Analyses of Highly Conserved Nucleotide Sequences within Protein Coding Regions of Eukaryotes

Rumiko Suzuki

Department of Genetics

School of life science

The Graduate University for Advanced Studies

2010

ABSTRACT

Nucleotide substitutions in the synonymous sites of codons do not alter amino acid sequences, therefore they are considered to be basically neutral. In some cases, however, synonymous sites accept selective constraints.

Requirement for translational efficiency or accuracy enhances the optimum codon usage and suppress the synonymous changes. Other than this, a certain region of a protein coding gene may function as exonic splicing signals, RNA editing targets, and RNA secondary structures that affect on gene expression. There is also possibility that messenger RNAs have interaction with non-protein coding transcripts. The existence of such functional regions would be detected from suppression of nucleotide substitution in the area.

Preceding studies have revealed many facts about codon biases and exonic splicing signals, however, other factors have not been extensively surveyed. The aim of this study is to explore unknown factors that affect on the nucleotide conservation in the coding regions in various taxa and to predict potential functionality of the conserved sequences.

For this purpose, I investigated significantly conserved coding sequences (SCCSs) in orthologous genes in seven taxa: mammals (*Homo sapiens, Macaca mulatta, Mus musculus,*

Rattus norvegicus, Bos taurus, and Canis familiaris), teleosts (*Tetraodon nigroviridis, Takifugu rubripes, Gasterosteus aculeatus, Oryzias latipes*), *Drosophilas* (*Drosophila melanogaster, D. simulans, D. sechellia, D. yakuba, D. erecta, D. ananassae*), nematodes (*Caenorhabditis elegans, C. briggsae, C. remanei, C. japonica*), dicots (*Arabidopsis thaliana, A. lyrata, Vitis vinifera*), monocots (*Oryza sativa japonica, O. s. indica, Sorghum bicolor, Brachypodium distachyon*), and budding yeasts (*Saccharomyces cerevisiae, S. paradoxus, S. mikatae, S. bayanus*). I analyzed the ratio of preferred codons, or the most frequently used codons for each amino acid, GC content, and codon degeneracy of SCCSs. The result clarified different characteristics of SCCSs among the seven taxa. The preferred codon ratio decreases as the conservation length elongates in the four animal taxa (mammals, teleosts, *Drosophilas,* and nematodes), while GC content and codon degeneracy do not show notable fluctuation. This result implies that selection toward optimum codons may not be the dominant factor in the above taxa.

To extract sequences whose conservation is significantly stronger than others, I took a permutation approach. I permuted codons of each alignment and surveyed the length and frequency of invariant sequences in the permuted alignment. In the mammals, the result of permutation showed significant deviation from the number of invariant sequences in the original alignments ($p < 2.2E-16$) but deviation is subtle in budding yeasts. This result implies that the distribution of conserved sites is skewed in mammals, while the distribution is rather homogeneous in the budding yeasts. I extracted invariant sequences that have significantly low expectancy ($P < 0.01$) in comparison with the permutation results and defined them as significantly conserved coding sequences (SCCSs).

These analyses revealed different characteristics of conserved nucleotide sequences among the taxa. In mammals and teleosts, it's not likely that long SCCSs have been retained solely by amino acid constraint judging from the codon degeneracy and negative correlation between the conservation length and preferred codon ratio. The sequence characteristics and skewed distribution of conserved sites predicted from the permutation result suggest that SCCSs of the above tow taxa have rather preferable traits as functional nucleotide elements.

There are cases that specific RNA secondary structures exert some functions. I computationally predicted RNA secondary structures of SCCS regions using Vienna RNA package and detected five SCCSs that form secondary structures of significantly low folding free energy ($P < 0.05$). The corresponding regions of platypus and opossum orthologs showed sequence similarity but the structures are more stable in the placental mammals. Although the roles of these structures are unknown, strong conservation and significantly low free energy suggest the possibility that these regions have some functions.

As for mammals, I investigated exonic splicing signals and non-protein coding RNAs that overlaps with SCCSs or non-SCCS coding regions. No significant difference is observed in splicing signal density between SCCSs and non-SCCS coding regions, however, the component of non-protein coding RNAs overlapped with SCCSs show difference from those overlapped with non-SCCS regions. This result suggests that non-protein coding RNAs may have some association with SCCSs in mammals.

Contents

Chapter 1

Introduction

The neutral theory of molecular evolution (Kimura 1983; Nei 1987) predicted that synonymous sites of codons are evolving faster than no synonymous sites because of the weaker selective pressure. This is true as a whole, however, synonymous sites also accept constraints in some cases.

