Codon Evolution

Mechanisms and Models

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CHAPTER 11

The future of codon models in studies of molecular function: ancestral reconstruction and clade models of functional divergence

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11.1 Introduction

Likelihood-based codon models of sequence evolution have been the focus of much excitement and development in recent years (Anisimova and Kosiol, 2009). Most attention has centred on the detection of positive selection in datasets (Yang and Bielawski, 2000; Bielawski and Yang, 2005; Yang, 2006), but unfortunately in many cases the link to adaptive causes can be tenuous at best (Nielsen, 2009). Given the steady proliferation of codon models, what novel approaches and insights can they offer for evolutionary studies of molecular structure and function? Ancestral reconstruction methods have proven to be a powerful and innovative approach for studying adaptive evolution of protein structure and function (Thornton, 2004). Recent advances in codon models incorporating more realistic assumptions about silent substitutions (Pond and Muse, 2005; Mayrose *et al*., 2007; Yang and Nielsen, 2008), however, offer the opportunity to reconstruct the evolution of synonymous substitutions, a promising but largely unexplored application of these models (Du, 2010). On the other hand, codon-based clade models of evolution (Forsberg and Christiansen, 2003; Bielawski and Yang, 2004), which were originally proposed years ago, are now gaining popularity for investigating changes in evolutionary constraint, and are increasingly being used to infer functional divergence in the evolution of gene families (for e.g. Hernandez-Hernandez *et al*., 2007; Liu *et al*., 2010). However, these models must be used with caution, particularly with respect to the specification of the null model in likelihood ratio tests (Weadick and Chang, in press). This chapter will consist of (1) a review of codon-based ancestral reconstruction methods, followed by an example of an application of their use in inferring synonymous evolution in mammalian rhodopsins, and (2) a review of clade models of molecular evolution, followed by a description of a recently proposed clade model likelihood test of divergence and its application to teleost short-wavelength visual pigments. Ultimately, the promise of both approaches lies in the possibility of generating specific hypotheses of molecular function, which can be then be interpreted in the context of data on molecular structure and function, particularly for genes for which a variety of biochemical assays and other functional data exist.

11.2 Ancestral reconstruction

Using comparative genomic information to infer sequences of ancestral proteins, and then resurrecting them in the laboratory for comparison to present day proteins, is a powerful way to study how the complex and intricate relationship of molecular structure and function may have evolved over

time (Liberles, 2007). This approach takes advantage of the wealth of data in the genome databases, providing for a more complete understanding of present-day molecular function in a unique historical context (Chang, 2003; Thornton, 2004). Studies in a variety of molecular systems show that not only is it feasible to reconstruct ancestral proteins in the laboratory, but that this can lead to insight into the evolution of protein function not easily attainable with other methods (Chang *et al*., 2002; Gaucher *et al*., 2003; Thornton *et al*., 2003; Ugalde *et al*., 2004; Chinen *et al*., 2005b; Thomson *et al*., 2005; Kuang *et al*., 2006; Skovgaard *et al*., 2006; Shimizu *et al*., 2007; Gaucher *et al*., 2008; Bridgham *et al*., 2009). Moreover, these studies can offer intriguing hints into the palaeobiology of the ancient organisms in which these resurrected molecules may have resided (Chang *et al*., 2002; Gaucher *et al*., 2003; Gaucher *et al*., 2008).

Codon-based ancestral reconstruction methods, the main focus of this review, are primarily carried out in a likelihood/Bayesian context. Ancestral reconstruction in a likelihood/Bayesian framework uses as an optimality criterion a likelihood score, calculated according to a specified model of evolution (Felsenstein, 2004). In phylogenetic reconstruction methods, the likelihood score associated with a particular topology is usually summed over all possible ancestral states. Bayesian methods can be used to infer the most probable ancestral reconstruction by calculating the posterior probabilities of particular ancestral states for a specified topology. This can be done using the maximum likelihood topology, branch lengths, and model parameters as priors in an empirical Bayesian approach (Yang *et al*., 1995; Koshi and Goldstein 1996), or alternatively the posterior probabilities can be calculated by taking into account the uncertainty in the maximum likelihood topology and parameters using Markov chain Monte Carlo simulations, if a full hierarchical Bayesian approach is adopted (Huelsenbeck and Bollback, 2001). Although the hierarchical Bayesian method has desirable statistical properties, it is not widely implemented as it is computationally much slower, and current implementations recommend only one ancestral node per run (Huelsenbeck and Bollback, 2001). In an empirical Bayesian approach, a phylogeny relating the extant sequences in the multiple sequence alignment must be specified. It can either be obtained from the published literature (e.g. an established species tree), or inferred from the data at hand using any number of phylogenetic methods (Salemi and Vandamme, 2003). In estimating posterior probabilities of ancestral sequences, either marginal (Yang *et al*., 1995; Koshi and Goldstein, 1996) or joint reconstruction approaches (Pupko *et al*., 2000; Pupko *et al*., 2002) can be used, although in practice these are generally assumed to give similar results (Yang, 2007). In experimentally resurrecting ancestral proteins, many studies have chosen to focus solely on the most probable ancestral sequence. However, this can introduce biases in amino acid composition, which may in turn alter the functional phenotype of a resurrected protein (Williams *et al*., 2006; Pollock and Chang, 2007). In recent years this concern has been addressed by a strategy of weighted random sampling of ancestral sequences from the posterior distribution, rather than only resurrecting the most probable ancestor, in order to assess the distribution of functional phenotypes of randomly sampled ancestors (Gaucher *et al*., 2008).

Many of the codon-substitution models currently available, including those described here and elsewhere in this book, can be used for ancestral sequence reconstruction (see Table 11.1). Early codon models accounting for variation in the nonsynonymous to synonymous substitution rate ratio (ω) (Nielsen and Yang, 1998; Yang and Bielawski, 2000; Yang *et al*., 2000) allowed for variation across sites and lineages (Nielsen and Yang, 1998; Yang and Bielawski, 2000; Yang *et al*., 2000; Yang and Nielsen, 2002; Yang *et al*., 2005; Zhang *et al*., 2005). Codon-based clade models (Bielawski and Yang, 2004), discussed in more detail in the following section, can also be used although they have not been widely implemented for this purpose. Finally, an alternative Markov-based likelihood method has also been developed to reconstruct ancestral codon sequences by first estimating a set of CodonPAM mutation matrices from a large empirical collection of vertebrate sequence data (Gonnet and Benner, 1996; Schneider *et al*., 2005; Cannarozzi *et al*., 2007), and using this in lieu of a parameterized codon substitution matrix (Goldman and Yang, 1994; Muse and Gaut, 1994). All of these models can be used for ancestral reconstruction, and are appropriate for studies interested in protein evolution, where the

