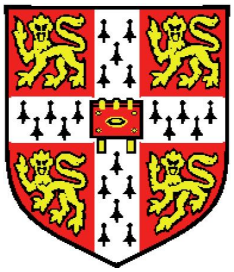
Sitewise error and constraint in mammalian comparative genomics



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Contents

Chapter 1

Patterns of sitewise selection in mammalian protein-coding genes

1.1 Introduction

1.1.1 The Mammalian Genome Project

A major goal of mammalian comparative genomics has been to quantify, identify and understand the fraction of the human genome that is under evolutionary constraint. The first non-human mammalian genomes showed at least 5% of the human genome to be under purifying selection [Lindblad-Toh *et al.*, 2005; Mouse Genome Sequencing Consortium & Mouse Genome Analysis Group, 2002; Rat Genome Sequencing Project Consortium, 2004], but the small number of genomes available limited the extent to which regions of evolutionary constraint could be identified. The Mammalian Genome Project, a coordinated set of genome sequencing projects initiated in 2005 and organised by the Broad Institute of MIT and Harvard, was designed with the primary purpose of increasing the accuracy and confidence with which regions of the human genome that have evolved under evolutionary constraint in mammals could be identified [TODO, 2011]. In line with this goal, 20 mammalian species were chosen for sequencing in order to maximise the amount of evolutionary divergence available for comparative analysis when combined with the 9 already available sequenced genomes [Margulies *et al.*, 2005]. To save on sequencing costs most of the 20 additional species were only sequenced to a target twofold coverage, meaning each genomic base pair would be

covered on average by two sequence reads and roughly 85% of genomic sequence would be covered by at least one read.

As the Mammalian Genome Project proceeded from its sequencing to analysis phase in late 2008, it became clear that the additional branch length afforded by the 29-species phylogeny would enable a number of improved evolutionary analyses beyond the identification of constrained noncoding regions. Among others, these included the evolutionary characterisation of gene promoters, identification of exapted noncoding elements, detection of evolutionary acceleration and deceleration in noncoding regions, and detection of purifying and positive selection in protein-coding genes. Given its prior involvement in analysing the ENCODE comparative sequencing data [TODO, 2011] and Massingham's work on a method and software program for sitewise evolutionary analysis [TODO, 2011], the Goldman group became involved in the protein-coding evolutionary analysis for the Mammalian Genome Project. This chapter describes my work on the project, which began in late 2008; the major results from the analysis are to be published in [TODO, 2011], and all of the work described below was performed by me in consultation with members of the Goldman group (Nick Goldman and Tim Massingham), EnsEMBL team (Albert Vilella, Javier Herrero, Ewan Birney) and organisers and members of the Mammalian Genome Project (Manolis Kellis, Kerstin Lindblad-Toh, Mike Lin, Katie Pollard).

1.1.2 The Sitewise Likelihood Ratio test

As described in Chapter **??**, differential survival of non-synonymous and synonymous mutations based on the degeneracy of the genetic code can be used as a source of information on the continued importance of mutations at a given protein-coding site over evolutionary time: a lower rate of non-synonymous substitution compared to synonymous substitution is indicative of purifying selection, or natural selection in favor of maintaining protein structure and function; equal rates of non-synonymous and synonymous substitutions is indicative of neutral selection, or no differential survival of protein-altering mutations; a greater rate of non-synonymous than synonymous substitution is indicative of positive selection, or natural selection in favor of protein-altering mutations.

Early evolutionary analyses of protein sequences showed large variation in the rates of amino acid change both within and between proteins [TODO, 2011]. This

variety results from the myriad structures and functions embodied by different proteins and protein domains [TODO, 2011]. Continued work suggested that the overall evolutionary rate TODO [2011]; **?** and the pattern of localised selective pressures TODO [2011] of a gene can reveal important insight into its role in the organism. Thus, the study of rates of non-synonymous and synonymous substitution in proteins became established as an effective method for using evolutionary information to investigate the functional characteristics of genes.

Maximum likelihood methods, introduced in Chapter **??**, are commonly applied to biological sequence analysis due to their desirable statistical features...

The Sitewise Likelihood Ratio (SLR) test is based on the mechanistic Goldman-Yang codon model of evolution, with an additional parameter for each site in the alignment representing the sitewise ω value. The inclusion of an additional parameter per alignment site makes the model extremely complex and difficult to optimise, but the dimensionality of the likelihood optimisation is reduced by making the assumption that the ω at each site does not contribute significantly to the overall likelihood, thus allowing for separate optimisation of the global parameters (including XYZ) across the whole alignment and the ω parameter at each site. In this way, SLR performs an approximate likelihood ratio test (LRT) for non-neutral evolution at each site...

1.1.3 Data quality concerns: alignment and sequencing error

The possibility that errors in the source alignments might cause false positives in the detection of sitewise positive selection was a major concern for this analysis. Although the SLR test and other sitewise maximum likelihoo methods have been shown to be conservative for detecting positive selection even when the amount of data is low or the null model is violated [TODO, 2011; **?**; **?**], most evolutionary analyses are based on the assumption that all sites within an alignment column are truly homologous. This assumption can be violated in a number of ways, some of which are described below.

Alignment error results from the difficulty of reconstructing the evolutionary history of sequences evolving with indels and can cause nonhomologous codons to be placed in the same alignment column. In Chapters **??** and **??** I explored the tendency of multiple aligners to produce such errors, showing

that PRANK_{C} alignments would be expected to introduce few falsely identified positively-selected sites resulting from alignment errors at mammalian-like divergence levels.

Errors resulting from incorrect genomic sequence was an additional concern. Twenty of the genomes under study were sequenced at low coverage and were not assembled into chromosomes or finished to completion, making the likelihood of miscalled bases, spurious insertions or deletions, or shuffled regions due to misassembly relatively high [TODO, 2011]. The potential effect of each of the aforementioned types of sequence errors on the detection of positive or purifying selection depends on the nature of the inference method, the type of sequencing error, and the branch length of the terminal lineage leading to the species containing the error.

As most codon-based inference methods assume independence between amino acid sites, I first consider the effect—in isolation—of a single spuriously-assigned homologous codon on the maximum likelihoo estimation of ω. Two cases can be considered: a single sequence error causing one spurious substitution within a codon, and one or multiple sequence or assembly errors causing multiple spurious substitutions within a codon. In the case of a single spurious substitution, if we assume no large difference between the natural mutational process and the process that caused the erroneous mutation, then the effect would be to shift the estimated ω in the branch containing the error towards 1. The sequence error would be incorporated into the maximum likelihoo optimisation as an additional neutral substitution, inflating the estimated substitution rate but not affecting the relative non-synonymous and synonymous rates. This effect may be biased towards higher or lower ω values if a significant difference exists between the neutral biological mutational process and the pseudo-mutational process causing the erroneous substitution. On the other hand, a codon with multiple erroneous bases may cause greater elevation of the inferred substitution rate and ω, due to the necessity of maximum likelihoo methods to infer a multi-step path of single substitutions between the two codons on either side of a given evolutionary branch. The path estimated between two completely nonhomologous codons depends on the estimated codon frequencies, the genetic code, and the pseudo-mutational process; while a detailed investigation of the expected effect on inferred ω values is beyond the scope of my analysis, it is not unreasonable to expect a greater

number of false positive PSSs resulting from codons with multiple erroneous bases than from codons with single errors.

TODO: quick randomisation experiment looking at elevated ω from multi-substitution codons?

Given the potentially greater impact of codons with multiple errors, the propensity of each of the common sequencing error types identified above (miscalled bases, spurious indels, and shuffled/repeated/collapsed regions due to misassembly) to cause single or multiple errors within codons could strongly affect its effect on the detection of positive selection. On its own, a miscalled base would obviously result in a single spurious substitution. However, low-quality bases tend not to be uniformly distributed among or within sequence reads, which makes for a larger probability of multiple errors within a codon resulting from miscalled bases. Spurious indels within coding regions may be even more likely than miscalled bases to cause multiple errors within a codon due to the potential alignment and frameshift effects (but see the discussion of Ensembl's frameshift filters for low-coverage genomes in Section **??**). Assembly errors, which result in larger-scale structural errors including missing, repeated, shuffled or inverted sequence regions, are most prone to produce codons with multiple erroneous errors due to the large amount of contiguous sequence data being misplaced.

I also note the impact of the inference method and terminal branch length on false positives resulting from sequence errors, which can be understood in terms of the information most directly affecting the inference of a positively selected site or a positively selected gene for a given detection method. Both the branch-site test and the sitewise tests (including SLR and PAML M8) are sensitive to substitutions at a subset of alignment sites, but the branch-site test is specifically sensitive to substitutions along the foreground branches of interest while the sitewise tests detect positive selection only throughout the entire tree. In the latter case, the effect of spurious synonymous and non-synonymous substitutions from sequence data depends on the ratio of the species' terminal branch length to the branch length of the entire tree: a longer terminal branch gives greater weight to the erroneous sequence data, making false positives more likely to result. In the former case of the branch-site test, the potential effect depends on the location and length of the foreground branches. If the terminal branch leading to the spurious sequence is within the foreground and the total foreground

branch length is small, then false positives could easily result; if, however, the terminal branch is outside of the foreground then it should have little to no effect on the FPR of the branch-site test. Interestingly, this suggests that branch-site tests where the foreground only consists of internal branches may be less prone to false positives from sequencing error than tests that include terminal lineages in the foreground model.

To summarise, the expected effect of alignment errors on the sitewise detection of positive selection should be minimal when using a good aligner and analysing data within vertebrate divergence levels, but the number of false positives resulting from sequence errors depends on a number of factors including the frequency, spatial clustering, and phylogenetic branch length associated with sequencing-based errors when applied to detecting sitewise positive selection. In some cases even a large amount of sequencing error should not produce a strongly elevated FPR (e.g., when the total branch length is large, when analysing all mammals or vertebrates) but in other cases it could potentially bias results (e.g., when the branch length is small and/or many low-quality genomes are included, as in the major mammalian sub-clades).

