttctcc cctggtgatt tgggggaggg aaatgttttt tctctcaaaa
ttgctttggg atgggggtga aggggtgagg aattctctgc tcaaatatcc catctttctg caacattccc aagaggtgtt gtttgaacac tcttttccca aggtcccgtc
ctgactcact agtctccctg tgcacccctc ccccaactcc ccagaggagc agaataaagg gggttccctg gggaaagcat atcgcttttg taaagcaagt ctattggagg
ccgtcttagc agtgaacctg cccctccatc cgcatttccc cccatgatcc acatcctttg actggatgga ctgatatccc aagagggaag attacccaca gtttggcaac
acaggccata ttccctttt ctctgcccct cctatgaagt tttcactggt gccagggagt gcattgaagg gggccaagag gaggaaaagt ttgtcccccg aggtgtctgt
gcctcagcca agtcgaatgc attgaatctt aattctgttg aaagtgggca tgattctgac acgggtagaa ccaacgagac tcagcatgat ccacctcggt cattgcaggg
gctttgtgct tcttcccagc atggctcaac tggaaccatt ctgtacatag tgtttgatac taaagcctgt tgtgtccctg ggacatctag ctagaaatgc aaaagggaaa
atgaccaaaa aaatctgcag cagtgagagt gagatagaga ataaagattt ttaacttctg tatataagtt ctgttcttat cctaccaaaa aaaaaaaatc aaaccctgat
acacccaaag aatgcccttt gcccagaagt gtttgctcta tatctatgca tgcgggtgag atggcggtga ctgcagtgga ggaagaggtt gggggtctgc ttggtgatta
cagactatgt ggagagaaga ctcag
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *Anolis* red visual pigment gene. The deduced amino acid sequence is written above each nucleotide triplet. A possible TATA box and two putative polyadenylation signals AATAAA are underlined. Introns 1, 2, 3, 4 and 5 consist of 424 bp, 497 bp, 608 bp, 755 bp and 333 bp, respectively.

diverged from that of R_{Ac} and R_{Gg}, and the common ancestor of these 6 genes diverged from the ancestor of G_{Gge} before that. The bootstrap resampling shows that Group IIa, and R_{Ac}, R_{Gg} and G_{Gge} (Group IIb) cluster with probability 0.95. However, Group IIa genes, R_{Ac} and R_{Gg} cluster with probability 0.48. Our previous phylogenetic analysis [18] strongly suggests that this low probability occurred because Group IIa and Group IIb are not treated as separate groups. Hence, we identified four tree topologies, one of which is the topology obtained by the NJ method and the other three assume that the Groups IIa and IIb form two separate clusters within the Group II, having different phylogenetic relationships among the three genes in the Group IIb. Then, the DNAML program [28] was applied to sequences with the bases at the third position of codons removed to reduce the noise caused by multiple substitutions.

In evaluating the log likelihoods for each tree topology, we assumed that Rh_{Hs} and Rh_{Mm} are most closely related, and Rh_{Bs}, Rh_{Gg}, Rh_{Xl}, Rh_{Pm} and Rh_{Lj} are more distantly related, in that order. One representative tree is given in Fig. 3, where R_{Ac} and G_{Gge} are most closely related. As we can see in Fig. 3, the 95% confidence interval of the branch for the common ancestor of R_{Ac} and G_{Gge} after its divergence from the ancestor of R_{Gg} is 0.0–0.019, showing that the phylogenetic relationship among G_{Gge}, R_{Ac} and R_{Gg} cannot be clearly established. The differences in the log likelihoods for the three tree topologies are not statistically significant, but the NJ tree is always significantly inferior to these tree topologies. Although the ML method does not provide a superior topology, the topology in Fig. 3 seems to be most likely because the insertion and deletion structures of N- and C-termini in G_{Gge} and R_{Ac} are most similar among the three Group IIb genes.

4. DISCUSSION

We have shown that there exists only one gene in the *Anolis* genome which is homologous to the LWS/MWS visual pigment genes in other vertebrates. Furthermore, R_{Ac} has Ser-138, Tyr-280 and Thr-258, which is the unique feature for all the red visual pigments studied so far [16,17,41,42]. All of these characteristics of R_{Ac} strongly suggest that it is the gene which encodes the visual pigment with maximum absorption at 625 nm.