Table 11.1 Programs for codon-based ancestral reconstruction

Method	Program	Substitution Model	Reference and Website
Empirical	**PAML**	One-ratio model	(Yang, 2007)
Bayesian	**Codeml**	Branch models	
		Branch-site models	http://abacus.gene.ucl.ac.uk/software/paml.html
		Random sites models	
		Clade models	
		Mutation-selection models	
	FASTML	Random sites model	(Pupko *et al.*, 2000, 2002)
		Empirical codon model	(Yang *et al.*, 2000; Schneider *et al.*, 2005)
			http://fastml.tau.ac.il/
	HyPhy	Constant model (one-ratio)	(Pond and Muse, 2005; Pond *et al.*, 2005)
		NonSynonymous model (random sites)	
		Proportional model	
		Dual model	http://www.datam0nk3y.org/hyphy/doku.php
		LineageDual model	
Hierarchical Bayesian	**MRBAYES**	One-ratio model	(Huelsenbeck and Bollback, 2001; Ronquist and Huelsenbeck, 2003)
		Random sites model	http://mrbayes.csit.fsu.edu/
Probabilistic Method	**DARWIN Ancestor**	CodonPAM Matrix	(Gonnet *et al.*, 2000; Schneider *et al.*, 2005; Cannarozzi *et al.*, 2007)
			http://www.cbrg.ethz.ch/darwin/index

Date last accessed for all websites is December 2010.

focus is the evolution of nonsynonymous substitution rates (d_N).

However, until recently, all of these codon-based likelihood phylogenetic methods assumed no selection acting on synonymous sites, and that the synonymous substitution rate (d_S) is constant among sites (Nielsen and Yang, 1998; Yang *et al.*, 2000), which is not necessarily true in many cases (Sharp *et al.*, 1995; Chamary *et al.*, 2006). This is particularly problematic for studies of synonymous evolution and codon bias. This has led in recent years to a number of codon models in which the assumption of constant d_S has been relaxed. Pond and Muse (2005) proposed the first set of random sites models that estimate d_S variation across sites by inferring d_N and d_S separately from discrete distributions of n categories. Pupko's group (Mayrose *et al.*, 2007) proposed a similar method accounting for rate dependency among adjacent sites, but did not allow for ancestral codon reconstruction. Both models can be used to test for significant variation in d_S across sites. Some of the most recent codon models have also incorporated selection on synonymous substitution rates. Yang and Nielsen (2008) modelled both selection and mutation effects in their estimations of synonymous substitution rates. This work is based on two separate parameters for a newly arisen mutant allele: the probability of mutation (incorporating the effects of mutation bias) and the probability of fixation (McVean and Vieira, 1999, 2001; Nielsen *et al.*, 2007). The fixation probability of a newly arisen mutant is determined by its fitness change (selective coefficients) and effective population size, which are concepts adapted from population genetic studies (Fisher, 1930; Wright, 1931; Kimura, 1957). In the Yang and Nielsen (2008) model, population size is assumed to be invariant, and the fitness of each codon is assumed to be the same among lineages and sites. A likelihood ratio test comparing models with and without selection can be used to test whether selection is acting on synonymous substitution rates. More recently, Zhou and colleagues (2010) took a different approach to incorporating selection on d_S, dividing synonymous rates into two categories, conserved and nonconserved, depending on whether shifts between preferred and unpreferred codons are inferred.

In contrast to earlier models, codon models developed in the past several years (Pond and

Muse, 2005; Yang and Nielsen, 2008) now allow for the detailed investigation of synonymous evolution, an advantage that has yet to be fully explored in the context of ancestral reconstruction. Until recently, ancestral reconstruction approaches have primarily been focused on resurrecting the evolution of proteins, as earlier methods did not explicitly model variation in synonymous rates. However, new developments in codon models have made possible evolutionary studies of codon usage bias, by allowing for selection not only at the protein coding level, but also on synonymous substitutions. In the following section we present work from our group illustrating the use of these methods in studies of synonymous evolution in vertebrate rhodopsins.

11.3 Reconstructing synonymous evolution in vertebrate rhodopsins

In mammals, natural selection has typically been assumed to play only a minor role in shaping codon usage bias (Sharp *et al*., 1995; Kanaya *et al*., 2001; Duret, 2002) due to small effective population sizes (Sharp *et al*., 1995), isochore effects (Bernardi *et al*., 1985; Eyre-Walker and Hurst, 2001), and mutation bias (Nagylaki, 1983; Galtier *et al*., 2001; Ponger *et al*., 2001). However, considerable evidence has since challenged this assumption, the bulk of which has focused on the population genetic, or genomic data from model organisms (for review see: Sharp *et al*., 1995; Duret, 2002; Chamary *et al*., 2006). A variety of plausible mechanisms have been proposed to explain selection on codon usage bias in mammals including increased translation efficiency/accuracy (Lavner and Kotlar, 2005; Kotlar and Lavner, 2006; Drummond and Wilke, 2008), mRNA stability (Chamary and Hurst, 2005), protein folding (Kimchi-Sarfaty *et al*., 2007; Drummond and Wilke, 2008), and splicing control (Willie and Majewski, 2004; Parmley *et al*., 2006).

The development of codon-based likelihood models that incorporate selection on synonymous substitutions (Yang and Nielsen, 2008) have made possible a broader, phylogenetic-based approach. In a recent study we implemented these new methods, focusing on a single gene —rhodopsin— for which sequence data exists from a large number of mammals, in order to determine if there is any evidence for selection on synonymous substitutions (Du and Chang, in press). Rhodopsin, a G protein-coupled receptor mediating dim-light vision in vertebrates (Menon *et al*., 2001), is an ideal system for studying synonymous evolution for a number of reasons. First, there is a strong codon usage bias in rhodopsin sequences (Chang and Campbell, 2000). Second, rhodopsin is a highly expressed gene (Pugh and Lamb, 1993), which has been associated with stronger selection at synonymous sites in previous studies in non-mammals (Sharp *et al*., 1995). Third, as the first step in visual perception, any effects of visual pigment adaptive evolution can be interpreted in light of organismal biology and visual ecology (Chang *et al*., 2002). In our recent work analysing a mammalian rhodopsin dataset, we detected a pervasive usage bias towards GC-ending codons, and that synonymous substitution rates are significantly variable among sites (Du, 2010). Moreover, we also found statistical evidence suggesting GC-ending codons are preferred by selection using the recent mutation–selection models. (Yang and Nielsen, 2008) This preference of GC-ending codons was found to be consistent with a variety of mechanisms such as maintaining mRNA stability, ensuring proper protein folding, and increasing translation efficiency/accuracy.