Simulation studies similar to those I performed in Chapters **??** and **??** could improve our understanding of the relative potential of different types of sequencing errors to introduce false positives in downstream analyses, but the absolute frequency and pattern of such errors would still difficult to predict without a reliable model for their generation. This is especially true for larger-scale errors from misassembly or misannotation, which are less easily modeled than base calling errors and could have potentially larger negative effects. For estimates of false positives resulting from these types of sequence errors, an empirical approach seems more appropriate.

Two empirical studies in mammals have provided convincing evidence that sequence, alignment and annotation errors can drastically increase the number of false positive PSGs in the branch-site test for positive selection.

Schneider et al. [**?**] performed a genome-wide scan for positive selection in the terminal branches of 7 mammalian genomes using the branch-site test and analysed the fraction of PSGs within subsets of high- or low-quality genes according to three sequence and alignment quality metrics. They found that the fraction of PSGs was significnatly higher for genes exhibiting lower quality se-

quence, annotation and alignment metric, with genes in the highest-quality and lowest-quality categories showing a 7.2-fold difference in the inferred fraction of PSGs [**?**]. This observation provided evidence of a correlation between the chosen quality metrics and the tendency of an alignment to exhibit positive selection. It did not necessarily imply causation, however, as the same result might have been observed—even in the absence of sequence error—if some biological properties of the true PSGs caused them to yield lower quality metrics than non-PSGs. Looking at the three metrics used in their study (sequencing coverage, gene annotation status, and alignment quality according to the heads-or-tails method), it is plausible that properties associated with elevated ω ratios and positive selection, such as recent gene duplication [TODO, 2011], high GC content [TODO, 2011] or functional shifts [TODO, 2011] might have had an error-independent effect resulting in a higher proportion of PSGs in low-scoring categories.

Mallick et al. [**?**] took a different approach to the same problem by performing a careful resequencing and reassembly of the chimpanzee genome (the initial assembly of which had lower coverage and lower quality than the human genome) and re-analysing the evidence for positive selection along the chimpanzee linegae in 59 genes which had previously been identified as chimpanzee PSGs. The authors, who were motivated by a concern that previous reports of a larger proportion of PSGs in chimpanzee than in human [TODO, 2011] were the result of its lower-quality genome rather than a biologically significant difference in levels of adaptation, found that the vast majority of PSGs identified in two previous studies showed no evidence for positive selection when using their reassembled and higher-coverage version of the chimpanzee genome [**?**]. This suggested that the original 4x coverage chimpanzee assembly contained a number of sequencing errors leading to false inferences of positive selection. A detailed analysis of 302 codons with multiple spurious non-synonymous substitutions in the original assembly showed roughly comparable effects of sequence error (explaining 23% of codons), assembly error (14% of codons) and local alignment error (30% of codons).

Taken together, the results of Schneider et al. [**?**] and Mallick et al. [**?**] provide strong evidence in support of the hypothesis that errors in sequencing, assembly, annotation and alignment can result in strongly elevated inferred ω values when using sensitive tests for detecting positive selection. The detailed

identification and quantification of error sources performed by Mallick et al. [**?**] is especially useful for designing filters to apply to an analysis based largely on low-coverage genomes; their observation that clusters of chimpanzee-specific mutations were responsible for many false positives motivated the window-based filter I applied here and in Chapters **??** and **??**.

TODO: Figure summarising the types of error and potential effects on the inference?

1.1.4 Low-coverage genomes in the Ensembl database

The prevalence of missing sequence data and fragmented contigs in low-coverage genomes presents a unique set of problems for the generation of transcript annotations. In recognition of these differences, the procedure used by the Ensembl database to annotate genomes assembled from low-coverage data is distinct from the usual gene-building pipeline [TODO, 2011; **?**]. Briefly, a whole-genome alignment is produced between the human genome and each low-coverage target, and gene models are projected from human to the target genome. Small frame-disrupting insertions or deletions within orthologous exons are corrected, and missing exons are padded with Ns in order to obtain the correct transcript length.

The inclusion of these error-correcting features allows intact, if not complete, coding transcripts to be generated for low-coverage genomes. The Compara gene family pipeline uses the set of transcripts from each species as its input [TODO, 2011; **?**], so the quality of the gene models from each species has a direct impact on the overall quality and accuracy of gene trees. Although the reliance on genome-wide alignments to, and gene annotations from, a reference genome could be criticised for potentially causing a bias towards the genomic properties of the reference, this approach is a reasonable workaround in the absence of higher-coverage sequence data or a painstakingly curated assembly. Furthermore, the gene model error-correcting features of the Ensembl pipeline are especially beneficial, making more complicated methods for correcting errors from low-coverage genomes such as those described by [TODO, 2011; **?**] seem largely unnecessary.

1.2 The Ensembl Compara gene tree pipeline

All genomic data and gene trees used for this analysis were sourced from version 63 of the Ensembl Compara database [TODO, 2011; **?**]. Although a complete description of the design, implementation, and validation of the pipeline behind the Ensembl database is beyond the scope of this thesis, I will briefly outline the major aspects of the approach, focusing on a few details which are relevant to the current sitewise analysis and the ensuing discussion.

The Compara pipeline begins with a set of protein-coding transcripts collected from each individual species' annotation database. This step is not exactly straightforward, as the prevalence of alternative splicing in Eutherian mammals makes it common for a single gene to harbor many different transcript structures. In terms of biology and evolution, alternative splicing is a very interesting phenomenon. Tightly linked to the evolutionary innovation of regulatory control and tissue-specific gene expression, the existence of multiple transcripts per gene is one of the likely substrates of biological and developmental complexity within vertebrates and mammals as compared to single-celled eukaryotes, which show less developmental complexity but largely similar numbers of genes [TODO, 2011]. Further evidence of the unique evolutionary characteristics of alternatively-spliced exons comes from molecular evolutionary studies which have shown such exons to show, on average, higher levels of evolutionary constraint, possibly owing to the importance of exonic splice enhancers in modulating the inclusion or exclusion of their associated exons [TODO, 2011].

However, in terms of organizing biological data, pervasive alternative splicing—with XYZ% of human genes containing at least two (and up to several dozen) transcripts per gene [TODO, 2011], showing tissue-specific and species-specific expression patterns, different levels of overall transcription, and sometimes comprising mutually exclusive exons—is somewhat burdensome. The first problem is the fact that primary data on alternative transcript structures (e.g., resulting from expressed sequence tags, RNA-seq, or proteomics experiments) are largely absent from most organisms with sequenced genomes. Even ignoring this lack of data, the task of incorporating multiple transcripts per gene into an evolutionary analysis is non-trivial, and leaves many unresolved questions open to debate: should all transcripts be treated as independent evolutionary entities, or should some form of meta-transcript be produced, comprising all possible transcripts for

a given gene? Should expression levels and tissue-specificity be taken into account (as both factors have been correlated with evolutionary rate, e.g. [TODO, 2011,?])? And what is the expected evolutionary impact of the loss, gain, or modulation of the prevalence or tissue-specificity of a given exon or transcript in one lineage? Even a fairly shallow consideration of the topic quickly reveals layers of complexity that would quickly hinder many large-scale evolutionary analyses such as the current one, whose main goals are to understand the levels of evolutionary constraint of some subset of genes (or protein-coding sites) within some subset of species.

As a result of these difficulties, the current design of the Compara pipeline only incorporates one 'canonical' transcript per gene into the evolutionary analysis and the resulting inferred gene trees. This reflects a conscious decision to sacrifice some biological fidelity for reduced design complexity and computational load (as the inclusion of multiple transcripts would inevitably require some amount of additional processing and/or calculation). Unfortunately, this only somewhat alleviates the problem, shifting the burden from "how to deal with multiple transcripts in a comparative setting" to "how to choose the best representative transcript for each gene." In the case of a gene with many transcripts of varying sizes containing many non-overlapping exons, the negative consequences of choosing a non-optimal transcript are clear: too short of a transcript could exclude important sequence information from the dataset, while transcripts with spurious exons (resulting from misannotation or erroneous experimental evidence for a transcript) could introduce potentially large amounts of non-orthologous, nonfunctional, or nonconserved sequence into the evolutionary analysis.

Fortunately, the consensus coding sequence (CCDS) project was initiated in 2005 to "identify a core set of human and mouse protein coding regions that are consistently annotated and of high quality" [TODO, 2011; **?**]. Although the transcripts that satisfy these two criteria will not necessarily be the same as those which meet the desired definition of "the best representative transcript for use in an evolutionary study," the confidence that one can have in the quality and consistency of CCDS transcripts helps to reduce the prevalence of potentially damaging errors in the Compara pipeline. Thus, in the current release (version 63), the "representative" transcript used for the Compara pipeline is chosen on the basis of (a) existence within the CCDS set of transcripts and (b) the total

length of the transcript's coding sequence. The combination of these two factors can be expected to identify a reasonably representative transcript, at least for the human and mouse genomes. The situation will be similar for genomes whose Ensembl annotation is derived largely from synteny and orthology to human and mouse annotated genes, but two classes of genomes—those resulting from low-coverage sequencing and those from more distant species whose annotations are derived from largely independent data sources—will still suffer from some amount error in the form of poor transcript choice.