This λ_{max} of the *Anolis* red visual pigment is uniquely long among terrestrial vertebrates and influences sexual, fighting, and feeding behaviors of this species. Acquisition of such a red vision is thought to be effective for this arboreal species to detect insect prey and to display its red dewlap of the throat during courtship and assertion against a background of green leaves and branches or the usually green surface of the rest of the body. Furthermore, display of the red dewlap has been shown to correlate with reproductive success [47,48]. As already noted, the *Anolis* employs the dehydroretinal as a chromophore which contributes to the big red shift of the λ_{max} value [9]. It is also possible that the opsin itself might have contributed to the red shift of the visual pigment.

Thus, we compared amino acid sequences of the rhodopsin and LWS/MWS visual pigments and, using the tree topology in Fig. 3, found nine amino acid changes which occurred in the R_{Ac} branch. Two of them, Asp-215 and Leu-220, are located in the second extracellular loop. Since the two residues are not critical for the rhodopsin function [49], these changes may not be important in the red shift of the *Anolis* vision. The other seven sites are all in transmembrane domains (Table I). Five of them, Ile-58, Ser-142, Val-191, Leu-239 and Val-289, are *Anolis*-specific. Among these, Val-289, an aliphatic amino acid, is derived from Phe, an aromatic amino acid. The other six substitutions are between amino acids with similar electrostatic properties: Ser-142 between hydroxyl-bearing amino acids, the remaining five between aliphatic ones.

Thus, most of these amino acid replacements do not change electrostatic properties. It should be noted, however, that the three amino acids Ser-183, Tyr-280 and Thr-288 of the LWS visual pigments replaced Ala, Phe, and Ala of the MWS visual pigments, respectively. These corresponding amino acids belong to the same physicochemical groups [50], but they are responsible for absorbing long and short wavelenghts. It will therefore be worthwhile to determine whether these seven *Anolis*-specific amino acid changes are important in absorbing the longer wavelength by using site-directed

Table I

Amino acid replacements in the *Anolis* red visual pigment

Residue	Transmembrane	G_{101}	G_{103}	R_{007}	R_{Ca}	G_{Gge}	R_{Ac}	R_{Gg}	G_{Hs}	R_{Hs}
58	I	**V**	L	L	L	L	**I**	L	L	L
142	III	T	T	**A**	**A**	T	**S**	T	T	T
149	III	I	I	**V**	**V**	I	**V**	I	I	I
191	IV	I	I	I	I	I	**V**	I	I	I
237	V	I	**V**	I	I	I	**V**	I	I	I
239	V	I	I	I	I	I	**L**	I	**V**	**M**
289	VI	**S**	**S**	F	F	**S**	**V**	F	F	F

Amino acid substitutions are in bold print.

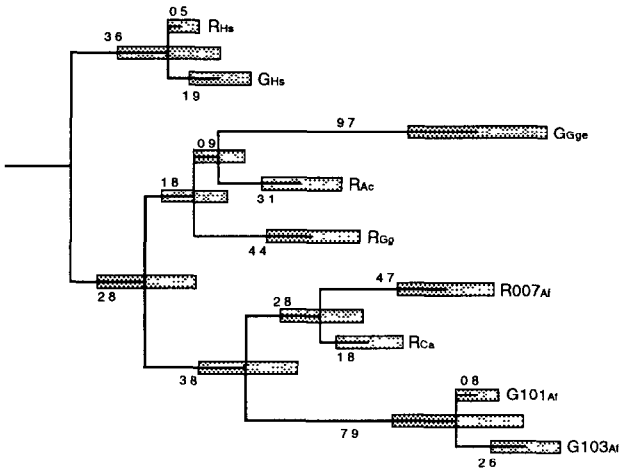

Fig. 3. A rooted phylogenetic tree for the LWS/MWS visual pigment genes based upon the first and second positions of codons, where rhodopsin genes were used as the outgroup. The numbers next to the different branches are branch lengths in 100 times the numbers of nucleotide substitution, as estimated by means of the ML method with the transition/transversion ratio set as 2 [28]. The stippled bars indicate the 95% confidence limits.

mutagenesis as has been done with other systems [31–36,38,39,45,49,51]. Furthermore, it will be of considerable interest to test such hypotheses in the presence of the dehydroretinal and 11-*cis*-retinal separately.

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