Here, we use codon-based ancestral reconstruction methods to examine when selection may have acted to increase GC-ending codons during early mammalian rhodopsin evolution in an expanded vertebrate dataset. Using the reconstructed ancestral mammalian sequences, we then quantify the intensity of departures at each node from equilibrium codon usage, using a method originally developed to measure evolution of codon usage bias in *Drosophila* (Akashi, 1995; Akashi *et al*., 2006). A control gene, isocitrate dehydrogenase (IDH), was also investigated in order to determine whether or not the unusual codon usage patterns found were unique to rhodopsin. Isocitrate dehydrogenase is a housekeeping gene involved in the regulatory control of mitochondrial energy metabolism, with the IDH1 subunit contributing to the regulatory properties of the enzyme (Panisko and McAlister-Henn, 2001). With its conserved func-

tion, sequence, and moderate expression levels (Warrington *et al*., 2000), we do not expect to see significant evidence for selection at synonymous sites in IDH1. We analysed a rhodopsin dataset of 27 vertebrate species, with a control gene IDH1 dataset of 19 vertebrate species due to limited data availability.

As our focus is on the evolution of synonymous substitutions, we implemented a variety of codon models, including recently developed mutation–selection codon models (Yang and Nielsen, 2008), and models incorporating among-site d_S variation (Pond and Muse, 2005) in our reconstruction of ancestral sequences. Ancestral reconstruction was performed in the codeml program of the PAML4.3b package (Yang, 2007) under six different models: M0, M0-FMutSel0, M0-FMutSel, M3, M3-FMutSel0, M3-FMutSel (Table 11.2). M0 refers to a one-ratio model, which assumes a single ω across branches and sites (Goldman and Yang, 1994), whereas M3 allows ω to vary across sites according to a discrete distribution with n categories ($n = 3$) (Yang *et al*., 2000). The recently developed mutation–selection models incorporate parameters for mutation bias, with (FMutSel) or without (FMutSel0) selection on synonymous rates (Yang and Nielsen, 2008). The mutation–selection models also allow for either a single ω ratio (M0-FMutSel/0), or variation in ω across sites according to a discrete distribution with two categories (M3-FMutSel/0). All analyses were run multiple times from different initial ω values to ensure convergence. Models were compared using likelihood ratio tests, LRT (Table 11.2). For both datasets, the best-fitting model was found to be M3-FMutSel. For comparison, ancestral reconstruction was also performed in HyPhy2.0 (Pond *et al*., 2005) under two different models (Table 11.2): the NonSynonymous model, which only considers d_N variation among sites (similar to M3 in PAML), and the Dual model, which considers d_S and d_N variation under two independent discrete distributions (three categories in this study). An LRT comparing the Dual and NonSynonymous models suggested significant variation in d_S across sites for the vertebrate rhodopsin dataset (Table 11.2).

After inferring the ancestral sequences of early mammalian lineages, we then investigated departures from equilibrium codon usage. This was done using a method originally developed to measure evolutionary shifts in codon usage bias in *Drosophila* (Akashi, 1995; Akashi *et al*., 2006). As our recent work suggests that GC-ending codons are under selection (Du, 2010), such codons were thus classified as 'preferred' (pu) in this analysis, with AT-ending codons as 'un-preferred' (up). Departures from equilibrium codon usage were measured by the $d_{pu,up}$ ratio, which is termed the Akashi ratio in this study (for detailed methods see Figure 11.1 legend). Using this approach, we found that many early mammalian lineages showed high positive $d_{pu,up}$ ratios, across different models, in the rhodopsin dataset (Figure 11.1, Table 11.3), indicative of strong substitution biases towards preferred codons. High positive ratios were found for the mammalian, therian, and placental ancestors. In contrast, the marsupial rhodopsin ancestor showed a bias towards unpreferred codons (negative $d_{pu,up}$ ratio), while the monotreme ancestor did not show a strong bias in either direction ($d_{pu,up} \sim 0$). These trends are *not* recovered in the IDH1 dataset. Similar patterns in Akashi ratios were also obtained with the NonSynonymous and Dual models (Table 11.3). Although these results are still preliminary, they suggest a major shift in codon usage toward GC-ending codons in early mammalian rhodopsin evolution.

What could be causing these evolutionary shifts in codon usage bias in early mammalian rhodopsins? We did not find any evidence for shifts in the IDH1 gene (Figure 11.1), which is located on a different chromosome from rhodopsin in mammals, making it unlikely to be due to genome-wide mutational pressure to increase GC-ending codons. Moreover, our recent studies comparing cognate tRNA abundances, predicted mRNA stabilities, and rhodopsin 3D structure suggested GC-ending four-fold codons in mammalian rhodopsin genes might be beneficial for a number of mechanisms, including increased translation efficiency/accuracy, increased mRNA stability, and possibly to maintain proper protein folding (Du, 2010). Given the critical role of rhodopsin in dim-light vision (Menon *et al*., 2001), the need to increase translation efficiency and generate greater numbers of rhodopsin proteins could be associated with changes in visual ecology in these lineages. For

Table 11.2 Likelihood analyses of the rhodopsin and IDH1 datasets

(a) PAML analyses

Model	np	lnL	AIC	κ	ω	π_C*	π_G*	π_T*	π_A*
					Rhodopsin				
M3	57	−10367	20848	2.3	$\omega_0 = 0.002,\ \omega_1 = 0.08,\ \omega_3 = 0.29$ $p_0 = 58\%,\ p_1 = 30\%,\ p_3 = 12\%,$		N/A		
M3- FMutSel0	77	−10186	20526	2.5	$\omega_0 = 0.008,\ \omega_1 = 0.27$ $p_0 = 67\%,\ p_1 = 33\%$	0.41	0.26	0.16	0.17
M3- FMutSel	118	−9979	20194	2.4	$\omega_0 = 0.005,\ \omega_1 = 0.19$ $p_0 = 66\%,\ p_1 = 34\%$	0.19	0.23	0.19	0.39
LRT M3-FMutSel Vs M3-FMutSel0: P value = 5.0×10^{-24} (df = 41)***									
					IDH1				
M3	41	−8770	17622	1.9	$\omega_0 = 0.006,\ \omega_1 = 0.1,\ \omega_2 = 0.47$ $p_0 = 64\%,\ p_1 = 29\%,\ p_2 = 7\%$		N/A		
M3-FMutSel0	61	−8661	17444	2.3	$\omega_0 = 0.02,\ \omega_1 = 0.33$ $p_0 = 79\%,\ p_1 = 21\%$	0.27	0.24	0.25	0.24
M3-FMutSel	102	−8492	17188	2.2	$\omega_0 = 0.014,\ \omega_1 = 0.26$ $p_0 = 79\%,\ p_1 = 21\%$	0.22	0.22	0.18	0.38
LRT M3-FMutSel Vs M3-FMutSel0: P-value = 1.8×10^{-17} (df = 41)***									

