

The spectrum of human rhodopsin disease mutations through the lens of interspecific variation[☆]

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Received 21 January 2004; accepted 13 February 2004

Received by S. Yokoyama

Available online 17 April 2004

Abstract

Mutations in rhodopsin, the visual pigment found in rod cells, account for a large fraction of genetic changes underlying the human retinal diseases, Retinitis Pigmentosa (RP). The availability of rhodopsin sequences from a large number of vertebrates has allowed us to investigate factors important in the development of RP by contrasting interspecific differences (long-term evolutionary patterns) with RP disease mutation data. We find that disease mutations in rhodopsin are overabundant in highly conserved sites and that amino acid positions with any potential of variability among vertebrates are likely to harbour disease mutations less frequently. At any amino acid position in rhodopsin, the set of disease-associated amino acids does not show any commonality with the set of amino acids present among species. The disease mutations are biochemically four times more radical than the interspecific (neutral) variation. This pattern is also observed when disease mutations are categorized based on clinical classifications that reflect biochemical, physiological and psychophysical traits such as protein folding, cone electroretinogram (ERG) amplitude, pattern of visual field loss, and equivalent field diameter. We also found that for artificial mutations (those not observed in nature interspecifically), there was a positive relationship between the biochemical distance and the magnitude of blue shift in the absorption spectrum maximum. We introduce the concept of the expected chemical severity based on the normal human codon at a position. Results reveal that the analysis of disease mutations in the context of the original codon is very important for the practical application of evolutionary principles when comparing original and disease amino acid mutations. We conclude that the analysis of rhodopsin data clearly demonstrates the usefulness of molecular evolutionary analyses for understanding patterns of clinical as well as artificial mutations and underscores the biomedical insights that can be gained by using simple measures of biochemical difference in the context of evolutionary divergence.

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Keywords: Visual pigment; Retinitis pigmentosa; Photoreceptor; Opsin; Neutral theory

1. Introduction

It is now well known that protein sequence defects are the cause of a large number of inherited diseases (Scriver et al., 2001). In particular, replacement mutations in the coding sequences of genes are thought to be responsible for the bulk of drastic human phenotypes (Krawczak et al., 2000). These disease-causing mutations are deleterious in effect and expected to be removed from the population by natural selection given sufficient time. However, not all positions in a protein are equally likely to harbor disease mutations (Miller and Kumar, 2001) and the disease propensity of a

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor-joining; ERG, electroretinogram; RP, retinitis pigmentosa; ADRP, autosomal dominant retinitis pigmentosa; ARRP, autosomal recessive retinitis pigmentosa; CSNB, congenital stationary night blindness; RPA, retinitis punctata albescens; ECS, expected chemical severity.

B Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.gene.2004.02.037](https://doi.org/10.1016/j.gene.2004.02.037).

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replacement mutation depends on its effect on protein function. Analysis of known disease mutations in the context of interspecific (neutral) variability provides unique opportunities to elucidate these patterns and understand factors important in the development of genetic diseases (e.g. Miller and Kumar, 2001; Greenblatt et al., 2003).

In this study, we have examined the relationship of genetic variation in the rhodopsin protein, the visual pigment found in rod cells, with the genetic differences observed between species. Rhodopsin is particularly suitable for this study because of the large number of mutant proteins (both natural and artificial) that have been constructed to elucidate the relationship between rhodopsin structure and function and the large number of clinical disease states that have been traced to mutations in this gene (see supplementary material available at <http://visiongene.bio.uci.edu/ABresearch.html>).

Rod cells make up 90% (~ 120 million cells) of the human retina (Nathans, 1992) and rhodopsin, the visual pigment found in rod cells, is their most highly expressed protein, accounting for 80% of their outer segment disk membranes (Dryja, 2001). Rhodopsin is replaced every 10 days throughout life (Dryja et al., 1991; Nathans, 1992). Mutations in rhodopsin account for many of the genetic changes underlying the human retinal diseases, Retinitis Pigmentosa (RP). Indeed, approximately 25% of autosomal dominant RP (ADRP) cases (Dryja et al., 1991; Macke et al., 1993), which account for an estimated 8–10% of all RP cases, are due to mutations of the rhodopsin gene (Dryja, 2001).

Retinitis pigmentosa (RP) affects the outer segment of the rod photoreceptor cell, a specialized cilium that is composed of stacks of microvillous membranes containing the phototransduction machinery. Morphologically, RP is identified by the presence of a black or brown star-shaped pigmentation of the fundus—normally orange–red in color—that is due to the budding off and settling of the pigment epithelium within the layers of the retina. In late stages, a thinning of the retinal blood vessels may be observed, resulting from the loss of retinal cells and the reduced need for blood. A progressive disease, the various forms of RP can lead to reduced contrast vision, night blindness, decreased peripheral vision, and in extreme cases, tunnel vision. Diagnosed typically in patients between the ages of 10 and 30, it affects over 50,000 people in the US alone (Dryja, 2001).

Rhodopsin molecules, expressed in cell culture, have quantifiable properties that relate to their wild type function *in vivo*. These include proper folding, proper trafficking from the endoplasmic reticulum, and reconstitution with the chromophore, 11-*cis*-retinal, which results in a characteristic absorption spectrum (wild type λ_{\max} = 500 nm). In a number of artificial mutant studies, amino acids that differ in size, polarity and chemical composition of their side group were introduced into the same site in the rhodopsin molecule, and their phenotype scored for the above-mentioned properties (e.g. Kaushal and Khorana, 1994; Han et al., 1996). Such experiments provide an important internal control for the

role of amino acid position effects on the rhodopsin phenotype. This allows us to determine the relationship between the biochemical differences of point mutations at the same sites and the properties of mutant rhodopsins expressed in cell culture. Such relationships, if found, may prove useful for understanding the effects of engineered proteins both *in vitro* and *in vivo*.

Therefore, we were interested in determining whether simple measures of the biochemical difference between the wild type and mutant amino acid could be used to understand the variety of clinical symptoms observed in RP in the context of rhodopsin interspecific differences. Such correlations might prove useful in predicting the clinical outcomes of patients with novel rhodopsin mutations. Further, rhodopsin may act as a model for how we can use interspecific variation to understand human protein-based diseases.