Several factors are known to affect on synonymous substitution. One of the well-known factors is the codon bias toward optimum codons. Optimal codons reflect the composition of the genomic tRNA pool. As optimal codons are advantageous for fast and accurate translation, highly expressed or biologically important genes would prefer optimal codons. Changes from an optimum codon to a non-optimal codon will be suppressed in these genes. Because optimal codons are similar among closely related species, highly expressed or important genes tend to have similar codons, therefore synonymous sites will show low substitution. Actually requirement for translational efficiency or accuracy are reported to reduce nucleotide changes through purifying selection (Ikemura 1985; Sharp, Li 1987; Akashi 1994; Kanaya et al. 2001; Akashi 2003). Codon optimization is strong in fast growing organisms, like Escherichia coli or

Saccharomyces cerevisiae, but generally weak in organisms that do not show high growing rates or species of small population size.

Splicing signals embedded in exons (exonic splicing enhancer or silencer) also suppress the synonymous substitution (Parmley, Hurst 2007; Takahashi 2009). In addition, messenger RNAs are targeted by various post-transcriptional modification (Licatalosi, Darnell 2010). RNA editing is one example of post-transcriptional modification, where the target region forms specific RNA secondary structure for recognition (Bhalla et al. 2004). RNA secondary structure is also known to associate with regulation of gene expression (Serganov, Patel 2007). Additionally, recent findings of various non-coding RNAs suggest possibility of interaction between coding and non-coding RNAs.

Other than the above factors, ultra conserved regions (UCRs) found in non-protein coding regions can extend to coding exons. In mammals, UCRs are reported to exist near to or overlap with genes associated with nucleotide binding, transcriptional regulation, RNA recognition motif, zinc finger domain, and homeobox domain (Bejerano et al. 2004; Schattner, Diekhans 2006; Lareau et al. 2007). The similar GO terms are reported to be enriched with low dS genes (Bejerano et al. 2004; Schattner, Diekhans 2006; Lareau et al. 2007). Extensively conserved nucleotide sequences are also found in Hox genes outside of the homeobox domain

(Lin, Ma, Nei 2008). Though the importance of highly conserved regions is assumed from evolutionary conservation, their functions are largely unknown.

In contrast with the suppressive factors mentioned above, GC rich regions are vulnerable to mutation through cytosine methylation. Cytosine methylation in vertebrates targets CpG dinucleotide and convert the cytosine to 5-methyl cytosine (Ticher, Graur 1989; Hurst, Williams 2000). Then 5-methyl cytosine turns into thymine by spontaneous deamination. This process causes transitional mutation from C to T. Cytosine methylation is observed in vertebrates and plants but absent or very weak in fruit flies, nematodes, and yeasts.

Thus, nucleotide conservation in the coding regions is affected by various factors. My hypothesis is that some fraction of the conservation is caused by the existence of regulatory elements within the coding regions. Although splicing signals and codon biases have been well investigated by the preceding studies, not many studies have conducted extensive survey on conserved sequences in coding regions or performed comparison among a wide variety of taxa.

This study focused on local and strong conservation within the coding regions in a wide variety of taxa and assessed potential functionality of the conserved sequences. Analyses on codon and nucleotide composition of conserved sequences revealed different characteristics among the taxa. This suggests the difference of factors that affect on codon conservation.

Additional analyses on exonic splicing signals and non-protein coding RNAs in mammals show little influence of exonic signals and possible contribution of overlapping non-coding RNAs to the local nucleotide conservation in the coding regions.

Chapter 2
Materials and Methods

2.1 Data preparation

I obtained peptide and nucleotide sequences of protein coding genes of six mammalian species (*Homo sapiens, Macaca. mulatta, Mus musculus, Rattus norvegicus, Bos taurus, and Canis familiaris*), four teleost species (*Tetraodon nigroviridis, Takifugu rubripes, Gasterosteus aculeatus, Oryzias latipes*), three dicot species (*Arabidopsis thariana, A. lyrata, Vitis vinifera*), and four monocot species (*0ryza sativa japonica, O. s. indica, Sorghum bicolor, Brachypodium distachyon*) from the Ensembl database (http://uswest.ensembl.org/index.html), six Drosophila species (*Drosophila melanogaster, D. simulans, D. sechellia, D. yakuba, D. erecta, D. ananassae*) from FlyBase (http://flybase.org/), four nematode species (*Caenorhabditis elegans, C. briggsae, C. remanei, C. japonica*) from Wormbase (http://www.wormbase.org:80/), and four budding yeasts (*Saccharomyces cerevisiae, S. paradoxus, S. bayanus, S. mikatae*) from Saccharomyces Genome Database (http://www.yeastgenome.org/). Phylogenies of the species are shown in Figure 3-1. These trees were drawn based on the averaged branch lengths of the all gene trees estimated by codeML.