(b) HyPhy analyses

				Rhodopsin		
np	lnL	AIC	ω	d_N	d_S	
NonSynonymous	61	−10190	20503	$\omega_0 = 0.003,\ \omega_1 = 0.13,\ \omega_2 = 0.40$ $p_0 = 62\%,\ p_1 = 30\%,\ p_2 = 8\%$	$d_{N0} = 0.003,\ d_{N1} = 0.13,\ d_{N2} = 0.40$	$d_S = 1$
Dual	65	−10179	20488	$\omega_0 = 0.003,\ \omega_1 = 0.12,\ \omega_2 = 0.38$ $p_0 = 62\%,\ p_1 = 30\%,\ p_2 = 8\%$	$d_{N0} = 0.003,\ d_{N1} = 0.12,\ d_{N2} = 0.38$	$d_{S0} = 0.31,\ d_{S1} = 0.98,\ d_{S2} = 4.35$
LRT: Dual Vs NonSynonymous; P-value = 0.027*						

NOTE np is the number of parameters in the model; lnL is the log likelihood score; AIC (Akaike information criterion; −2*lnL + 2*np) is a measure of the goodness of fit of an estimated statistical model; κ is the transition/transversion ratio; ω is the nonsynonymous/synonymous substitution ratio; π_N^*(N = C, G, T, A) are mutational bias parameters; P-value is the result of likelihood ratio tests (LRTs); df is the degree of freedom in LRTs; *** highly significant (P-value < 0.0001), * significant (P-value < 0.05).

example, a diurnal to nocturnal niche transition has been proposed early in the ancestral mammalian lineage (Jerison, 1971; Crompton *et al*., 1978). However, the association between GC increase of four-fold codons and visual ecology change along mammalian and placental lineages is, of course, speculative and in need of further investigation. In addition, it is surprising that, although the evolutionary patterns of synonymous substitutions differ widely, both the rhodopsin and IDH1 datasets showed significance in the LRT comparing the FMutSel and FMutSel0 models (Table 11.2). This would suggest that synonymous substitutions might be under selection in two genes, or that, alternatively, there might be a sensitivity issue with respect to the LRT in mutation–selection models. Further power analyses of mutation–selection models will be required to address this concern.

In conclusion, new developments in codon-based ancestral reconstruction methods have allowed us to investigate changes in codon usage bias in rhodopsin that may be linked to shifts in the visual ecology in early mammals. We have inferred an obvious evolutionary trend towards increased GC-ending codons at three early mammalian (mammalian, therian, and placental) lineages of rhodopsin. This is consistent with our recent work suggestive of selection acting on synonymous sites in order to increase mRNA Stability and translation efficiencies in mammalian rhodopsins (Du, 2010). This work is just an initial step towards exploring selection on synonymous substitutions

Table 11.3 Akashi ratios* calculated for reconstructed mammalian rhodopsin ancestors

	Akashi Ratio ($d_{pu,up}$)			
Rhodopsin	**M3**	**M3-FMutSel**	**NonSynonymous**	**Dual**
Ancestors	(PAML)	(PAML)	(HyPhy)	(HyPhy)
Mammalian	0.5	0.43	0.68	0.74
Monotreme	0.11	0.1	0.12	−0.02
Therian	0.23	0.38	0.33	0.50
Marsupial	−0.62	−0.92	−0.67	−0.84
Placental	0.64	0.67	0.69	0.47

* (Akashi *et al*., 2006)

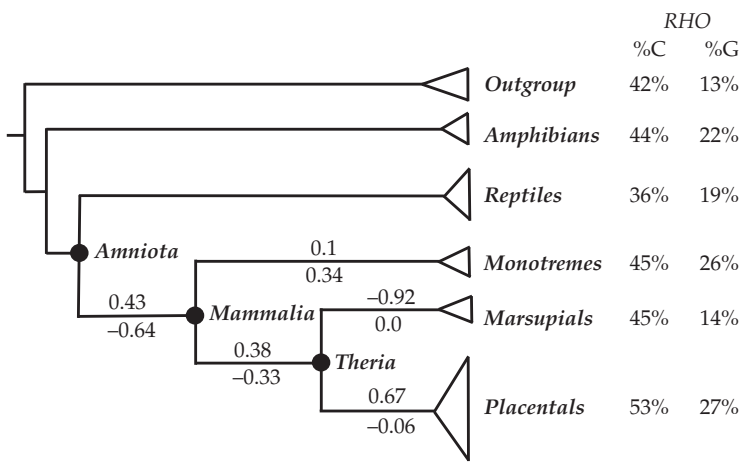

Figure 11.1 Schematic vertebrate phylogeny showing calculated Akashi ratios for reconstructed early mammalian ancestral sequences. Akashi ratios (Akashi *et al*., 2006) for the rhodopsin (RHO) dataset are shown above the nodes, for the control gene (IDH1) below the nodes. These ratios were calculated based on ancestral sequences reconstructed under the M3-FMutSel model for both datasets. For synonymous substitutions, changes from un-preferred to preferred codons were classified as preferred ('up'), and the reverse as un-preferred ('pu'). For the Akashi ratio, the difference in the proportion of 'up' and 'pu' changes is used to measure the departures from equilibrium codon usage, $d_{pu,up} = (up - pu)/(up + pu)$. The maximum value for $d_{pu,up}$ is 1, which would indicate that all the synonymous substitutions are preferred changes, with a minimum value of −1 indicating un-preferred changes. A value of 0 would indicate no departures from equilibrium codon usage. Outgroup sequences for the RHO dataset are the coelacanth and lungfish, and for the IDH1 dataset are the zebrafish and pufferfish. Average percentages of C and G nucleotides at 3rd positions of four-fold codons in extant rhodopsin sequences are shown to the right of each vertebrate clade.

in early mammals. Future investigations might involve incorporating a Bayesian distribution of different ancestral states into the calculation of the Akashi ratio in order to better estimate deviations from equilibrium codon usage, and simulations to assess the significance of the deviations observed for rhodopsin. Our studies highlight some of the many as yet unexplored possibilities that new developments in codon-based ancestral reconstruction methods offer for evolutionary investigations of codon bias and other effects on synonymous substitutions.

11.4 Clade models of functional divergence

Functional divergence of proteins following events such as gene duplications can result in complex patterns of sequence evolution poorly described by current implementations of the widely used branch-site codon models (Anisimova and Liberles, 2007; Levasseur *et al*., 2007; Hahn, 2009). In contrast, clade models, which until recently have received much less attention, are a collection of flexible codon-substitution models incorporating both among-site and among-lineage variation in selective pressure, that can be useful for detecting signatures of functional divergence among clades (Forsberg and Christiansen, 2003; Bielawski and Yang, 2004). In this section, we briefly review the development and use of clade models, and discuss some of the pitfalls of clade model usage that led to our recent work specifying an improved null model for use with a popular clade model, Clade model C (CmC) (Weadick and Chang, in press). We apply these methods in analyses of a fish opsin data set as a case study, and suggest directions for future development and validation of clade models.