Once the set of canonical transcripts is chosen, the Compara pipeline performs an all-against-all protein BLAST search (using the Washington University variant of BLAST) and clusters genes into groups of evolutionarily-related sequences using *hcluster_sg*, an implementation of a hierarchical clustering algorithm for sparse graphs. Sequences are aligned using MCoffee, a meta-aligner algorithm which combines the results from different aligners into one alignment using a maximum-consistency criterion. The aligners used for the M-Coffee alignment include XXX, YYY, and ZZZ. Finally, the aligned sequences are input to TreeBeST, which infers a gene tree (including gene duplication and loss events) given a set of aligned sequences and a known species tree [TODO, 2011]. The type of the homology relationship between each pair of genes (e.g., one-to-one ortholog, one-to-many ortholog, within-species paralog) is determined using a simple set of rules based on the structure of the inferred gene tree and the annotation of ancestral nodes where a duplication event has likely occurred.

The Compara pipeline has been a part of the Ensembl ecosystem at least since its first mention in [TODO, 2011]. Remarkably, aside from slight tweaks to the protein clustering method and some changes in the exact aligners used, the pipeline has changed little from its original published form [TODO, 2011]. In part, this lack of change reflects the ease with which sets of vertebrate orthologs can be identified using the existing methodology, lying in stark contrast to the equivalent task in sets of insect or fungal genomes where divergence levels between extant sequences are much larger [TODO, 2011] and the shape of the underlying species tree may be uncertain and/or unknown [TODO, 2011], making the development of specialized methods or extensive manual annotation necessary [TODO, 2011]. This is equivalent to saying that Ensembl's pipeline, while not perfect in its orthology predictions or tree inferences (as indicated in a series of back-and-

forth papers between Ensembl scientists and XYZ, [TODO, 2011]), has proved sufficiently accurate enough that an extensive reworking of the system has not yet been deemed necessary. Additional validation of this approach comes in the form of Treefam [TODO, 2011], a database of animal gene trees which applies a similar set of tools to infer gene trees from a more diverse set of genomes, with largely similar results.

[Something about Ensembl being directed at inferreing gene tree topologies, and not being vetted for use in estimates of selective constraint]

[Introduce the structure of the next few subsections: ways of massaging / filtering the Ensembl data to fit with the needs of the current project]

1.3 Identifying orthologous subtrees within large mammalian gene families

The first task in preparing the Ensembl data for sitewise analysis was to identify and extract a biologically meaningful set of orthologous mammalian subtrees from the set of gene trees within the Compara database. This was necessary because many Compara gene trees contain multiple sets of Eutherian orthologs linked by ancient gene duplication events, while I wished to study the evolution of each individual set of Eutherian orthologous genes. In other words, Compara gene trees are over-clustered with respect to the core set of Eutherian orthologs.

Evidence for this over-clustering comes from Table **??**, which shows the number of root Compara gene trees which contain zero, one, or multiple genes in human, zebrafish and drosphila, as well as Figure **??**, which shows the distribution of gene counts in the set of root Compara gene trees. The percentage of Compara trees with 2 or more human genes is strikingly high, at XYZ%. If each Compara tree contained one single set of Eutherian orthologs, then the proportion of trees with multiple human gene copies could only be explained by an unrealistically high rate of gene duplication. A more parsimonious explanation would be that many Ensembl trees represent not one group of Eutherian orthologs, but two or more sets of Eutherian orthologous gene trees joined by one or more ancient duplications. This explanation is further supported by Figure **??**, which shows concentrations of gene counts centered roughly around whole-integer multiples of the number of vertebrate species present in the Ensembl database (shown as gray

dotted lines).

The prevalence of over-clustered Eutherian orthologs in the Compara database is easily explained by a combination of the *hcluster_sg* algorithm used for the hierarchical clustering step, which uses only protein distances as its source of clustering information, and the wide range of protein evolutionary rates in the vertebrate genome. As I mentioned in the previous subsection, the Compara pipeline uses all-by-all protein BLAST E-value scores and the *hcluster_sg* algorithm to produce sets of sequences containing minimal average within-group E-values. No additional biological information, such as the source species of each sequence or the overall taxonomic coverage of each cluster, is used in identifying clusters, and no attempt is made to fit clusters to an expected model of orthologous gene evolution. On the one hand, the lack of additional information and assumptions allows the algorithm to remain simple and the clustering behavior to remain consistent across different groups of genomes; on the other hand, a number of technical (in the sense of non-biologically meaningful) parameters and thresholds must be tuned in order to result in the desired cluster sizes and contents. Importantly, even after these parameters are tuned to perform well on the dataset as a whole, the reliance on protein distances alone means that fast-evolving proteins will be more likely to be under-clustered and slow-evolving proteins will be more likely to be over-clustered. Given that the protein evolutionary rate varies widely within a genome (in a study of vertebrate genes, XYZ et al. found K_a values ranging from ZZZ to YYY, TO-DO), the excess of over-clustered orthologs in the Compara database is understandable and even somewhat expected.

I should note that my use of the phrase "over-clustered" refers only to over-clustering with respect to the current goal of analyzing independent sets of orthologous genes within Eutherian mammals. Certainly these large "over-clustered" trees, which represent a more distant evolutionary history than a single Eutherian orthologous group, are just as accurate with respect to the true evolutionary history of the genes as more narrow groupings would be. Furthermore, the inclusion of a deeper evolutionary context may sometimes be more useful to users of the Compara database, for whom an understanding of the overall evolutionary history of a gene may be the topic of primary interest.

Take for example the gene *NBEAL2* and its human paralogs, whose gene trees, exon structures and domain classifications were extracted from Ensembl

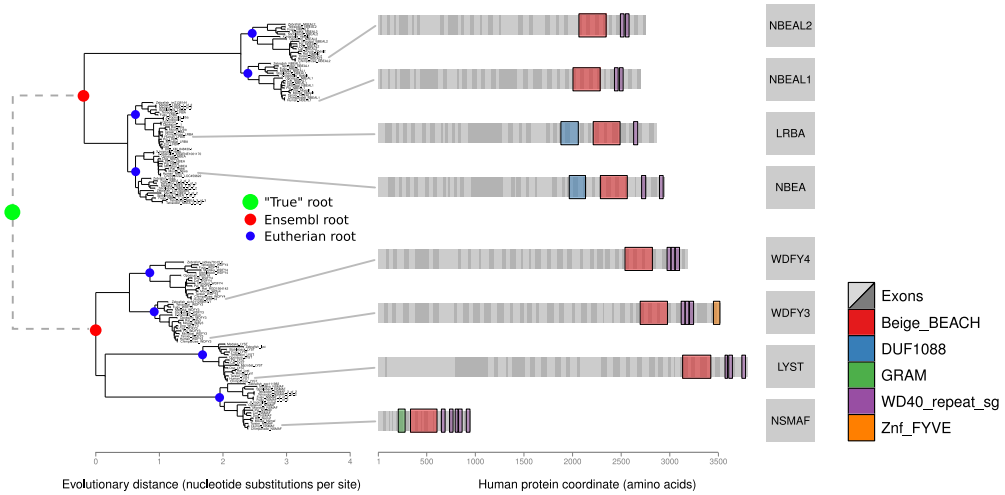

Figure 1.1: The evolutionary history of the human *neurobeachin-like 2* gene (*NBEAL2*) and its paralogs. Left, two phylogenetic trees from Ensembl Compara (release 60) are shown, summarizing the evolution of *NBEAL2* and its three paralogs (top) and *LYST*, a presumed distant paralog of *NBEAL2*, and its three paralogs (bottom) in 15 vertebrate species. The phylogeny shows that *NBEAL2* is taxonomically conserved and distinct from its paralogs. Red dots highlight the root nodes of Ensembl gene trees, blue dots highlight the root nodes of Eutherian orthologous subtrees, and a dashed line with a green dot represents the putative paralogous relationship (with a hypothetical root) between the two Ensembl gene trees. Right, the exon and domain structure of each human gene is shown: exons are displayed alternating shades of gray, and Pfam domain annotations are colored according to their Pfam identifier.

v62 and summarized in Figure 1.1. A recent medical sequencing project identified *NBEAL2*, a gene of previously unknown function, as the putative causative gene for gray platelet syndrome, a predominantly recessive platelet disorder resulting in moderate to severe bleeding [TODO, 2011]. It was important for the authors of this study to ensure that the *NBEAL2* gene is well-conserved across mammals and distinct from its paralogs. The Compara pipeline clustered *NBEAL2* with three of its closest paralogs into one tree (and similarly clustered four more distant *NBEAL2* paralogs into a separate tree), yielding two views which together showed both the full taxonomic coverage of the *NBEAL2* subtree and the large amount of separation between paralogs. Had each Eutherian ortholog been displayed independently in Ensembl (using the blue "Eutherian root" nodes in Figure 1.1), it would have been more difficult to make such claims regarding the evolutionary history of *NBEAL2* without further analysis. Conversely, had the Compara pipeline been even more inclusive in its clustering step and identified a

hypothetical deeper root connecting these two sets of trees (represented by the green node in Figure 1.1), the connection between these eight genes would have been more immediately apparent.

For the purposes of the current mammalian sitewise analysis, however, it was important to isolate individual mammalian gene trees for further processing and sitewise analysis. To this end, I designed a simple scheme for splitting gene trees into non-overlapping subtrees based on flexible taxonomic coverage criteria.