2. Materials and methods

2.1. Rhodopsin mutation data

Rhodopsin disease mutations were obtained from the literature and from databases maintained by Retina International (<http://www.retina-international.com/sci-news/rhomut.htm>) and the University of Texas Houston Health Science Center (RetNet; <http://www.sph.uth.tmc.edu/RetNet/disease.htm>). A table of these compiled mutations is available as supplementary material (<http://www.visiongene.bio.uci.edu/ABresearch.html>). The final set contained 93 unique nonsynonymous disease mutations that cause autosomal dominant retinitis pigmentosa (ADRP), congenital stationary night blindness (CSNB), autosomal recessive retinitis pigmentosa (ARRP), and retinitis punctata albescens (RPA). All nonsense, deletion, as well as truncation mutations were excluded from our analyses. Frequencies of most of the disease mutations are not known. Therefore, we included each mutation only once in our analyses; this prevents biases resulting from the overrepresentation of commonly observed mutations over those reported less frequently.

2.2. Sequence alignment

Vertebrate rhodopsin amino acid sequences were downloaded from GenBank and aligned using ClustalW (Thompson et al., 1997) in the MEGA3 software (Kumar et al., 2003). The ClustalW alignment of the full-length rhodopsin sequences resulted in a less optimal alignment, where amino acids that are known to be critical for rhodopsin transport were not conserved. To better align the C-terminus, which is the only portion of the rhodopsin protein that varies in length between species, we used only the last 30 amino acids in ClustalW. In all analyses, the human sequence was used as the reference sequence. Fig. 1 shows the set of observed amino acids at different positions in the human rhodopsin cDNA.

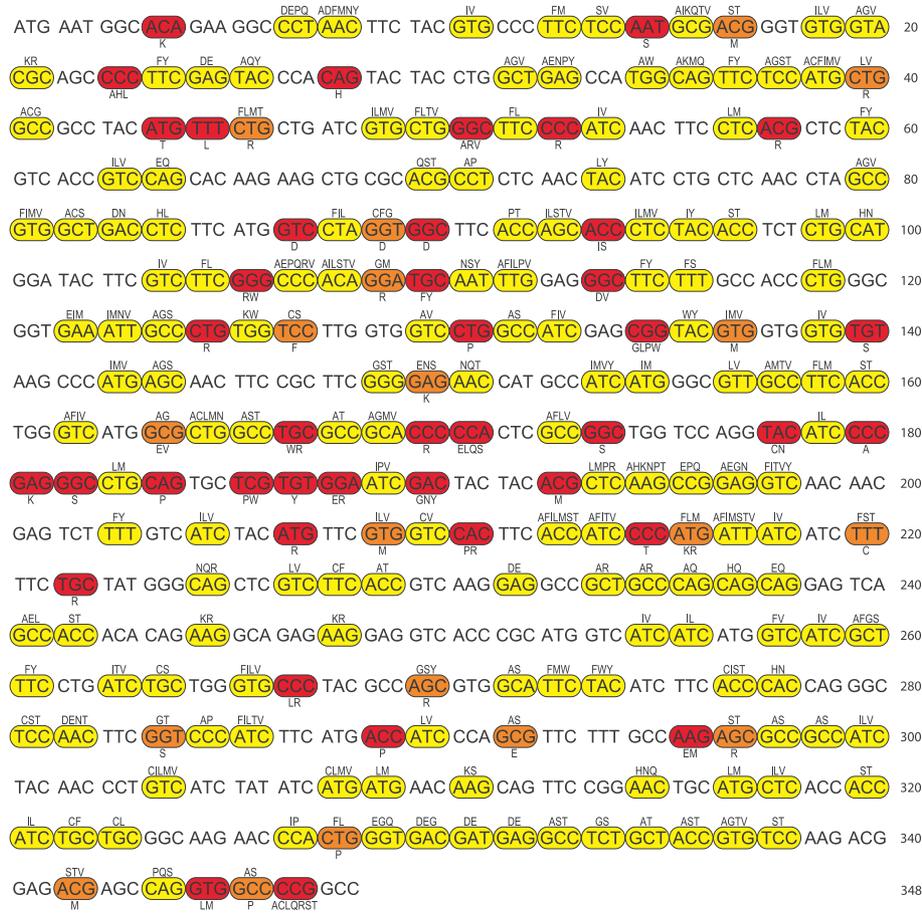


Fig. 1. A schematic showing the observed sets of amino acids at each human codon in among species alignment (above) and disease mutations (below). Red background marks codons harboring only disease mutations, yellow marks codons showing interspecific variation at protein sequence level but no disease mutations, and orange background marks codons with interspecific variability as well as disease mutations. Species used for identifying interspecific variants are given in Fig. 2 legend.

2.3. Interspecific site variability estimation

The maximum likelihood method (Yang, 1993) was used to determine the variability of each position in the amino acid sequence alignment. The vertebrate rhodopsin phylogenetic gene tree reported by Chang et al. (2002) was used (Fig. 2); the green anolis sequence was excluded because this branch was highly unstable in phylogenetic analysis and showed a higher rate of mutation. (However, even if it was included, all reported results reported remain the same). Maximum likelihood estimates of ancestral states assuming this topology were reconstructed in PAML (Yang, 1997) under a simple Poisson model of amino acid evolution. Each site was assigned an interspecific variability category based on the number of differences between the ancestor–descendent comparisons throughout the tree. This was done to conduct the Miller and Kumar (2001) tests.

2.4. Interspecific substitution severity

Grantham (1974) chemical distance between amino acids was used to quantify their biochemical differences. A large

value refers to a more radical difference. This measure was selected because it has been shown to discriminate clearly between disease mutations and neutral substitutions (Miller and Kumar, 2001). For a given amino acid site, the average chemical severity of interspecific substitutions is a simple average of the severity of all ancestor–descendent amino acid differences throughout the tree.

2.5. Expected chemical severity of replacement mutations

The expected chemical severity at each site in the human rhodopsin reference sequence was computed as the average severity over all non-synonymous single mutations in the human codon. Hence, for each codon there are nine possible codons to which it can mutate by a single nucleotide change. Out of these nine codons, only the codons that result in non-synonymous changes are considered. The chemical severity of the changed codon (i.e. amino acid) was calculated with reference to the original codon. This procedure was repeated for all the non-synonymous changes at that codon and an average expected chemical severity was calculated.