Two patterns of amino acid sequence variation are commonly treated as evidence of protein functional divergence (Gu, 2006; Studer and Robinson-Rechavi, 2010). Functional divergence along a particular evolutionary lineage may result in descendents fixed for one state, and relatives fixed for another. This pattern, which has been termed 'conserved-but-different' (CBD) or 'Type-II divergence', is consistent with purifying selection acting throughout the history of the protein, except for a brief period where positive selection adaptively fixes functionally important substitutions. Alternatively, functional divergence along a given lineage may result in descendents that, as a group, display altered sequence conservation compared to relatives. This pattern, referred to as 'covarion-like' or 'Type-I divergence', is the predicted result of increases or decreases in the strength of purifying selection.

Codon-based tests of selection, with their focus on ω, the relative rate ratio of nonsynonymous to synonymous substitutions (d_N/d_S), can be thought of in terms of CBD vs. covarion-like substitution patterns. The Branch-Site Model A of Zhang *et al*. (2005) assumes that some sites can switch from purifying or neutral selection regimes ($0 < \omega_0 < 1$, and $\omega_1 = 1$, respectively) to positively selected ($\omega_2 > 1$) along a pre-defined foreground branch; on all other branches (background branches), positive selection is not allowed. In this way, branch-site tests for positive selection are analogous to tests for CBD substitution patterns, but at the codon rather than amino acid level. Less commonly employed are the clade models, which are codon-based tests for covarion-like substitution patterns (Forsberg and Christiansen, 2003; Bielawski and Yang, 2004). Such models assume a class of sites that experience divergent selection pressure in different *a priori* defined partitions, or clades (i.e. $\omega_A \neq \omega_B$ for clades A and B). By focusing on entire clades, rather than individual branches (as is typical for branch-site models), clade models provide estimates of long-term divergence in strength of constraint following functional divergence, much like tests of covarion-like amino acid patterns. Furthermore, as their focus is not solely on detecting $\omega > 1$, clade models are useful for identifying more subtle signatures of divergence.

Bielawski and Yang (2004) proposed two codon models for describing functional divergence among clades, Clade model C (CmC) and Clade model D (CmD), both of which are implemented in the codeml program of the PAML software package (Yang, 2007). Both models assume that some sites evolve consistently across the entire phylogeny (i.e. ω in clade A = ω in clade B) and some sites evolve divergently (i.e. ω in clade A $\neq \omega$ in clade B). CmC, which was modified slightly by Yang *et al*. (2005),

models this process using three site classes, applying to proportions p_0, p_1, and p_2 of the dataset. The first and second site classes, respectively, consider sites that uniformly experience either purifying selection ($0 < \omega_0 < 1$) or neutral pressure ($\omega_1 = 1$), while the third models divergently evolving sites (ω_2, $\omega_3 > 0$, for two *a priori* defined clades, referred to as the foreground and background; note that more than two tree partitions can be defined, though two is typical). The recommended LRT for establishing CmC's goodness-of-fit uses the M1a random-sites model as its null; M1a lacks the third (divergent) site class. CmD is more flexibly designed, and can be applied assuming either two or three site classes, none of which have constrained ω parameters. Assuming three sites classes for CmD, the first two capture consistently evolving sites ($\omega_0 > 0$, $\omega_1 > 0$) while the third applies to divergently evolving sites (ω_2, $\omega_3 > 0$). The LRT used to determine the goodness-of-fit of CmD uses the M3 random-sites model as its null; under M3, ω_2 and ω_3 are constrained to be equal. For both CmC and CmD, empirical Bayes' (EB) methods can be used to identify specific codons as members of a particular site class; under CmC, for instance, it might be found that codon X is placed in the purifying selection site class with high posterior probability, whereas codon Y is placed in the neutral site class, and codon Z in the divergent site class. For CmC, but not CmD, the initial naïve EB (NEB) approach has since been replaced with a Bayes' EB (BEB) approach (Yang *et al*., 2005), which is designed to be less sensitive to sampling error.

A similar clade model was proposed by Forsberg and Christiansen (2003). Their codon model assumes among-site variation in ω using three site classes, each with an unconstrained ω (ω_0, ω_1, $\omega_2 > 0$). Divergence is modelled by allowing a proportion of the dataset, p_d, to be re-fit to this ω distribution in the two pre-specified clades of the phylogeny, with the rest having consistent ω estimates regardless of clade. Goodness-of-fit is established via a LRT against a null model with the constraint $p_d = 0$. An EB approach is used to identify specific codons as either 'divergent' or 'consistent'. Compared to Bielawski and Yang's (2004) CmC and CmD, which assume that divergent sites simply have ω_2 in clade A and ω_3 in clade B, Forsberg and Christiansen's (2003) model can accommodate more complex forms of among-clade divergence; a divergently evolving site can be in any of the three site classes (evolving with either ω_0, ω_1, or ω_2) in clade A, and then switch to any of the other site classes in clade B, implying six possible ω transitions between the two clades. A possible tradeoff with this increased ability to model complex forms of divergence is reduced interpretability, as finding a codon is a high probability member of the divergently evolving class of sites does not provide information on the strength of selection affecting the codon in the two clades. In any case, the clade model of Forsberg and Christiansen (2003) has not been widely implemented.

Codon-based clade models, particularly CmC and CmD, have proven useful for testing for functional divergence in a growing number of datasets (see Table 11.4), particularly among duplicated genes. For example, Hernández-Hernández *et al*. (2007) used CmD to test for functional divergence following gene duplication in angiosperm B-class MADS-box regulatory genes, where they found significant evidence in favour of functional divergence among the PI and AP3 paralogs. These duplicates form obligate heterodimers that regulate meristem differentiation during flower development; duplication and divergence in this gene family thus appears to have resulted in both molecular and morphological novelty. While first designed to test for divergence associated with gene duplication, the clade models of Bielawski and Yang (2004) have also been employed to examine evolution associated with niche evolution (as initially proposed by Forsberg and Christiansen (2003)). Liu *et al*. (2010), for instance, used CmC to study functional divergence in Prestin, a motor protein gene expressed in the inner ear. They reported large, statistically significant increases in ω when comparing either echolocating bats or whales to a background partition of non-echolocating mammals, suggesting convergent changes in selection pressure associated with the adaptation to new sensory niches.