I hypothesized that a relatively simple set of rules based on taxonomic coverage would be sufficient to identify most largely orthologous mammalian subtrees. This hypothesis was based on two well-established observations in mammalian genomes. First, the existence of two rounds of whole-genome duplication preceding the evolution of vertebrates [TODO, 2011] suggested that many of the ancient duplication events contained within Ensembl gene trees occurred before the divergence of mammals, making it possible to cleanly separate out taxonomically complete mammalian subtrees in the majority of cases. This would not be possible if duplication events were common and spread evenly throughout the mammalian tree; if that were the case, many duplication events would have occurred after the divergence of some or all of the major mammalian groups, resulting in a larger proportion of mammalian genes with "internal" duplications and, thus, fewer singly orthologous trees with high taxonomic coverage. Second, the overall low rate of gene duplication and loss in mammals [TODO, 2011; **?**] (excluding, of course, the aforementioned whole-genome duplication events) predicts that few mammalian gene trees will be subject to one or more gene duplication or loss events. In other words, most mammalian gene trees should contain sequences from a majority of mammalian species, so the effectiveness of using taxonomic coverage to identify mammalian subtrees should be largely unaffected by individual (i.e., post-2R) gene duplication or loss events. The potential utility of taxonomic coverage was further bolstered by the star-like shape of the mammalian tree: star-like trees contain more branch length within terminal lineages than ladder-like trees with an equivalent total branch length, making it less likely that a gene duplication or loss event (if such events occurred randomly throughout the mammalian tree) would result in a significant disruption to the taxonomic coverage of the gene tree.

The taxonomic-based tree splitting scheme works as follows. For every in-

ternal node N of each Compara gene tree, the taxonomic coverage (TC) was calculated for several vertebrate clades. The TC for node N and clade C is given by $TC(N, C) = species(N)/species(C)$, where $species(N)$ is the number of unique species represented by the sequences beneath node N and $species(C)$ is the number of species within the vertebrate clade C. The tree is traversed from root to tip, and if a given set of TC constraints (referred to as the subtree constraints) are satisfied by both subtrees below node i, then the tree is split into two subtrees at node i (with the new trees having root nodes placed at the two child nodes, i_a and i_b). The traversal continues recursively until every node is tested. If only the original root node satisfies the subtree constraints, then the entire Compara tree is included in the resulting tree set; if the entire Compara tree fails to satisfy the subtree constraints, it is excluded altogether.

I chose a variety of subtree constraints based on the structure of the vertebrate phylogeny, all of which were run against the 18,613 gene trees within the Compara database to generate several genome-wide sets of subtrees. Table 1.1 shows the details of the various subtree constraints I used; the clade names (e.g., $TC(Primates)$) are used to refer to sets of species contained within the Ensembl database, as defined by the NCBI taxonomy. The NCBI taxonomy of species contained in Ensembl is shown in Figure 1.2.

For the Ingroup and Outgroup categories of subtree constraints, a TC value of greater than 0.6 was required for a single taxonomic clade. If the required TC value for a clade were set to 1, then all subtrees containing deletions in any species within the clade of interest would be rejected. On the other hand, requiring a TC value of less than 0.5 would allow for a truly singly-orthologous tree to be split into two subtrees, with one tree having a TC below 0.5, and the other tree (containing the other half of the species) also having a TC below 0.5. Thus, 0.6 seemed to be a reasonable TC requirement for isolating subtrees with reasonable taxonomic coverage while allowing for some amount of gene deletion.

Two additional types of constraints were designed for use in the MammalSubgroups and MammalSubgroupsPlusOutgoup methods. Inspired by the alignment filtering method from Pollard et al. [2011], which required sequence data from all three major mammalian clades (Primates, Glires, and Laurasiatheria) to be present for a column to pass through the filter, the TC_{all} constraint requires that the TC for all of the included clades is above a given threshold. To complement

the TC_{all} constraint, the TC_{any} constraint requires that the TC for any of the included clades is above a given threshold. These more complicated methods were included in the analysis in case the simpler TC constraints within the Ingroup and Outgroup categories did not perform satisfactorily.

The methods within the Orthologs category of subtree constraints were implemented separately from the rest. Instead of splitting Compara trees based on taxonomic criteria, the subtrees in the Orthologs category were defined from the sets of genes annotated by Ensembl as orthologs to each gene from a given source species. Thus, for each gene from the source species, the Compara subtree containing all of the Ensembl-annoated orthologs was extracted and stored; this was guaranteed to yield exactly one subtree for every gene in the source species. I chose to include human, mouse, zebrafish, and drosophila were chosen as source species for testing. This approach differs from the tree-splitting strategy in two ways: first, it makes use of the orthology annotations resulting from Ensembl's orthology pipeline, and second, it does not guarantee that each subtree contains a completely unique set of genes. For example, a gene which was recently duplicated in humans would yield two subtrees, one for each human paralog, with identical sets of non-human genes in each tree. Although the orthology-based might be useful when an evolutionary study is focused on a specific target or reference species, as is often done with human and mouse due to their finished genome sequence and high-quality annotation, I considered it to be less applicable to the current study due to the potential for introducing reference genome-specific biases, such as over-representation of genes with gene family expansions in the reference species or non-representation of genes which have been deleted in the reference species. Still, I expected that the sets of subtrees resulting from the Ensembl ortholog annotations would serve as a useful reference with which to compare the other TC-based methods.

1.4 Analysis of genome-wide sets of orthologous mammalian trees

The subtree splitting schemes described in the previous subsection were each applied to the 18,607 root gene trees from the Ensembl database. In this and the next section I will describe the resulting sets of trees and subtrees, discuss

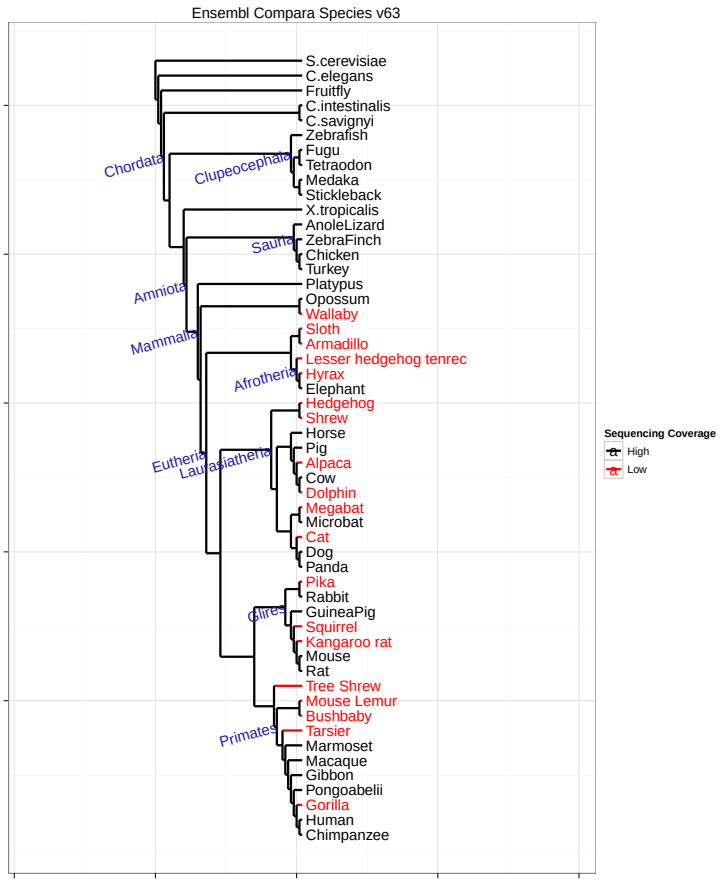

Figure 1.2: The NCBI taxonomy of species within the Ensembl Compara database. Note that branch lengths are not drawn to scale. Low-coverage genomes are labeled in red, high-coverage genomes are in black. Selected internal nodes, used are labeled in blue.

what they reveal about the evolutionary history of vertebrates and the feasibility of using taxonomic coverage to isolate orthologous trees for sitewise analysis, and finally, explain my reasoning for deciding to use the subtrees based on the Eutherian taxonomic coverage for the subsequent sitewise analysis.

1.4.1 The set of root Compara gene trees

Table 1.2 presents a summary of the set of root Compara gene trees and the subsets of trees with more or fewer than 15 sequences.

It is somewhat surprising that nearly half of all Compara gene trees contain few sequences: 9,378 out of 18,607 root trees constitute fewer than 15 sequences.

Method		
Category	Name	Constraints
Ingroup	Primates	$TC(Primates) > 0.6$
	Glires	$TC(Glires) > 0.6$
	Laurasiatheria	$TC(Laurasiatheria) > 0.6$
	Sauria	$TC(Sauria) > 0.6$
	Fish	$TC(Clupeocephala) > 0.6$
Outgroup	Eutheria	$TC(Eutheria) > 0.6$
	Amniotes	$TC(Amniota) > 0.6$
	Vertebrates	$TC(Vertebrata) > 0.6$
	Fungi/Metazoa	$TC(Fungi/Metazoa) > 0.6$
Subgroups	MammalSubgroups	$TC_{all}(Laur., Glires, Primates) > 0.1$
	MammalSubgroupsPlusOutgroup	$TC_{all}(Laur., Glires, Primates) > 0.1$ AND $TC_{any}(Sauria, Clupeo., Ciona, Marsup.) > 0)$
Orthologs	Human Orthologs	
	Mouse Orthologs	
	Zebrafish Orthologs	
	Drosophila Orthologs	
Root Nodes	Ensembl Roots	

Table 1.1: Subtree constraints used for identifying Eutherian orthologous subtrees. Ensembl gene trees were split into subtrees based on taxonomic coverage (TC) requirements at internal nodes. Laur. - Laurasiatheria; Clupeo. - Clupeocephala; Marsup. - Marsupiala

Given the protein-based clustering performed by the Compara pipeline, one might expect many of these small trees to represent portions of larger fast-evolving gene trees whose high sequence divergences made the BLAST search step inaccurate or caused clustering via the *hcluster_sg* algorithm to be ineffective. Alternatively, these small clusters might have resulted from exceptional lineage-specific gene duplications or pseudogenes mis-annotated as genes, creating tight clusters of

Tree		Med. Size		Human Content			Human	Med.	Med.
Set	Count	(Min / Max)	N50	0	1	2+	Total	MPL	Species
All	18607	15 (2 / 400)	139	0.50	0.30	0.20	19995	0.55	8
($\leq$ 15)	9378	3 (2 / 15)	5	0.92	0.08	0.00	809	0.04	2
($>$ 15)	9229	54 (16 / 400)	146	0.07	0.53	0.40	19186	1.04	47

Table 1.2: Summary of the set of Ensembl Compara root trees. The 'Human Content' columns represent the fraction of trees which contain the indicated number of human genes, and 'Human Total' is the total number of human genes contained within the tree set. 'Med. Species' is the median species count across all trees. Med. - median, MPL - mean path length

very closely-related transcripts that were identified by *hcluster_sg* as independent gene trees. Some evidence for the latter scenario comes from the median species counts and mean path lengths of the smaller versus larger trees. The subset of small root trees has a median species count of 2 compared to 47 for the large subset, indicating that the smaller trees encompass sequences from a very small taxonomic range. Furthermore, the median MPL for small trees is 0.04 compared to 1.04 for the large subset, revealing a much smaller level of sequence divergence within each tree. Together, these summary statistics indicate that the smallest trees in the Compara database consist of highly species-specific, closely-related proteins that are likely artifactual gene annotations.