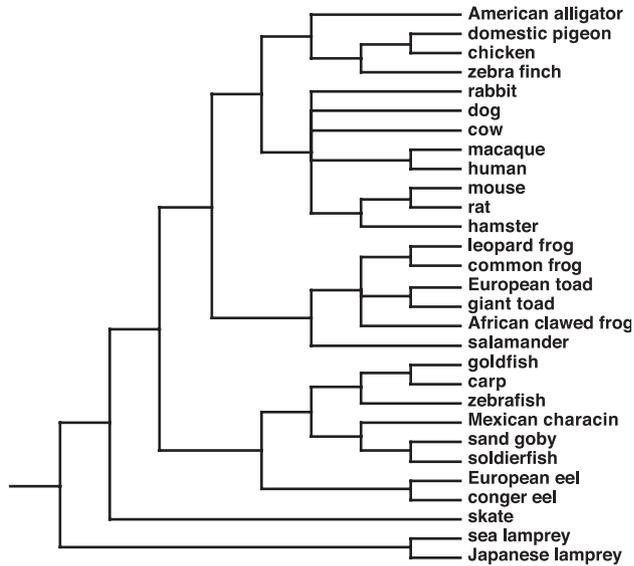


Fig. 2. Evolutionary relationships used to determine the number of mutational events that have occurred at each amino acid site in the rhodopsin gene throughout evolutionary history. GenBank accession numbers for sequences used in the analysis are as follows: American alligator, *Alligator mississippiensis* (P52202); domestic pigeon, *Columbia livia* (AAD32241); chicken, *Gallus gallus* (S29152); zebra finch, *Taeniopygia guttata* (AAF63461); rabbit, *Oryctolagus cuniculus* (P49912); dog, *Canis familiaris* (P32308); cow, *Bos taurus* (P02699); macaque, *Macaca fascicularis* (Q28886); human, *Homo sapiens* (P08100); mouse, *Mus musculus* (P15409); rat, *Rattus norvegicus* (P51489); hamster, *Cricetulus griseus* (P28681); northern leopard frog, *Rana pipiens* (P31355); common frog, *Rana temporaria* (P56516); European toad, *Bufo bufo* (P56514); giant toad, *Bufo marinus* (P56515); African clawed frog, *Xenopus laevis* (P29403); salamander, *Abystoma tigrinum* (Q90245); goldfish, *Carassius auratus* (P32309); carp, *Cyprinus carpio* (P51488); zebrafish, *Danio rerio* (NP571159); Mexican characin, *Astyanax fasciatus* (P41590); sand goby, *Pomatoschistus minutus* (P35403); soldierfish, *Myripristis berndti* (P79798); European eel, *Anguilla anguilla* (Q90214); conger eel, *Conger conger* (O13227); skate, *Raja erinacea* (P79863); sea lamprey, *Petromyzon marinus* (Q98980); Japanese lamprey, *Lethenteron japonicum* (P22671).

2.6. Frequencies of different amino acid changes in disease and interspecific substitutions

In order to evaluate the extent to which observed disease mutations can be compared with neutral substitutions, we compare the frequencies of different amino acid changes in disease-associated mutations with those observed among species, following the procedure of Miller and Kumar (2001). In this case, the relative frequency of mutations from amino acid residue i to j is given by $M_{ij} = N_{ij}/N_i$, where N_{ij} is the observed number of i to j mutations and N_i is the sum of all N_{ij} 's for all changes from residue i to residues that are possible as a result of a single nucleotide mutation. This was done because all disease mutations in our database (except two) were single point mutations. We also calculated M_{ij} 's for the amino acid substitutions observed among species.

3. Results

3.1. Overabundance of disease mutations in conserved sites

There were a total of 176 variable sites out of the 354 amino acids in the alignment, and the amino acid sites with the highest levels of variability (estimated using the phylogeny in Fig. 2) were in the C-terminus portion of the protein (Fig. 1). The evolutionary variability at these sites also was reflected in the fact that protein length varies among species in the C-terminal domain. The 93 unique nonsynonymous disease mutations mapped to a total of 61 human codons in the rhodopsin alignment (Fig. 1). This included the mutations that cause (in descending order of frequency) autosomal dominant retinitis pigmentosa (ADRP), congenital stationary night blindness (CSNB), autosomal recessive retinitis pigmentosa (ARRP), and retinitis punctata albescens (RPA). The vast majority of disease mutations (77.4%) were found at 42 fully conserved sites, which constitute 12% of all human rhodopsin amino acids (Macke et al., 1993). Therefore, the invariant sites harbor a much larger number of disease mutations than would be expected to occur randomly; this difference is statistically significant ($P < 0.001$). Fig. 3A shows the expected and observed numbers of disease mutations at amino acid positions with different levels of variability. This pattern is similar to that observed for other disease genes, such as the *CFTR* and *G6PD* (Miller and Kumar, 2001).

In fact, only the fully conserved sites show a significant overabundance of disease mutations (Fig. 3A). The propensity of disease mutations to occur preferentially at invariant sites is particularly evident in Fig. 1; disease mutations in the region 170–200 (extracellular loop E-II) are found only on sites that have never changed in vertebrate evolutionary history. While these sites are interspersed with variable positions, no disease mutations have been reported for variable positions, which lie adjacent to the disease mutation harboring sites. The high propensity for disease-causing sites in this region may reflect the fact that it is both extensively in contact with extracellular regions of rhodopsin and forms part of the 11-*cis*-retinal binding pocket (Palczewski et al., 2000).

Alternatively, disease sites themselves may be non-randomly distributed among interspecific variability classes. When we remove the effect of multiple hits at the same site, the number of completely conserved disease-harboring sites is also significantly higher than those expected by chance ($P < 0.05$) (Fig. 3B). Therefore, the overabundance of disease mutations in invariant sites is caused by multiple disease mutations at disease-prone sites and by the fact that invariant sites are more prone to harbor disease mutations. This difference is not caused by differences in mutation rates among regions, as the analysis of available (but limited) synonymous single nucleotide polymorphism (SNP) data did not show an overabundance of mutations in the invariant