However, as with any inference method, care must be taken to ensure the results of clade model tests are both statistically reliable and biologically informative. We recently reported a problem with the LRT commonly used to establish the

Table 11.4 Studies that employed either Clade model C (CmC) or D (CmD)

Study	Data set	Test for divergence among. . .	Model(s) used
Li *et al.*, 2011	Feline cauxins (urinary proteins)	Orthologs	CmC, CmD
Liao *et al.*, 2010	Rhododendron small heat shock proteins	Paralogs	CmD
Liu *et al.*, 2010	Mammalian prestins (auditory motor proteins)	Orthologs	CmC
Wang *et al.*, 2010	Vertebrate plasma membrane transport proteins	Paralogs	CmC
Wei *et al.*, 2010	Feline major-histocompatibility complex peptide binding regions	Paralogs	CmC
Hughes *et al.*, 2009b	Mammalian UCP mitochondrial anion carriers	Paralogs	CmC
Hughes *et al.*, 2009a	Primate melanocortin receptors	Orthologs	CmC
Li *et al.*, 2009	Cyprinid cone opsins (visual pigment proteins)	Paralogs	CmC
Mondragon-Palomino *et al.*, 2009	Orchid class-B MADS-box transcription factors	Paralogs	CmC, CmD
Zhao *et al.*, 2009	Mammalian rod opsins (visual pigment proteins)	Orthologs	CmC
Des Marais and Rausher, 2008	Morning glory acanthocyanin pigment pathway reductases	Paralogs	CmC
Haudry *et al.* 2008	A variety of gene fragments from selfing and non-selfing grasses	Orthologs	CmC
Summers and Zhu, 2008	Cichlid prolactin hormones	Paralogs	CmD
Alverson, 2007	Diatom silicon transporters	Orthologs	CmD
Hernandez-Hernandez *et al.*, 2007	Eudicot class-B MADS-box transcription factors	Paralogs	CmD
Li *et al.*, 2007	Bat FoxP2 transcription factors	Orthologs	CmC
Balakirev *et al.*, 2006	Drosophila β-esterase enzymes	Paralogs	CmD
Bielawski and Yang, 2004	Primate RNases and globins	Paralogs	CmD

goodness-of-fit of CmC (Weadick and Chang, in press). Briefly, CmC and its typically used null model, M1a, differ not just in whether ω is heterogeneous between among clades, but also in how they model among-site ω variation. M1a possesses two site classes ($0 < \omega_0 < 1$; $\omega_1 = 1$), whereas CmC possesses three ($0 < \omega_0 < 1$; $\omega_1 = 1$; ω_2, $\omega_3 > 0$). Consequently, a significant LRT could occur simply because among-site d_N/d_S variation is better described by three rather than two site-classes, and not due to $\omega_2 \neq \omega_3$, as originally intended in this test. Among-site heterogeneity is widespread (Yang *et al.*, 2000), and this confounding difference appeared likely to cause false-positive LRT results. In our recent paper we used simulations to investigate whether the CmC versus M1a LRT can handle modest among-site variation in d_N/d_S (Weadick and Chang, in press). Our simulations assumed (1) three site classes, and (2) among-clade homogeneity. The results strongly indicated an extremely high false-positive rate: 99% of the LRTs produced significant results at $\alpha = 0.05$, even though d_N/d_S was equivalent among clades. While we did not explore the performance of CmD using null simulations, this problem should not arise with the CmD versus M3 test as it does not confound clade-divergence and number of site classes.

To address this issue, we implemented a new null model for comparison with CmC, the M2a_rel model (Weadick and Chang, in press). Like CmC, the M2a_rel model possesses three site classes. The first two M2a_rel site classes include sites experiencing purifying ($0 < \omega_0 < 1$) or neutral ($\omega_1 = 1$) pressures, and are equivalent to the first two site classes of CmC. The third site class corresponds to CmC's divergent site class, but is represented by a single d_N/d_S parameter ($\omega_2 > 0$). Since the only difference between the CmC and M2a_rel models is whether the third site class is represented by one (ω_2) or two (ω_2, ω_3) d_N/d_S parameters, comparing these models tests whether $\omega_2 \neq \omega_3$. Applying this new LRT (with one degree of freedom) to the same simulated datasets described above showed much improved results; only 4% of the LRTs were significant at $\alpha = 0.05$. The results of our simulations show that M2a_rel is a more appropriate null model.

Although clade models show much promise for investigations of functional divergence (Table 11.4), their statistical properties have received much less attention than other more widely used codon

models such as the branch-site models. Work from our group shows that while some of the earlier clade model tests tended to have extremely high false-positive rates, our recently proposed null model has corrected this problem (Weadick and Chang, in press). In the following section we present new analyses applying this new clade model test to a data set of duplicated teleost fish opsin genes, and point out some of the issues that can arise when interpreting the results of various parameter estimates in a biological context.

11.5 Testing for functional divergence among teleost SWS2 opsins

Short wavelength-sensitive visual pigments in the SWS2 class absorb light maximally in the violet-to-blue portion of the visual spectrum (~ 400–470 nm). Many fish species possess duplicated SWS2 opsins that are functionally divergent; SWS2a opsins tend to be most sensitive to blue light, whereas SWS2b opsins are most sensitive to violet light (reviewed in Hofmann and Carleton, 2009). To test for functional diversification associated with this duplication event, a collection of teleost SWS2 opsin sequences was obtained from Genbank, amino acid-translated and aligned using MEGA4 (Tamura *et al*., 2007), and phylogenetically analysed using MrBayes (Ronquist and Huelsenbeck, 2003). A Bayesian estimate of phylogeny is presented in Figure 11.2 (see figure legend for further details on tree estimation). According to this tree, the SWS2a/SWS2b duplication event is restricted to fish of the Acanthopterygii superorder (a taxonomic group containing model systems such as pufferfish, cichlids, and the medaka).

Branch-site analyses (Zhang *et al*., 2005) were carried out to examine whether positive selection affected either the SWS2a or SWS2b opsins along the lineage immediately following gene duplication (Table 11.5). When applied to the SWS2a post-duplication branch (PDB), the Branch-site Alternative model (BrS-A) collapsed to the null (BrS-N) (P = 1.000). When applied to the SWS2b PDB, conversely, BrS-A significantly improved on BrS-N (P = 0.012), indicating that a small proportion of sites (1.57%) were evolving with d_N/d_S well above one (ω_2 = 10.54). Three sites were identified by BEB analysis as members of this positively selected site class; the inferred substitutions (bovine rhodopsin numbering) and posterior probabilities for these three sites are A94C (PP = 0.974), F103M (PP = 0.987), and N195G (PP = 0.976). Site 94, notably, has been implicated in spectral tuning (Takahashi and Ebrey, 2003; Chinen *et al*., 2005a; Yokoyama *et al*., 2007), and appears to be a major contributor to the spectral sensitivity difference between SWS2a and SWS2b paralogs. Whether or not the other two sites, both of which are located in the opsin's extracellular loops, affect any aspects of opsin function is not currently known.

CmC analyses (Table 11.6) provided further evidence for significant among-clade divergence when the SWS2b clade, but not the SWS2a clade, was considered separate from the remainder of the data set (CmC vs. M2a_rel LRT results: SWS2b P = 0.0118; SWS2a P = 0.1716). These results appear to complement those of the branch-site analyses, where post-duplication adaptive evolution was detected for SWS2b but not SWS2a. However, the divergent d_N/d_S estimates of CmC are quite low for both clades, indicative of strong purifying selection ($\omega_2 \approx 0.02$, $\omega_3 \approx 0.04$), and it is difficult to interpret the difference between these parameter estimates as biologically meaningful. Presumably the small difference in d_N/d_S between alternative and null models leads to a significant LRT because the divergent site class is large ($p_2 \approx 60\%$); the divergence may be slight, but, over such a large proportion of sites, its effect on overall likelihood is substantial. Since for most real datasets the majority of sites experience strong, pervasive purifying selection (Yang *et al*., 2000), the presence of slight among-clade variation in d_N/d_S at generally conserved sites could obscure the detection of biologically meaningful divergence.