Despite the existence of many small trees in the Compara database, they comprise only a small fraction of all protein-coding sequences. Only 4% of the human gene set—which we expect to be well-annotated and to contain few false positive genes due to the high level of manual curation and the large amount of continued scrutiny—is contained within the subset of small trees. This indicates that whatever process is causing the Compara pipeline to yield such a high number of small gene trees has not had too much of an impact on the placement of the most confident set of protein-coding genes within the database of root gene trees.

A closer examination of the distribution of tree sizes in the set of root Compara trees presents a clear view of the over-clustering of mammalian orthologous trees. The black bars in Figure 1.5 show the distribution of sequence counts for all trees with more than 15 sequences, with vertical dashed lines overlaid at multiples of 48 (the number of vertebrate species in Ensembl release 63). The highest peak of the histogram is at or slightly above 48 sequences, with the tree counts quickly diminishing at larger sizes. Weaker, but still discernable, peaks appear at larger tree sizes, with the location of these echo-like peaks corresponding closely to the second, third and fourth multiples of 48. The pattern of recurring peaks becomes indistinguishable at sizes above 200, but there is still a long tail of large trees extending out to a maximum size of 400 sequences. Overall, the distribution of tree sizes provides good support for the situation described above, with the Compara pipeline often clustering together two or more largely-orthologous gene trees sharing ancient homology.

It is also interesting to characterize the set of Ensembl trees by the propor-

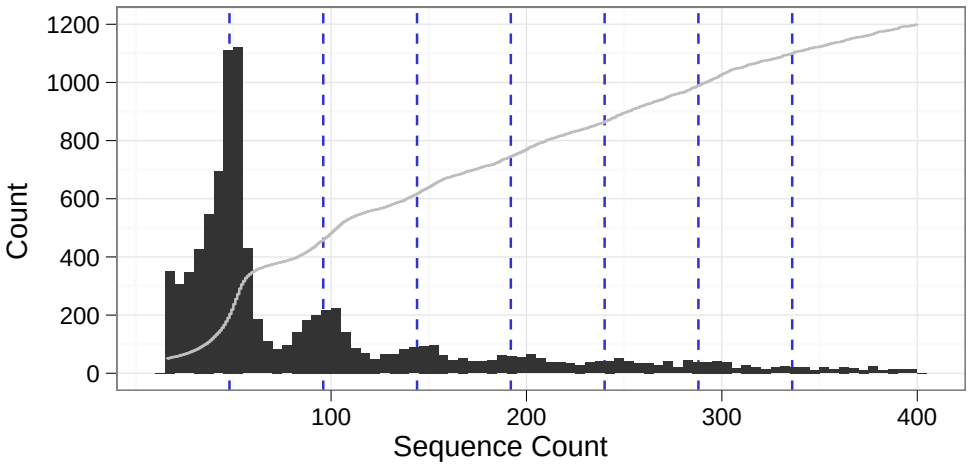

Figure 1.3: Sequence counts for the set of root Compara trees. Black bars show a histogram of sequence counts in bins of width 5, and the gray line shows the cumulative fraction of sequences contained within trees of that size or smaller. For clarity, 9,378 trees with 15 or fewer sequences are not shown. Dashed green lines are drawn at integral multiples of 48, the number of vertebrate species within Ensembl.

tion of all sequences which are covered by trees of a given size or smaller. This value is plotted in Figure 1.5 as a gray line. First, one can see that trees with fewer than 15 sequences (which were excluded from the plot but inlcuded in the calculation of the cumulative fraction of sequences) represent a trifling fraction of the sequences within Compara; this is similar, but not identical, to the above-mentioned calculation that 4% of human genes are contained within these smaller trees. Second, the steady slope of the cumulative curve contrasts with the declining height of the histogram. This results from the increasing number of sequences encompassed by each of the larger trees: although there are relatively few trees with more than 300 sequences, together they contain around 10% of all protein-coding genes in Ensembl. Two points along this cumulative plot are of particular interest. First, one can identify the fraction of vertebrate proteins which exist as identifiable paralogs. Looking at the value along the x-axis where the largest bump in the histogram ends, at around 75 sequences, one can see that in total around 30% of proteins are covered by trees of 75 sequences or fewer. Since the pattern of bumps in the histogram correlate well with the number of Ensembl vertebrate species, it would be reasonable to state that 70% of vertebrate proteins are contained within large gene trees containing sequence-based evidence

of ancient paralogy. Second, a look-up in the reverse direction can identify the tree size at which 50% of sequences are clustered. This value represents the size of tree that an "average" protein might be clustered in, and in some ways is a more accurate characterization of the set of gene trees than the median tree size. A similar calculation is often performed to characterize the size distribution of contigs (contiguous sequence blocks) within a genome assembly. This statistic, referred to as the N50 length, is the contig length for which 50% of bases are contained in contigs of that size or larger [TODO, 2011]. For the Ensembl root trees, the N50 tree size is 139, slightly less than three times the number of vertebrate species. The N50 tree size is shown for the root trees in Table 1.5 and in the table for taxonomically-defined tree sets below.

Another way to characterize the distribution of gene trees is across the taxonomic space. A question of particular interest to the identification and analysis of mammalian orthologs is whether levels of gene presence and absence are consistent across different species and different levels of assembly quality. To investigate this question in the context of the root Ensembl trees, data were collected by counting the number of sequences from each species contained within each gene tree. Results were tabulated for each species and are presented in Figure 1.4, showing the number of trees containing 0, 1, 2, or more than 3 genes from each of the 53 species in Ensembl. Comparing the range of values in the panels for each copy count (labeled 0, 1, 2 and 3+), one finds that most trees (8,000-11,000 within vertebrates) contain zero copies from a given species, fewer trees contain one copy (4,000-6,000) and several thousand contain two, three or more copies (ca. 1,000-1,500 for 2 copies and 1,500-2,000 for 3+). The plethora of trees with zero copies from a given species is again a result of the existance of many small, species-specific trees within the root Ensembl set. Similarly, the high number of trees with many copies from each species reflects the clustering of multiple orthologous sub-trees together.

A comparison of values across the range of species in Figure 1.4 reveals that the zero-copy count tends to increase along with evolutionary distance from human, while the 1, 2 and 3+ copy counts tend to decrease as the distance from human increases. Both trends are most striking at the distant end of the tree where the five non-vertebrate species begin. For the increase in zero-copy trees and the decrease in single-copy trees, the strength of the trend at the highest

level of divergence can be partly explained by the very long branch lengths connecting those species to each other and to the more well-represented vertebrate clade: the distance-based clustering algorithm might reasonably be expected to produce more false negatives in longer branches for a number of reasons including the behavior of the *hcluster_sg* algorithm, inaccurate BLAST E-values at larger distances, and heterogeneity in evolutionary rates across lineages [TODO, 2011]. However, the dearth of 2 and 3+ copy counts in non-vertebrates is most likely a signal resulting from the 2R event at the basal vertebrate lineage, with the non-vertebrate species strongly depleted of multi-copy duplicates compared to their vertebrate relatives.

It is slightly concerning that human and its close primate relatives contain fewer zero-copy genes and more one-copy and two-copy genes than any other group of vertebrates in the set of Ensembl trees. There is no *ab initio* biological reason to expect this to be the case, and I suspect that the existence of such a pattern, which is fairly small in effect, is due to the widespread reliance on human annotation and protein experimental data in the annotation of non-human genomes. There is one region where this trend does not appear to be the case: in the 3+ copy count for the fish species, which is instead a result of gene duplicates retained after the third round of genome duplication which occurred in the teleost ancestor [TODO, 2011]. The signal resulting from the teleost genome duplication event is clearer in the sets of taxonomically-defined subtrees, so I will defer its discussion to the next subsection where those sets of trees are described.

Finally, the differences in copy counts between species with low- and high-coverage genome sequences show the tendency of low-coverage genome sequences to yield false negatives in the gene annotation, as low-coverage species contain more zero-copy, roughly the same number of one-copy, and noticeably fewer multi-copy genes than high-coverage species. These clear effects of low sequencing coverage show that gene absence in low-coverage genomes should not be taken as evidence for actual gene loss and that gene duplications are systematically underrepresented in low-coverage genomes. The former point was emphasized in a recent critical analysis of the effect of low-coverage genomes on gene duplication inference [TODO, 2011; **?**], but the latter point was largely ignored. Again, this signal is also stronger in the more stringent set of mammalian orthologous subtrees and will be revisited in the next subsection.