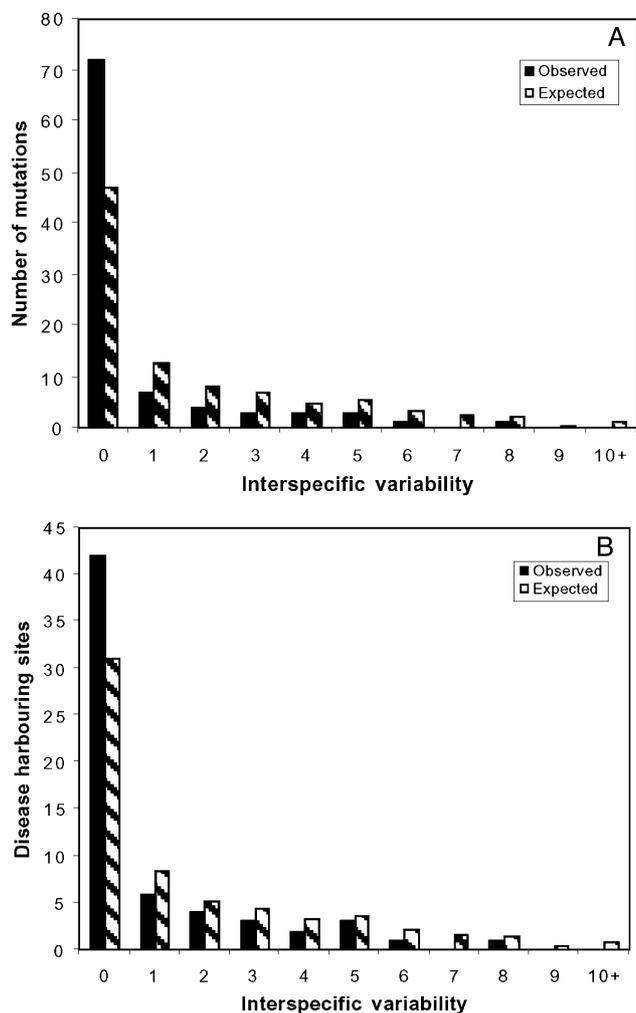


Fig. 3. (A) Counts of observed (black bars) and expected (striped bars) numbers of rhodopsin disease mutations at sites that have undergone different numbers of substitutions among species. Interspecific site variabilities were calculated using maximum likelihood methods. The expected number of mutations was computed using Eq. (1) in Miller and Kumar (2001). The number of disease mutations observed at completely conserved sites (0–class) is significantly higher than expected by chance ($P < 0.001$). (B) Counts of observed and expected numbers of sites harboring disease mutations. The number of disease mutations observed at completely conserved sites (0–class) is significantly higher than expected by chance ($P < 0.05$).

amino acids, as only one of six rhodopsin SNPs in the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) was found in the invariant sites (see also Miller and Kumar, 2001). Therefore, while the overabundance of disease mutations in conserved sites is predicted by the Neutral Theory, we find that this is true only for the most slowly evolving sites (that is, those that have never changed) in the present case. Interestingly, all other sites, even those that have changed only once in a lineage distantly related to humans, show an under-abundance of disease mutations (Fig. 3). This finding shows how even a slight potential for variability over a long term period (which indicates some relaxed functional

constraints) can decrease the disease potential of a position significantly. Therefore the class of invariant (or the slowest evolving) amino acid sites appear to be the most important in disease mutation analysis.

3.2. Biochemical severity of disease mutation is higher than interspecific variation

We examined whether the biochemical attributes of disease mutations differ significantly from naturally occurring variation observed among species. The chemical severity of the 93 disease mutations relative to our human rhodopsin reference sequence was assessed using Grantham's (1974) distance, which takes into account differences in the atomic composition, polarity and molecular volume of the compared amino acids. The average severity of rhodopsin disease mutations (91.3) is almost twice that observed among species (57.4). This pattern is very similar to those observed in more drastic diseases, such as *cystic fibrosis* (Miller and Kumar, 2001), and is even more drastic (91.3 vs. 22.3) when only sites containing disease mutations are considered (Table 1). The observed difference is statistically significant in both cases. From a neutral theory perspective, the direction of difference in the chemical severity of disease mutations is expected, but the magnitude of this difference is not predicted by any existing theory. As mentioned above our empirical analyses show that this difference is rather large (four times larger than for interspecific substitutions).

3.3. Disease chemical severity is driven by the intrinsic properties of codons, not the interspecific variation

Fig. 4A shows the relationship of the severity of disease mutations with the severity observed for interspecific (neutral) variation at each position. There appears to be a significant correlation between the two quantities ($R^2 = 0.22$), but a closer inspection reveals that it is caused by two sets of outliers: one with a very low value for interspecific variation and the other with a very high value. The removal of these three points makes the correlation very small ($R^2 = 0.03$). This absence of correlation is expected because the chemical severity of interspecific variation is largely determined by natural selection, whereas we expect that the radical nature of disease-causing mutations to be determined by the involved codon. To test this effect, we calculated the expected chemical severity (ECS) of replacement mutations for each human rhodopsin codon harboring

Table 1
Comparison of average interspecific and disease Grantham distance

	Interspecific	Disease
All sites	57.4	91.3
Only disease sites	22.3	91.3

The observed differences are significant in Mann–Whitney U tests.