By focusing on slight divergence at sites that are largely conserved across clades, CmC may have ignored more biologically meaningful divergence in the other, smaller site classes. The question then becomes, can the analyses be forced to focus on biologically meaningful divergence patterns despite their lesser contribution to overall likelihood? One possible approach would be to change the boundaries that constrain ω estimation, such that the non-divergent ω_0 parameter is forced to apply

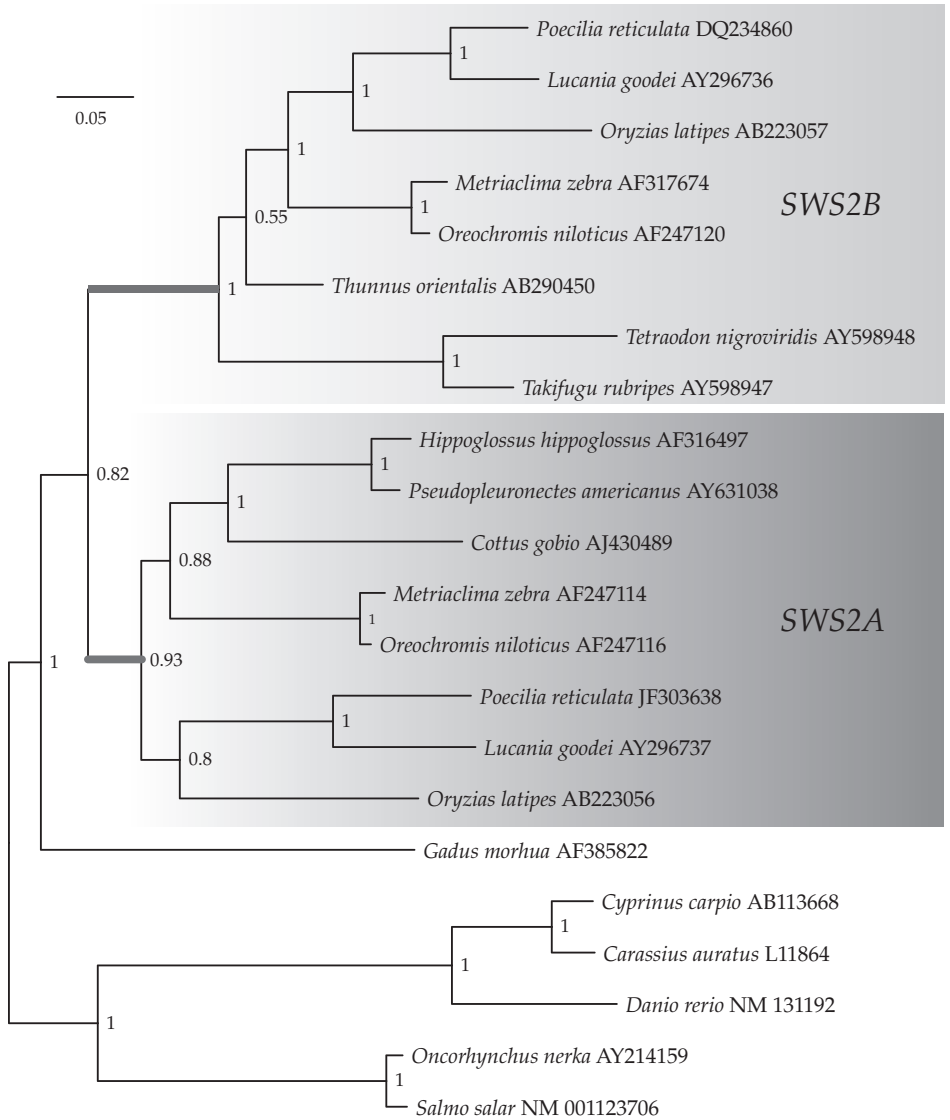

Figure 11.2 Phylogeny of selected teleost SWS2 opsins, highlighting the duplication event that produced the SWS2a and SWS2b paralogs. Branch-site analyses were performed to investigate positive selection along the (SWS2a) and (SWS2b) post-duplication branches. Clade model analyses were carried out to detect divergent evolution between either of these two clades (shaded boxes) and the remaining SWS2 sequences. The tree was estimated using Bayesian analysis of codon-partitioned nucleotide sequences. Node support values (posterior probabilities) are provided on the tree. The scale bar indicates the inferred number of substitutions per site. All sequences were obtained from NCBI Genbank, with the exception of the guppy (*Poecilia reticulata*) SWS2A sequence (Genbank Acc #JF303638), which we cloned from a guppy cDNA library by PCR with degenerate primers.

to sites with very low d_N/d_S across the entire phylogeny ($0 < \omega_0 < 0.1$), leaving the divergent ω_2 and ω_3 parameters free to apply to sites with higher d_N/d_S ($\omega_2,\ \omega_3 > 0.1$). Doing so would mean that sites that are generally subject to strong constraint, but for which slight, biologically uninterpretable differences in d_N/d_S exist between clades, would no longer be fit to the divergent site class.

By adjusting the starting parameter values during ML optimization, one can explore 'sub-

Table 11.5 Branch-site analyses of SWS2a and SWS2b post-duplication branches

	Model	lnL	np*	p_0	ω_0	p_1	ω_1	p_2	ω_2	LRT P**
SWS2a	Alt.	−9588.0634	46	0.7780	0.0764	0.2220	1	0.0000	1.0000	1.0000
	Null	−9588.0634	45	0.7780	0.0764	0.2220	1	0.0000	1	–
SWS2b	Alt.	−9582.1639	46	0.7663	0.0747	0.2180	1	0.0157	10.5355	***0.0118***
	Null	−9585.3336	45	0.7391	0.0748	0.2104	1	0.0505	1	–

*np = Number of parameters. ** LRT's have 1 degree of freedom.

Table 11.6 Clade model C (CmC) analyses* of SWS2a and SWS2b clades

Model	lnL	np	p_0	ω_0	p_1	ω_1	p_2	ω_2	ω_3	P (M2a-rel)**	P (M1a)***
CmC-SWS2a	−9500.6930	47	0.3276	0.3190	0.0664	1	0.6061	0.0297	0.0197	0.1716	$< 10^{-30}$
CmC-SWS2b	−9498.8440	47	0.3237	0.3220	0.0655	1	0.6108	0.0209	0.0408	0.0183	$< 10^{-30}$
M2a-rel	−9501.6275	46	0.3314	0.3155	0.0671	1	0.6015	0.0256	–	–	–
M1a	−9588.0634	44	0.7780	0.0764	0.2220	1	–	–	–	–	–

*CmC results are from the best of 20 separate analyses, carried out using different ω starting values (see Table 11.7).