The preceding analysis of the set of root Ensembl trees, in which I characterised the distribution of trees with respect to size (i.e. sequence count) and across the taxonomic space, showed that despite the over-representation of small, species-specific trees, most sequences are contained in trees with biologically plausible sizes given the history of vertebrate genome duplications. The tree-based equivalent of the N50 statistic was developed for summarizing the distribution of differently-sized trees, and two main views of this distribution were introduced (in Figures 1.4 and 1.5), providing evidence for the clustering of paralogous mammalian sub-trees and for species-based and genome coverage-based trends in the breakdown of gene copy counts within these trees.

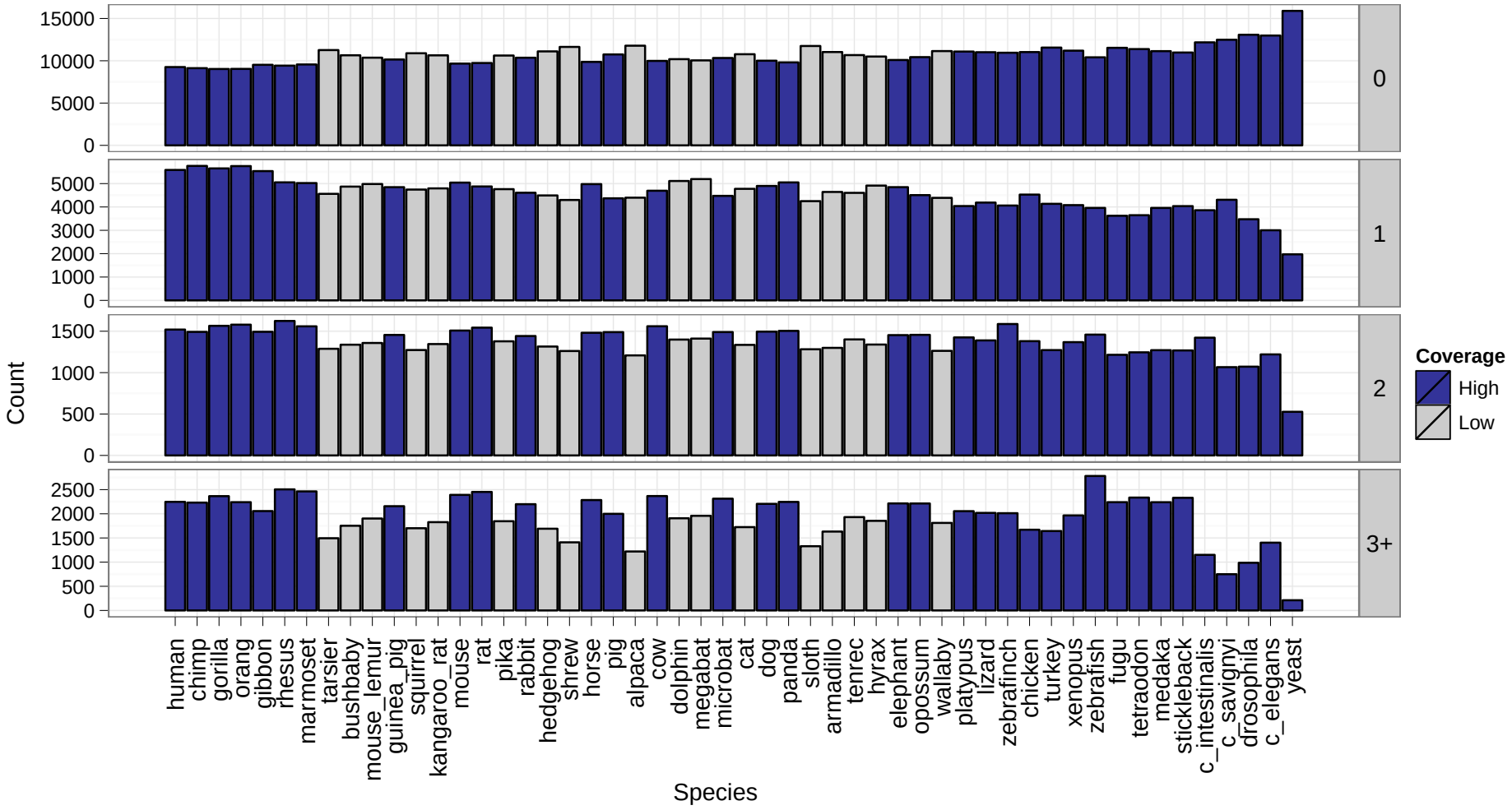

Figure 1.4: Taxonomic distribution of gene copy counts for the root Ensembl trees. The number of trees containing 0, 1, 2 or more than 3 sequences from each species is shown. Bars are colored blue and gray for species with high- and low-coverage genomes, respectively. Note that the y-axis scale is not the same for each panel.

1.4.2 Sets of subtrees defined by taxonomic coverage and orthology annotation

The sets of trees resulting from applying the various subtree extraction methods to the root Ensembl gene trees are summarised in Table 1.3, with the original Ensembl trees included at the bottom for comparison.

The Ensembl Roots and Drosophila Orthologs sets are two clear outliers, with much higher N50 values than any other set (139 and 125 vs. the next highest value of 56) and more trees with multiple human copies (0.20 and 0.43 vs. the next highest value of 0.14). In fact, the major differences between these sets are all attributable to the excess of small species-limited trees in the Ensembl Roots group: the Drosophila Orthologs set contains fewer trees than the Ensembl Roots (9,210 vs. 18,607) and a larger average tree size (60 vs. 15), closely resembling the set of Ensembl Roots with small trees removed as summarised in the third row of Table 1.2.

Within the Ingroup category of methods, the methods based on mammalian TC values (Primates, Glires and Laurasiatheria) produced largely similar sets of trees, with the Primate set containing around 2,000 more trees and covering around 1,000 more human genes than the other two sets. A reason for the higher number and human coverage of Primate trees is not immediately apparent, although it may speculatively be due to an excess of primate-specific gene trees that are not captured by non-primate TC-based criteria. Further investigation of the trees unique to this set might reveal the root cause of this slight discrepancy.

The Sauria and Fish tree sets stood in strong contrast to the mammal-based methods from the Ingroup category. The Sauria clade is represented by only four Ensembl species and diverged from the mammalian ancestor at an early point in the evolution of amniotes. The moderately lower number of trees (13,046 vs. 15,764 for Laurasiatheria) and the increased proportion of trees containing multiple human genes (0.14 vs. 0.09 for Laurasiatheria) are presumably consequences of the lower clade size, which could affect the TC calculation, and the long branch separating Sauria from the other vertebrate clades. The fish-based subtree constraint produced a strikingly different set of trees resulting from the impact of the teleost-specific whole genome duplication on the structure of fish gene trees. Although the Fish tree set contains a N50 value of 49 which is no different from the N50 of the other Ingroup sets, Table 1.3 highlights three major differences in

the Fish set: it contains many more trees, a higher proportion of trees with zero human copies, and a lower total human gene count than the other Ingroup sets.

The reason for the drastically different Fish tree set is that the tree splitting procedure identifies largest non-overlapping subtrees that satisfy the given TC criteria. Genes that were duplicated in the teleost lineage and retained in duplicate form (as opposed to one or both copies being lost in either of the descendant duplicate chromosomes) would result in a gene tree with two teleost-specific subtrees, each containing a high TC value for the Clupeocephala clade. In this case, the splitting procedure would result in two small Fish subtrees, "missing" the single subtree of mammalain orthologs because two non-overlapping trees already exceeded the TC threshold of 0.6. If, however, one of the duplicate gene copies were lost, then the tree would resemble a typical singly-orthologous vertebrate gene tree, and the splitting procedure would select a subtree encompassing the entire vertebrate clade. It follows that the presence of small, teleost-specific gene trees in the Fish set is a signal of retained duplicate copies, and the size distribution of trees from the Fish set, shown in Figure **??**, shows that several thousand trees fit the expected model. If we assume that all trees from the Fish subset which contain zero human copies, span 5 or fewer species, and contain 40 or fewer sequences are likely retained duplicate genes, a total of 6,980 retained duplicates are identified, yielding a retention rate of 17.5%, which is very much in line with a previously published estimate of 15% based on a comparison of tetraodon, fugu and zebrafish genes [TODO, 2011; **?**].

The sets of subtrees resulting from the Outgroup methods were of special interest, as the clades used to define these TC constraints contained all or nearly all of the mammalian species whose orthologous genes I wished to study. The resulting sets of subtrees show little variation, owing perhaps to the large sizes of the clades and their similar composition. Each set contained between 15 to 17 thousand trees, N50 values of around 49, and greater than 90% of trees containing exactly one human sequence. These measures provided good evidence that the tree-splitting method was effectively isolating singly orthologous mammalian trees. Some slight trends were apparent, however, with the tree count decreasing, the proportion of trees with human duplications increasing, and the overall human gene coverage decreasing as the clade size used for the TC calculation increased. These trends could understandably be the result of the minimum re-

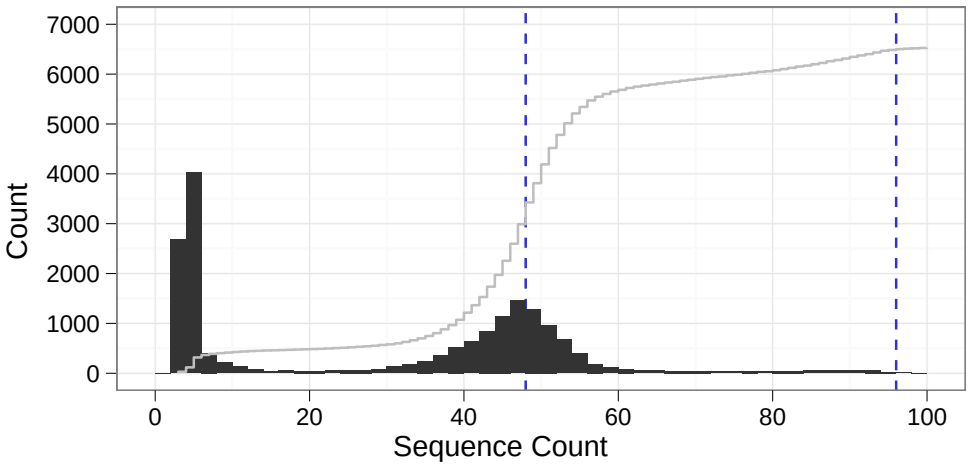

Figure 1.5: Sequence counts for the set of subtrees identified using the Fish clade taxonomic coverage constraint. Black bars show a histogram of sequence counts in bins of width 2, a gray line shows the cumulative fraction of sequences contained within trees of that size or smaller, and dashed blue lines are drawn at integral multiples of 48, the number of vertebrate species within Ensembl. The 255 trees with more than 100 sequences are not shown.

quired tree size increasing along with the clade size, ranging from 21 for Eutheria to 32 for Fungi/Metazoa.