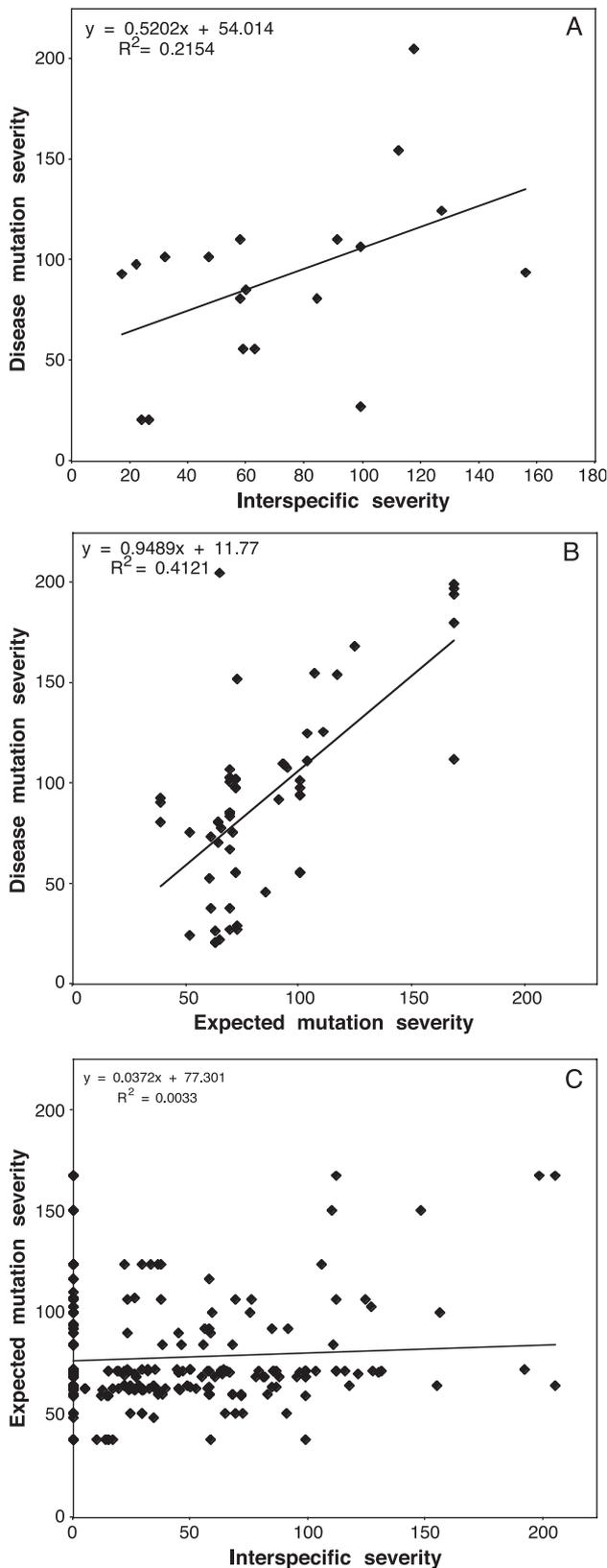


Fig. 4. (A) Scatterplot showing the relationship between interspecific severity at a site and chemical severity of disease mutations. (B) Scatterplot showing positive relationship of observed disease chemical severity and expected chemical severity at a codon. (C) Scatterplot showing no correlation between expected chemical severity and interspecific chemical severity.

disease mutations and contrasted it with the chemical severity of the disease mutations. Fig. 4B shows a direct and linear relationship between these two quantities ($P < 0.001$). Therefore, the mutational processes primarily shape disease mutation chemical severity. This is also evident from the number of rhodopsin disease mutations found at CpG dinucleotides (which are mutational ‘hotspots’ in humans). Almost 31% (19 out of 61) of disease mutation sites in the human rhodopsin reference sequence are at CpG sites, which is consistent with the overabundance of CpG mutations in the HGMD database of disease mutations (Stenson et al., 2003). These patterns contrast with the chemical severity of the interspecific variation, which shows no correlation with the expected chemical severity (Fig. 4C). This directly demonstrates that the process of mutation generation is not in itself shaping interspecific amino acid substitutions’ biochemical severity. The neutral theory of molecular evolution predicts that purifying selection will remove the vast majority of deleterious mutations from a population. This process should be reflected in the amino acid variation observed among species.

3.4. Disease-causing amino acids do not overlap with the amino acid variants observed among species

The results mentioned above show that amino acid substitutions causing human rhodopsin diseases have more significant biochemical differences than those among species. Since there is only a very small set of possible amino acids that can be produced by a single mutation at a given codon, we were interested in examining the extent to which the amino acids causing RP disease were shared with the set of neutral amino acids (observed among species) at the same position in human rhodopsin. The primary data used for this comparison is shown in Fig. 1, with the set of interspecific variation shown above the codon and the disease mutation types shown below. It is clear that there is never an overlap with the exception of site 137. Site 137 contains a V to M substitution that was originally reported to be associated with mild symptoms of ADRP (Ayuso et al., 1996). This substitution appears twice within vertebrates in lineages that are distantly related to humans (within the bonyfish lineage and in the branch leading to skates). The Retina International’s Rhodopsin Mutation website, however, notes that “members with mild symptoms... may not be caused by these mutations or not fully penetrant.” Therefore, while this mutation is the exception, it is possible that the V137M mutation may not be the cause of RP symptoms in those families. Alternately, the structure and/or function of rhodopsin in the fish and skate lineages containing this substitution have shifted to tolerate this substitution. (More extensive pedigree analyses in families displaying symptoms of RP will need to be performed to assess the importance of the V to M substitution in the etiology of this disease). Still, remarkably, only one out of 93 disease mutations shows an overlap with interspecific variation!

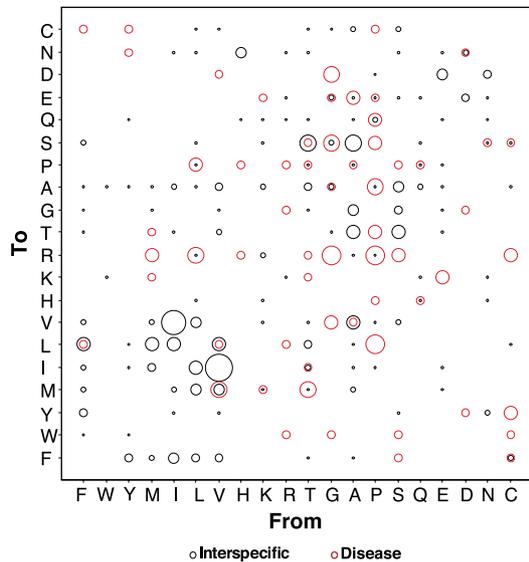


Fig. 5. Plots of the relative frequencies of amino acid changes observed in interspecific comparisons and those detected in disease patients.

In addition to the codon specific observations made above, we examined whether the disease mutations differ from global patterns of amino acid substitution across the rhodopsin protein. The frequency of amino acid changes is compared in Fig. 5. Only 43% (26 of 61) of the classes of disease-causing mutations are observed to have occurred as substitutions between species; that is, a majority of disease-causing mutation types are never observed as neutral variation between species, regardless of their position in the protein. This suggests that they probably have highly deleterious effects on protein structure and functions and are, thus, eliminated by natural selection. We note that there are many more types of interspecific amino acid substitutions that do not cause diseases. The vast majority of these are presumably neutral mutations with little effect on rhodopsin structure and function. A good example of these are I to V or V to I substitutions, which we observe to be the most common class of substitution within vertebrate rhodopsins and, as noted above, occur as a polymorphic variant in humans, unassociated with disease or known phenotypic effect. An even smaller number of interspecific substitutions may cause spectral tuning shifts that are fixed between species as the result of natural selection (Yokoyama et al., 1999; Briscoe, 2001, 2002). Interestingly, none of these classes of amino acid substitution (F to Y, S to A) are disease-causing but rather are commonly observed as interspecific variation (Fig. 5).