**LRTs against M2a-rel have 1 degree of freedom.

***LRTs against M1a have 3 degrees of freedom.

optimal' CmC results that resemble this constrained scenario; we took this approach for the SWS2 data set (Table 11.7). As intended, the majority of the dataset ($p_0 \approx 60\%$) fit to the strong purifying selection class ($\omega_0 \approx 0.03$). However, the divergent d_N/d_S estimates, which applied to most of the remainder of the dataset ($p_2 \approx 33\%$), were nearly identical ($\omega_2,\ \omega_3 \approx 0.30$). Effectively, the sub-optimal CmC run in which low d_N/d_S sites were prevented from being treated as divergent collapsed to the M2a_rel null model ($P = 0.9723$). Using the CmC versus M2a_rel test, we thus found no evidence for biologically meaningful d_N/d_S divergence among duplicated teleost SWS2 opsins. Divergence among paralogs in this dataset appears to have involved the adaptive replacement of critical amino acids immediately following duplication, but not in a manner that adjusted the strength of constraint over the long-term. This example illustrates the care that must be taken in interpreting significant results in a biological context.

The flexibility and power of clade models make them ideal for detecting site-specific divergence in selection pressure among clades, and they hold promise for functional studies of gene duplication and divergence. Combined with EB site assignment methods, these models may be able to help illuminate the molecular bases of functional diversification, and guide biochemical analyses of homologous yet functionally divergent proteins. Typically these models have been used to study divergence within individual gene families, but they may also be useful for studies at the genomic scale (Studer and Robinson-Rechavi, 2010). However, the limitations of clade models have not been fully explored, and future research is needed to firmly establish the power and accuracy of current clade model LRTs when faced with complex, biologically realistic forms of divergence. One obvious limitation of current clade models is the lack of incorporation of among-site rate variation in d_N/d_S. Additionally, it would be helpful to know how CmC and CmD perform when faced with complicated forms of divergence among clades. CmC and CmD both assume one class of sites for which d_N/d_S either increases or decreases (but not both), but more complex divergence patterns are possible. For instance, some sites in the divergent clade may switch from purifying to neutral classes, while others could switch in the opposite direction. If such patterns are expected for

Table 11.7 Global and local optima from CmC analyses of the SWS2 opsin data set, with the SWS2b clade set as the foreground clade

Optima	lnL	Parameter estimates	Initial ω starting value(s)
Global	-9498.8440	$p_0 = 0.3237$, $p_1 = 0.0655$, $p_2 = 0.6108$, $\omega_0 = 0.3220$, $\omega_1 = 1$, $\omega_2 = 0.0209$, $\omega_3 = 0.0408$	0.0, 0.1
Local 1	-9501.6269	$p_0 = 0.6016$, $p_1 = 0.0671$, $p_2 = 0.3313$, $\omega_0 = 0.0256$, $\omega_1 = 1$, $\omega_2 = 0.3162$, $\omega_3 = 0.3147$	0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4
Local 2	-9586.9720	$p_0 = 0.7771$, $p_1 = 0.2196$, $p_2 = 0.0033$, $\omega_0 = 0.0767$, $\omega_1 = 1$, $\omega_2 = 12.1034$, $\omega_3 = 1.0384$	1.5, 2.0, 3.0, 4.0
Local 3	-9588.0634	$p_0 = 0.7780$, $p_1 = 0.2220$, $p_2 = 0.0000$, $\omega_0 = 0.0764$, $\omega_1 = 1$, $\omega_2 = 13.8968$, $\omega_3 = 10.5185$	5.0

a given dataset, alternative approaches to detecting divergence may be necessary, such as the 'switching' codon models of Guindon *et al*. (2004).

11.6 Conclusions

A major goal of molecular evolutionary biology is to identify and understand changes in gene function, such as the functional diversification of proteins. Exciting developments in likelihood-based codon models of evolution hold great promise to offer new insights into the evolution of molecular function. Clade models, which can be used to reveal site-specific differences in d_N/d_S among clades, represent powerful yet underused tools for documenting functional divergence in protein families. These models appear to be experiencing a recent surge in popularity, and developments in new likelihood ratio tests promise to make them even more useful in identifying instances of functional divergence. Moreover, innovative ancestral reconstruction approaches, which have already proven so useful in evolutionary studies of protein structure and function, can now be expanded to the evolution of silent sites. The incorporation of recently developed models that explicitly model variation in synonymous rates now offer the opportunity to identify and reconstruct synonymous substitutions that may be under positive selection, and to investigate their effect on translational efficiency, splicing control, and protein folding.

Ultimately, the real power of codon models for investigations of molecular evolution lies in their ability to generate hypotheses linking certain substitutions to specific shifts in molecular function, which can be then be tested experimentally. Despite the enormous popularity and widespread use of codon-based phylogenetic methods of detecting selection, functional verifications of d_N/d_S-based site predictions have been lagging behind, with the vast majority of claims of positive selection based on comparative sequence analyses alone (MacCallum and Hill, 2006; Hughes, 2008; Nielsen, 2009). Only a handful of studies have functionally tested d_N/d_S site predictions, in many cases finding functional divergence suggestive of adaptive evolution (Sun *et al*., 2002; Ivarsson *et al*., 2003; Sawyer *et al*., 2005; Norrgard *et al*., 2006; Weinberger *et al*., 2009). A notable exception is an often cited experimental study of rhodopsin evolution in which positively selected sites were found to have minimal effect on spectral tuning (Yokoyama *et al*., 2008). However, this study assumed that the only adaptive reason for positive selection in visual pigments would be for spectral tuning. In fact, *in vitro* assays have found significant variation in other aspects of visual pigment function that may have equally important implications for visual behaviour and ecology (Imai *et al*., 2007; Sugawara *et al*., 2009).

Their study does, however, highlight the necessity for further experimental validation of these approaches. Forging a link at any level between positive selection and adaptive evolution is never an easy task (Gould and Lewontin, 1979; Nielsen, 2009). At the very least, it requires rigorous experimental investigation into the underlying molecular mechanisms by which particular substitutions may result in functional change. And for most

experimental studies, it must be remembered that functional assays of particular mutations are often conducted in the background of extant proteins, and not, in fact, the ancestral proteins in which the adaptive evolution might have occurred. Ancestral reconstruction approaches offer us the exciting opportunity to recreate in the laboratory the positively selected substitutions in the context of the ancestral molecules in which they occurred, and to investigate possible associations with interesting functional shifts. The real promise of the future may lie in experimentally recreating the past adaptive history of genes, in order to avoid merely telling just-so-stories of molecular adaptation.

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