The Subgroups methods did not appear to produce subtrees of any higher quality or more biological interest than the Outgroup methods. The MammalsSubgroups set was more numerous than the Outgoups sets, but the N50 was slightly lower (46 vs. 49) and the proportion of zero-humany trees was higher (0.18 vs. 0.01), suggesting that the additional trees were spurious results containing fragmented species coverage. The addition of an outgroup requirement to the MammalSubgroupsPlusOutgroup method produced a tree set more closely resembling the Outgroup methods, but the human gene coverage was lower than that for any Outgroup method despite the overall higher tree count.

Finally, the ortholog annotation-derived subtrees provided for an interesting comparison between three different ortholog sources and between the overlapping and non-overlapping sets of subtrees. As I mentioned at the beginning of this section, the *Drosophila* ortholog set was highly contrasted with the vertebrate sets due to the two rounds of whole genome duplication. There was minimal variation among the other ortholog sets, although it is interesting to note that Ensembl

contained 21,873 mouse protein-coding genes while human contained only 19,991. Zebrafish, on the other hand, contained 24,540 genes, in line with the 17.5% rate of duplicate gene retension I estimated earlier. Overall, 76% and 81% of mouse and zebrafish genes have an apparent one-to-one ortholog in human, which is slightly lower than the 92% of Eutheria subtrees containing one human sequence.

Method			Med. Size		% w/ Human Count			Human	Med.	Med.
Category	Name	Count	(Min / Max)	N50	0	1	2+	Total	MPL	Species
Ingroup	Primates	17673	46 (6 / 388)	48	0.02	0.93	0.05	19024	0.68	42
	Glires	15786	48 (8 / 391)	49	0.02	0.90	0.08	17904	0.73	44
	Laurasiatheria	15764	48 (8 / 391)	49	0.01	0.90	0.09	17952	0.73	44
	Sauria	13046	49 (3 / 391)	51	0.06	0.80	0.14	14988	0.78	45
	Fish	18291	40 (3 / 391)	49	0.43	0.52	0.06	12183	0.58	38
Outgroup	Eutheria	16477	47 (21 / 391)	49	0.01	0.92	0.07	18343	0.71	43
	Amniotes	15899	48 (26 / 391)	49	0.01	0.91	0.08	18094	0.73	44
	Vertebrata	15634	48 (29 / 391)	49	0.01	0.91	0.08	17938	0.74	44
	Fungi/Metazoa group	14957	48 (32 / 391)	50	0.01	0.90	0.09	17623	0.76	44
Subgroups	MammalSubgroups	21179	40 (4 / 159)	46	0.18	0.79	0.03	18595	0.54	37
	MammalSubgroupsPlusOutgroup	17155	46 (5 / 159)	48	0.05	0.90	0.05	17640	0.71	43
Orthologs	Human Orthologs	19991	49 (2 / 367)	52	0.00	1.00	0.00	19991	1.07	44
	Mouse Orthologs	21873	50 (2 / 352)	54	0.10	0.81	0.09	28256	1.01	43
	Zebrafish Orthologs	24540	51 (2 / 392)	56	0.11	0.76	0.13	30063	1.14	46
	Drosophila Orthologs	9210	60 (2 / 399)	125	0.08	0.49	0.43	17625	1.22	50
Root Nodes	Ensembl Roots	18607	15 (2 / 400)	139	0.50	0.30	0.20	19995	0.55	8

Table 1.3: Summary of Ensembl subtrees identified using taxonomic criteria or Ensembl ortholog annotations. The set of Ensembl root trees ("Ensembl Roots") from Table 1.2 is included for comparison. Cells in numeric columns are shaded according to their value relative to other rows, with low values in white and high values in blue. The 'Human Content' columns represent the fraction of trees which contain the indicated number of human genes. 'Med. Species' is the median species count across all trees. Med. - median, MPL - mean path length

Figure 1.6 shows the taxonomic distribution of gene copy counts for the trees resulting from each of the subtree methods tested. By way of reference, the values shown in the separate panels of Figure 1.4 appear in Figure 1.6 as different-colored bars in the bottom panel. Although the various characteristics of each of the subtree methods have already been discussed at length, the taxonomic view reveals some salient features of the patterns of gene deletion and duplication within the tree sets and shows the pervasive impact of genome-wide duplications on the evolution of vertebrate genes. The large fraction of species with multiple copies in Drosophila Orthologs subtrees is a result of the two rounds of vertebrate genome evolution, while the elevated fraction of multi-copy fish trees in the Outgroup subtrees shows the impact of the teleost-specific duplication event.

Furthermore, the relative prevalence of zero-copy and multi-copy trees can provide some indication of whether gene deletion or gene duplication is a more common process in vertebrate genomes. Focusing on the four Outgroup subtree methods, the observation of a greater number of multi-copy trees than zero-copy genes, valid across all four subtree methods and throughout all mammalian species except platypus, can be interpreted as tentative evidence for a greater number of gene duplications than gene deletions in the evolution of mammalian genomes. This pattern does not hold for vertebrates more distantly related to human, however: vertebrates beyond opossum show a distinct and consistent increase in zero-copy trees, and birds appear to exhibit a slight clade-specific drop in the proportion of multi-copy trees. Of course, both of these trends could be methodological artifacts related to the *hcluster_sg* algorithm or to the methods used to assemble and annotate more distantly-related genomes.

The distributions in Figure 1.6 also reveal the pig to harbor a very high number of apparent gene deletions, unmatched by other mammalian species and nearing the proportion of zero-copy trees seen in platypus and more distant vertebrates. Given the consistently low proportion of zero-copy trees for other closely-related species, I would expect this number to change once a finished-quality pig genome sequence is included in the Ensembl pipeline **??**.

In the end, the set of Eutheria subtrees was chosen as the final set for use in the downstream evolutionary analysis, due to the slightly larger number of trees and better coverage of human genes in the Eutheria set compared to the other Outgroup methods. The distribution of tree sizes for the Eutheria set of

subtrees is shown in Figure **??** and the full taxonomic distribution of copy counts is included in Figure 1.7.

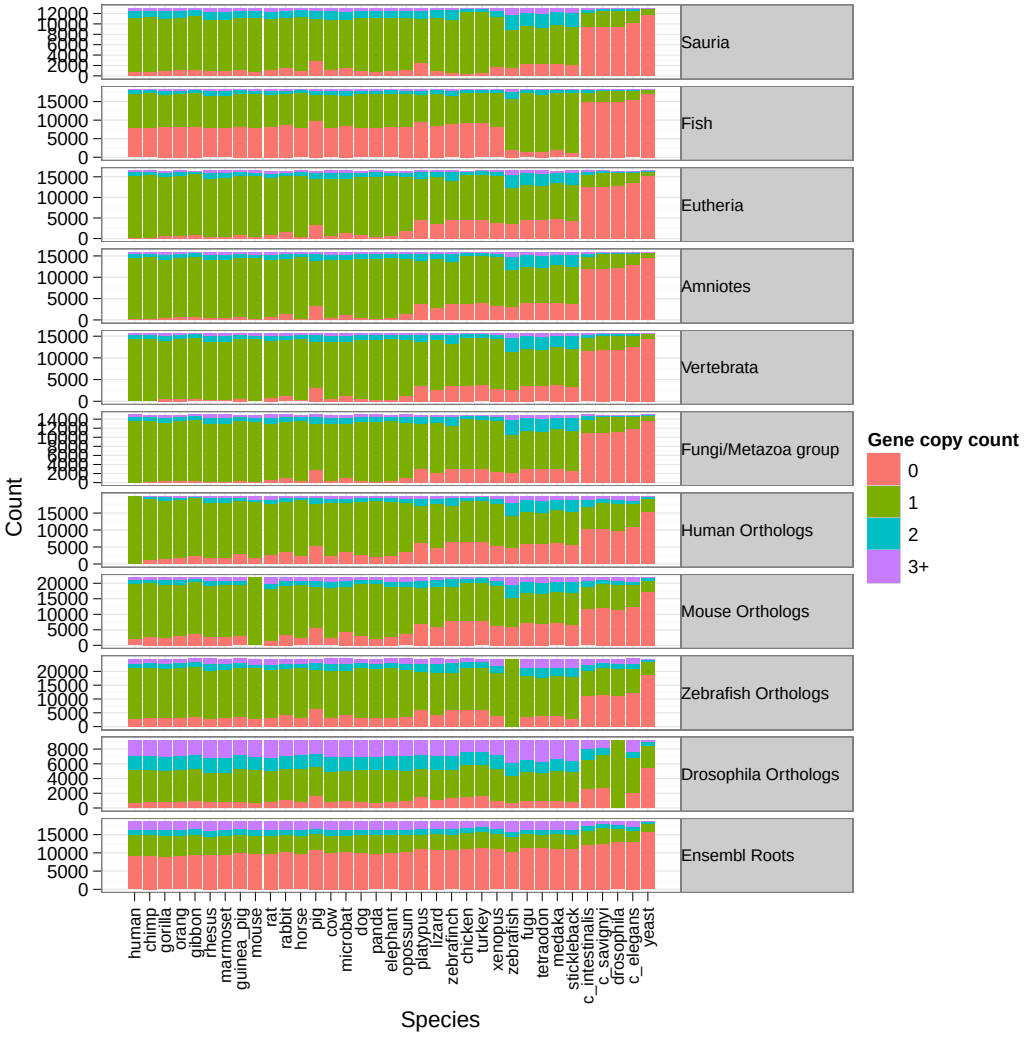

Figure 1.6: Taxonomic distribution of gene copy counts for different subtree methods. The numbers of trees containing 0 (red), 1 (green), 2 (blue) or more than 3 (purple) sequences from each species are shown as stacked colored bars. The Ingroup and Subgroups methods were omitted for clarity, as were species with low-coverage genomes. Note that the y-axis scale is not the same for each panel.