3.5. Phenotype and genotype of rhodopsin disease mutations in different domains

Berson et al. (2002) recently reported a clear-cut difference in the clinical expression of RP in patients with mutations located in the C-terminus (324–348; Fig. 1) versus patients whose mutations occurred in the plug

(110, 173–198) or globule (1–33) domains. The mean annual rate of field loss in the C-terminus group (7.4%) was reported to be significantly faster than in globule (1.7%) and plug (1.1%). Also, the ERG decline was faster in the C-terminus group (13.5%) than in the globule (8.5%) or plug (3.7%) groups. We were interested in examining whether there were any differences in the chemical severity of the disease mutations in these domains. We found that the seven mutations in the C-terminus (amino acids 324–328) had a significantly lower disease chemical severity than the 15 mutations Berson et al. (2002) studied in the plug domain (aa 110, 173–198, Fig. 1, Table 2). This appears to be due to a difference in expected chemical severity (ECS) of the disease mutation harboring sites in these domains, rather than in the difference in natural selection on these two domains. These results suggest that some sites have a lower ECS, are more critical for proper rhodopsin function, and result in more severe phenotypes, despite having milder mutations. Consistent with this are the following observations. It has been suggested that the severity of RP tends to be worse for cytoplasmic mutations than intradiscal, with intermediate phenotypes associated with transmembrane domain mutations (Sandberg et al., 1995). We categorized all sites in rhodopsin into these domains according to the crystal structure of Palczewski et al. (2000) and calculated the associate ECS of each. We found that average ECS was inversely correlated with the severity of RP: cytoplasmic sites and transmembrane sites had the lowest (76.0) and intradiscal mutations had the highest (86.3). The difference between the ECS of transmembrane and intradiscal sites was statistically significant (two-tailed *t*-test, $P < 0.006$).

3.6. Magnitude of spectral shifts is correlated with Grantham distance

Bovine rhodopsin has long been used as a mainstay of rhodopsin structure-function studies. A large number of mutations, sometimes several different ones at the same site, have been introduced into the gene encoding bovine rhodopsin and have been expressed in cell culture (see

Table 2
Comparison of disease mutation chemical severity in different rhodopsin domains

Domain	No. of sites	ECS	No. of mutations	Observed	P^a
C-terminus vs. plug + globule	2	67.3	7	61.6	0.04
	15	95.2	22	101.7	
Globule vs. plug	5	68	7	61.6	0.01
	10	107.9	15	120.5	

Berson et al. (2002) categorized the observed mutations according to their locations in the following domains: Globule (amino acids 1–33), plug: (110, 173–198), C-terminus: (324–348).

^a *t*-test (two-tailed) is a comparison of the observed Grantham distances between the different domains. Similar *t*-tests between the expected chemical severity (ECS) of different domains are even more significant (data not shown).

supplementary material for references). We were interested in the relationship between spectral tuning effects and the biochemical dissimilarity of the introduced mutation. Such a relationship, if present, may be useful for predicting the

Table 3
Artificial mutations, their spectral shifts, and their biochemical difference

Amino acid	Mutation	Grantham distance	nm shift
86	M86L	15	-2
90	G90S	56	-11
90	G90D	94	-20
90	G90E	98	-20
94	T94S	58	-6
94	T94D	85	-4
94	T94I	89	-22
117	A117G	60	-4
117	A117F	113	-8
118	T118A	58	-16
121	G121S	56	-3
121	G121A	60	-2
121	G121T	59	-17
121	G121V	109	-23
121	G121I	135	-25
121	G121L	138	-25
121	G121W	184	-39
122	E122Q	29	-16
122	E122D	45	-22
122	E122A	107	-22
122	E122L	138	-5
124	A124T	58	-3
124	A124R	112	-5
125	L125F	22	0
125	L125A	96	+3
126	W126F	40	0
126	W126L	61	-8
126	W126A	148	-22
134	E134Q	29	-2
134	E134L	138	-3
161	W161L	61	-3
211	H211F	100	-3
211	H211C	174	-5
265	W265Y	37	-15
265	W265F	40	-18
265	W265A	148	-28
267	P267A	27	+2
267	P267G	42	0
267	P267S	74	-3
267	P267N	91	-3
268	Y268F	22	-5
269	A269T	58	+14
292	A292S	99	-9
292	A292E	107	-20
292	A292D	126	-10
295	A295S	99	-2/-5
299	A299C	195	-2

All mutations were introduced into bovine rhodopsin template. Lin et al. (1998) speculate that 117 and/or 122 have synergistic or cooperative effects with other residues, unlike the other sites they study, which appear to be additive in effect—our results favor 122 as having synergistic effects. L125R (Grantham distance=102) fails to form chromophore with 11-*cis*-retinal (Garriga et al., 1996). References for data in Table 3, 4, 5 are available as supplementary material at <http://visiongene.bio.uci.edu/ABresearch.html>.

photochemical effects of novel artificial mutations. There were 13 transmembrane sites (amino acids 90, 94, 117, 121, 122, 124, 125, 126, 134, 211, 265, 267, 292) with multiple mutants. For these sites, it was possible to examine if the larger Grantham distances between (or among) mutations show a parallel trend in nm shift. We found that in almost all cases, a higher Grantham distance led to a larger blue nm shift (Table 3). While the correlation between the chemical severity of a mutation and the extent of its observed phenotypic effects is expected, the direction of this effect, in terms of wavelength, cannot be predicted under the neutral theory. Such direct relationships are rarely observed (even though they are always expected) and this observation was possible because the rhodopsin phenotype is measurable, allowing us to infer the observed pattern. The few exceptions to this pattern involve Threonine, Leucine or Aspartic Acid. Their observed deviation could arise from the fact that Grantham distance is a simple measure that considers only three attributes, size, polarity and composition, while there are many other attributes could be important in accounting for the functionality of these amino acids.

4. Discussion

We have found that disease mutations in rhodopsin are overabundant in highly conserved sites. While the presence of disease mutations in functionally important sites is expected (because mutations at these highly conserved sites are deleterious and therefore selected against by natural selection), the pattern of disease mutation frequency at positions with different interspecific variability reveals an interesting fact: sites with even a single substitution (that is, sites with any potential of variability among vertebrates) show an under-abundance of disease mutations. This is quite unexpected and appears to be a general pattern for disease mutation types (Miller and Kumar, 2001). On the other hand, there are a large number of disease mutations found at sites that vary, sometimes extensively (Fig. 3). In these cases, we have found that the disease mutation type almost never overlaps with the interspecific variation at that site (Fig. 1). The analyses presented suggest that novel disease mutations can be quickly identified by conducting an extensive sample of naturally occurring variation among species, which is by its very nature, neutral. Once a SNP is identified in an individual, it is straight-forward to examine whether that replacement mutation appears as a substitution among species at that position. If so, then it is unlikely that it is disease-causing. Otherwise, there is a high probability that it may be associated with the disease phenotype. Accuracy of this inference can be improved by conducting an extensive gene sampling from diverse species.

We have also found that the disease mutations are biochemically four times more radical than the interspecific variation (Table 1). These statistically significant and clear-cut results are particularly interesting when we consider that

rhodopsin defect-based diseases are mild as compared to many other genetic diseases (e.g., *cystic fibrosis*; Miller and Kumar, 2001). Patients with retinitis pigmentosa rarely have associated systemic or extraocular abnormalities (Dryja, 2001).

We also have examined the expected chemical severity for each codon and found that the distribution of observed rhodopsin disease mutations is highly correlated. In fact, the difference between the severities of disease mutations among different rhodopsin domains is mediated by the expected chemical severity intrinsic to the disease mutation sites in those domains (Table 2; see also Fig. 4). Also, the phenotype (absorbance spectrum maximum) of artificial mutations shows a clear-cut trend with the biochemical severity of the mutation. Mutations with small differences show smaller blue shifts. Some of this difference may be mediated by the size of the introduced mutant (Han et al., 1996), which is a component of Grantham distance. This pattern is observed for a majority of sites for which multiple artificial mutations were available (Table 3). Therefore, there is a direct relationship between the genotype and phenotype in this gene.

While the vast majority of artificial mutations reported result in loss-of-function phenotypes and blue shifts in absorption spectrum maximum (see also Andres et al., 2001; Bosch et al., 2003), there may be other phenotypic properties that can be related to Grantham distance. The size of the amino acid side chain at site 51 has been observed to correlate with the rate of metarhodopsin II decay (Bosch et al., 2003). We calculated the Grantham distances (60, 109, 138) for the three mutants (G51A, G51V and G51L) with increasing order of higher Meta II decay. We found that that the faster the rate-of-decay the higher the Grantham distance. Moreover, the size of the side chain at 135 may affect protein folding (Min et al., 1993; Andres et al., 2003). Therefore there may be a significant relationship between Grantham distance and some of the other quantifiable measures of rhodopsin function. Clearly, more work needs to be done in order to understand the relationship between Grantham distance and these other measures. The construction of artificial rhodopsins that exhibit maximal blue shift while remaining stable in terms of structure and function has been proposed in order to create a molecule that is less likely to bleach (and therefore easier to study) under standard laboratory light conditions (Janz and Farrens, 2001). The choice of amino acids with large Grantham distances at appropriate sites might usefully aid in this procedure.

In line with the close mapping of artificial mutant rhodopsin genotype and phenotype, we have also found that a relationship exists between the mutation chemical severity and the clinical rhodopsin phenotypes. Table 4 shows results from an analysis of all the schemes (Sung et al., 1991; Kaushal and Khorana, 1994; Gal et al., 1997; Cideciyan et al., 1998; Berson et al., 2002) used to classify rhodopsin disease mutations clinically into more

Table 4
Average disease mutation chemical severity according to biochemical or clinical subtype

Phenotype	Subtype	n	mean	<i>P</i> *
Biochemical ^a	Type I vs.	9	75.8	0.20
	Type IIa,b	28	102.8	
Onset, rod b-waves, psychophysics, histopathology	Type A ^b vs.	7	86.9	0.39
	Type B1, B2	8	100.75	
Cone 30-Hz flicker amplitude	Normal vs.	4	69.8	0.17
	Reduced	20	102.3	
Pattern of visual field loss ^c	Altitudinal vs.	8	66.4	0.03
	Constricted	18	104.4	
Pattern of rod sensitivity loss	Diffuse vs.	8	80.9	0.76
	Regional	12	87	
Clinical ^d	Type 1 vs.	22	97.1	0.97
	Type 2	10	97.7	
Equivalent field diameter ^e	Normal vs.	4	58.8	0.19
	Reduced	24	94.4	

^a Type I: in vitro resemblance to WT in folding, cell membrane targeting and reconstitution with retinal; Type IIa: misfold in cell culture, get stuck in ER and do not form chromophore; Type IIb: less severe than Type IIa in folding, and can bind retinal to varying degrees (Sung et al., 1991).

^b Type A: severely abnormal rod function early in life; Type B: compatible with normal rods in adults in some regions, and slow disease sequence (Cideciyan et al., 1998).

^c Altitudinal pattern of visual loss, usually only in depression of the superior field, having better preservation of peripheral vision than constricted (Gal et al., 1997).

^d Clinical type 1: early and diffuse loss of rod function, followed by loss of cone function; type 2: later onset, better visual field preservation, regionally progressive loss of rod function, concomitant loss of rod and cone function (Gal et al., 1997).

^e Equivalent visual field diameter in degrees (normal $\geq 120^\circ$).

* Two-tailed *t*-test.

severe or less severe phenotypes. For five of the seven classification schemes, the average Grantham distance was much lower for disease mutations associated with milder disease phenotypes than for those associated with more severe phenotypes. This pattern was observed for diverse biochemical, physiological and psychophysical traits such as protein folding, electroretinogram (ERG) amplitude, pattern of visual field loss, and equivalent field diameter.

As an example, Sung et al. (1991) proposed distinct classes for synthetic rhodopsins bearing disease-causing mutations as defined from the functional expression of the proteins in cell culture. Type I mutants resulted in wild-type yield, normal plasma membrane localization, and regenerability with 11-*cis*-retinal. Type II mutants resulted in lower yield, inefficient plasma membrane transport, retention in the endoplasmic reticulum and variable or no regenerability. Class II mutants result in disease through the slow death of photoreceptor cells due to the secondary metabolic costs of getting rid of the bad proteins, impaired transport to the outer segment, and active interference with the phototransduction pathway, which may be the cause of the decrease in dark adaptation rate observed in some disease patients

(Nathans, 1992). The accumulation of mutant rhodopsin in regions other than the outer segment is detrimental to the photoreceptor cell because rhodopsin is catabolized normally by the retinal epithelium cells (Dryja, 2001). Type I mutants had an average Grantham distance of 75.8, which was much lower than the average of Type II mutants at 102.8 (Table 4). Both types of mutations, however, were statistically much higher than the observed interspecific Grantham distances at the same sites (Table 5).