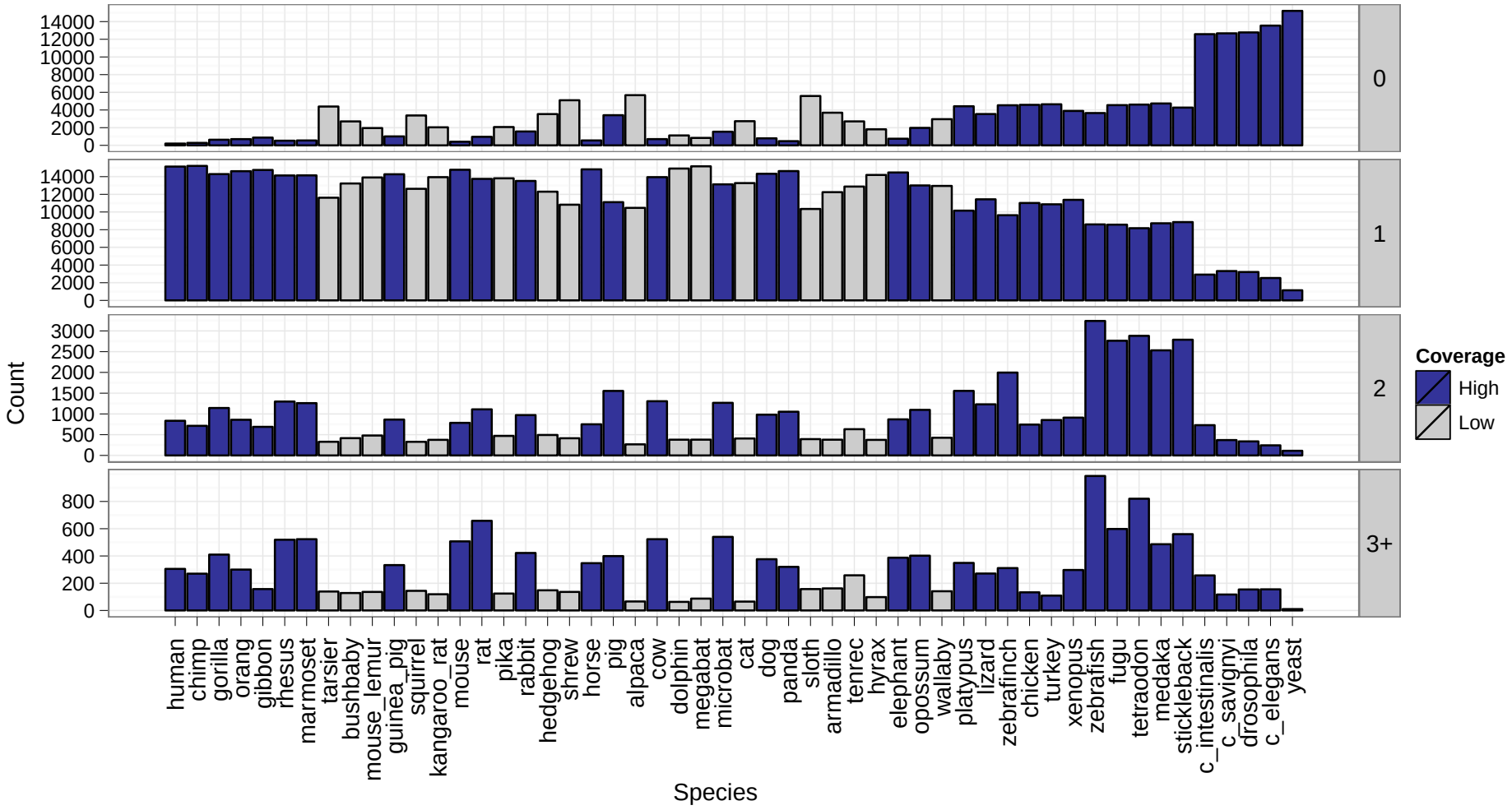

Figure 1.7: Taxonomic distribution of gene copy counts for the Eutheria subtrees. The number of trees containing 0, 1, 2 or more than 3 sequences from each species is shown. Bars are colored blue and gray for species with high- and low-coverage genomes, respectively. Note that the y-axis scale is not the same for each panel.

1.5 Preparing mammalian alignments for site-wise analysis

As discussed in the introduction to this chapter, the effects of sequencing, annotation and alignment error on the results of comparative evolutionary analyses can be severe [TODO, 2011], with a high potential for false positive results when using sensitive evolutionary models [TODO, 2011]. In order to minimize the potential for false positive results in this study, sequences were prepared for input to SLR with a series of filters and realignment steps designed to (a) remove low-quality sequences prone to high error rates, (b) realign sequences using an aligner with better performance for detecting sitewise positive slection, and (c) mask out short alignment regions with dubious elevated rates of nonsynonymous substitution.

1.5.1 Filtering out low-quality genome sequence

Due to the presence of several low-coverage genome assemblies in the set of available mammalian genomes and the elevated sequencing error rates in such assemblies [TODO, 2011; **?**], I applied a conservative filter to the set of input sequences based on sequence quality scores where available.

Most automated genome assembly pipelines, such as the Arachne tool used to sequence many of the low-coverage mammalian genomes included in Ensembl [TODO, 2011; **?**], output a set of Phred quality scores alongside the genome sequence, with one Phred score per base ranging from 0 to 99. A Phred score represents the probability, calculated by the sequencing and/or assembly program, that a given base call is incorrect. This probability is usually concisely expressed as the negative logarithm of the probability of an error multiplied by ten, or $Q = -10log_{10}P$ where Q is the Phred score and P is the probability of an incorrect base call [TODO, 2011; **?**].

Unfortunately, Ensembl does not store quality scores from its source genome assemblies, so Phred quality scores were downloaded for all genomes with readily available Phred-like quality scores (**TO-DO: make a list of genome assemblies and quality score sources**). Most quality scores were provided as a single file in FASTA format with one string of numerical scores per assembled contig. Since the process of filtering a mammalian coding alignment required

collecting scores from many different species at many disjoint genomic locations, an index file was created for each quality score string based on the contig name and sequence position to allow for quicker score lookup and retrieval.

A suitable score threshold for filtering coding regions was chosen based on the work of Hubisz et al. [2011], who performed a detailed analysis of Phred quality scores and observed error rates in low-coverage mammalian genome assemblies by comparing the low-coverage assemblies to matched regions of high-quality sequence from the ENCODE comparative genomics dataset [TODO, 2011]. The authors identified a strong correlation between Phred scores and error rates for scores below 25, indicating that the scores were accurate in this range. Error rates did not decrease significantly at scores above 25, however, suggesting that the value of using an extremely high Phred score threshold would be minimal. Furthermore, Hubisz et al. noted that 85% of bases in the low-coverage mammalian genomes contain very high Phred scores (¿45) and only 4% have low scores (¡20).

Based on these considerations, a threshold Phred score of 25 was chosen as a reasonable trade-off between the potential benefit of avoiding miscalled bases and the potential cost of masking out correctly sequenced bases. For each coding sequence (CDS) with quality scores available, a "minimum score" approach was used to filter codons: all codons containing one or more nucleotides with a score below 25 were masked out with three ambiguous nucleotides, 'NNN'.

The expected proportion of filtered nucleotides can be calculated from the fraction of bases below the Phred score threshold of 25. According to the cumulative distribution of quality scores found in Hubisz et al. [2011], approximately 5% of bases in low-coverage mammalian genomes contain Phred scores below 25. The worst case scenario, in terms of high-quality bases being masked as a result of using the minimum score, would be if only one base per codon had a score below the threshold. Were that the case, an expected 15% of nucleotides would be filtered, since 3 bases would be masked for every low-quality base. However, the distribution of low-quality bases is likely highly clustered, due to the uneven distribution of repetitiveness and GC content as well as the tendency for uncertain base calls to occur towards the end of sequence reads (all of which are known to affect read coverage and assembly performance, e.g. TODO [2011]; **?**). A more clustered distribution of low-quality bases would cause fewer high-quality bases

to become masked by the minimum score approach, reaching the limit of 5% total filtered bases if they always occurred in codon triplets. Thus, anywhere from 5% to 15% of low-coverage nucleotides were expected to be filtered by this approach.

TO-DO: Collect the filtering results and show a graph / some numbers on the amount of filtered material. How many genes had lots of filtered stuff? None at all? Which species were filtered the most?

Bibliography

TODO (2011). Citation will be inserted at a later point in time. 1, 2, 3, 4, 7, 8, 9, 10, 11, 12, 14, 15, 16, 22, 23, 27, 34, 35

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