More importantly, in our analysis of available clinical data one measure of the severity of retinitis pigmentosa, pattern of visual field loss, shows a statistically significant relationship with biochemical distance. Patients with an altitudinal pattern of loss had a better preservation of peripheral vision than patients with the more severe constricted field phenotype (Gal et al., 1997). The average Grantham distance of disease mutations causing altitudinal loss was 66.4, which is similar to the average observed for interspecific variation (57.4; Table 4). By contrast, the average Grantham distance of disease mutations causing a constricted pattern of visual field loss was almost twice as high (104.4; Table 4) and was statistically significantly higher ($P < 0.03$) than that observed in patients with the less severe outcome.

We also observed a pattern with regard to the relationship between equivalent field diameter and Grantham distance. Those patients classified as having a normal equivalent field diameter had mutations with an average chemical distance of 58.8. This is nearly identical to the average Grantham

distance of all interspecific variation at all sites (57.4). We also note that when we only consider the observed interspecific variation at disease sites that produce a normal cone ERG amplitude (as opposed to an overall average), then interspecific and disease mutation Grantham distances are not statistically distinguishable (Table 5). Taken together, these preliminary results suggest that the closer the biochemical severity of a disease mutation is to the average interspecific biochemical severity, the milder the retinitis pigmentosa phenotype. Put conversely, the greater the disease mutation difference, the more severe the disease. Consistent with this is the report by Kremmer et al. (1997) of two patients with a C110F (Grantham distance=205) mutation displaying “severe functional loss of rods and cones in the ERG,” and another patient with an R135G (Grantham distance=125) with “concentrically narrowed visual fields and nondetectable ERG responses.” Both classes of mutations are two to four times higher than interspecific variability.

We note that the exceptions to this observation are the classification schemes of Gal et al. (1997), clinical Type 1 vs. 2 and pattern of rod sensitivity loss, where phenotypes closer to wild type have a nearly identical Grantham distance to the more severe phenotypes. We speculate that the trends we have observed so far will be strengthened by additional observations, as only about a quarter of all known disease-causing mutations have been classified according to any of the criteria (Table 4).

The magnitude of biochemical difference between disease mutations and interspecific variation at disease sites is most strikingly shown in Table 5, where even broken down into more or less severe clinical phenotypes, Grantham distances of diseases are always higher than interspecific variation. Collectively, our results show that simple measures of biochemical difference distinguish between neutral and disease-causing variation (see also Miller and Kumar, 2001).

Finally, we note that many of the disease-causing mutations are in the C-terminal domain, which contains indels. Is it significant from the point of view of evolution and the specialization of function given that this region contains many disease-causing mutations? Amino acids 344–348 have been shown to be essential in opsin transport and sorting to the outer segment (see Concepcion et al., 2002). This process occurs by the movement of vesicles containing rhodopsin along microtubules. The dynein light chain, Tctex-1, binds rhodopsin at the terminal amino acids and mediates this process (Tai et al., 1999). Therefore it is not surprising that two of the residues involved in dynein binding, Valine 345 and Proline 347, are conserved among both vertebrate rod and cone pigments and harbor nine disease-causing mutations (Rattner et al., 1999). Phosphorylation of rhodopsin by protein kinase-C also occurs at the Serine and Threonine residues at amino acid positions 334, 335, 336, 338 and 343, and is involved in the desensitization of receptor visual transduction (Sakmar et al., 2002). Many of the indels in the C-terminus are due to the loss or gain of

Table 5
Comparison of interspecific and disease mutation chemical severity at disease sites

	Subtype	n	Mean	P	
All disease sites	Interspecific vs.	62	22.3	3.18×10^{-15}	
	Disease	62	91.3		
Biochemical	Interspecific vs.	5	5.3	0.002	
	Type I	9	75.8		
	Interspecific vs.	19	11.3	8.95×10^{-10}	
	Type IIa,b	28	102.8		
Cone 30-Hz flicker amplitude	Interspecific vs.	4	24.8	0.097	
	Normal	4	69.8		
	Interspecific vs.	17	9.7		2.7×10^{-7}
Reduced	20	102.3			
Pattern of visual field loss	Interspecific vs.	8	7.3	0.001	
	Altitudinal	8	66.4		
	Interspecific vs.	12	6.6		4.67×10^{-9}
	Constricted	18	104.4		
Clinical	Interspecific vs.	16	4.91	1.9×10^{-9}	
	Type 1	22	97.1		
	Interspecific vs.	7	8.3		0.0007
	Type 2	11	90.9		
Equivalent field diameter	Interspecific vs.	4	0	0.036	
	Normal	4	58.8		
	Interspecific vs.	21	15.8		1.2×10^{-8}
	Reduced	24	94.4		

additional Serine residues, which presumably reflects evolutionary changes in the patterns of phosphorylation. No RP-causing disease mutation has yet to be reported for any of the aforementioned phosphorylation sites, perhaps because of the redundancy of multiple sites in the same region.

In conclusion, numerous genes have been found to be responsible for eye diseases such as retinitis pigmentosa (RP) and allied diseases including congenital stationary night blindness (CSNB) and retinitis pigmentosa albescens (RPA). These include rhodopsin, arrestin, *RDS*, *CRALBP*, *ROM1*, *RPE65*, *TULP1*, *ABCR*, *RPGR*, *RP2* (Dryja, 2001; Farrar et al., 2002). We have examined the disease mutation patterns in one of these genes, rhodopsin, and found that they can be clearly understood in the context of short-term and long-term evolutionary processes. The majority of disease-causing mutations occur at completely conserved sites. An analysis of rhodopsin data clearly demonstrates the usefulness of molecular evolutionary analyses for understanding patterns of clinical as well as artificial mutations and underscores the biomedical insights that can be gained by using simple measures of biochemical difference in the context of evolutionary divergence.

Acknowledgements

We thank Dana Desonie and the Briscoe Lab for comments and Renee Grothe for help with drawing Fig. 1. This research was supported in part by research grants from UCI and NSF (IBN-0082700) to A.D.B. and NIH (HG02096), NSF (DBI-9983133), and the Burroughs-Wellcome Fund (BWF-1001311) to S.K.

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