Orthology information of each taxon is also obtained from the corresponding databases. I eliminated one to many and many to many type orthologs and selected 10,790, 11,604, 9,328, 7,102, 3,297, 6,647, and 11,754 single copy ortholog sets for mammals, teleosts, Drosophilas, nematodes, budding yeasts, dicots, and monocots, respectively. First, multiple alignments of peptide sequences are constructed using ClustalW (Thompson, Higgins, Gibson 1994), and nucleotide alignments are constructed based on the peptide alignments. From the nucleotide multiple alignments I extracted sequences that are invariant for 9 nucleotides (3 codons) or longer. Gene and sequence data were stored and managed in a database constructed by MySQL software package.

2.2 Identification of significantly conserved coding sequences (SCCSs)

I performed permutation simulation to identify significantly conserved coding sequences (SCCSs). In this process, I narrowed down the targeted to ortholog sets that contain invariant sequences longer than 30 nucleotides (2,309 ortholog sets containing 4,575 SCCSs in mammals). This is to confine the run time required for the statistical correction within a feasible number. For an N-codon long alignment, I generated a non-redundant series of random numbers from 1 to N and permuted the codon sites according to the random numbers. Gap sites are fixed and the rest of the sites are permuted. Then the length and numbers of invariant sequences in the permuted alignment is surveyed and stored in the memory. This process was repeated 500,000 times per ortholog set and the result gives a distribution of the length and relative frequency of invariant sequences. The p-value of an invariant sequence in the original alignment is evaluated based on the distribution predicted for that alignment. This approach helps identify sequences whose conservation is rare to occur in the substitution background of each alignment. Multiple testing correction of the p-values is done by FDR (False Discovery Rate)(Benjamini et al. 2001). Then I identified invariant sequences with $p < 0.01$ as significantly conserved coding sequences (SCCSs).

2.3 Analysis on codons and GC content

For each SCCS, I calculated preferred codon ratio, GC content and average codon degeneracy. A preferred codon here refers to the most frequently used codons for a given amino acid. Preferred codons are determined according to codon usage tables provided by Kazusa DNA Research Institute (http://www.kazusa.or.jp/e/index.html). Because the codon usage is similar among the species in a taxon, codon tables of *H. sapiens, T. nigroviridis, D. melanogaster, C. elegans, A. thaliana, O. s. japonica*, and *S. cerevisiae* are used as representatives.

2.4 GO term enrichment

For mammals, Drosophilas, nematodes, dicots, monocots, and budding yeasts, we used Fatigo web service (http://babelomics.bioinfo.cipf.es/functional.html) to identify gene ontology (GO) terms that are significantly enriched with genes that contain SCCSs (SCCS genes) compared to genes that do not (non-SCCS genes). Fatigo accepts a list of Ensembl gene IDs as input and provides p-values for enrichment of a GO term in the gene group. The p-values are calculated by Fisher's exact test and corrected by FDR (false discovery rate). I used Ensembl gene IDs of *H. sapiens*, *D. melanogaster*, *C. elegans*, *A. thaliana, O. s. japonica*, and *S. cerevisiae* as input. Because Fatigo does not deal with the teleost and monocot species I investigated, I performed the same procedure as Fatigo, i.e. Fisher's exact test and FDR correction by software package R (Ihaka 1996), to GO terms of *T. nigroviridis* and *O. sativa japonica*,

2.5 Prediction of RNA secondary structures

I computationally predicted secondary structures and free folding energy of SCCSs using Vienna RNA software package (Hofacker 2009) (http://www.tbi.univie.ac.at/~ivo/RNA/). Because folding free energy varies depending on the sequence length, I constructed free energy distribution by 1000 randomly chosen sequences for each length (12 to 246 nucleotides). The p-value for a given free energy was evaluated based on these distributions. Multi testing correction for the p-values is done by FDR.

2.6 Evaluation of exonic splicing enhancers

As for mammals, I obtained 238 hexamers from RESCUE-ESE Web Server (Fairbrother et al. 2002) as candidates of exonic splicing enhancers. I counted the number of these hexamers in SCCS genes and non-SCCS genes, as well as the total nucleotide numbers of the both regions. The hexamer counting allows overlaps. Then I applied the Fisher's exact test to the obtained numbers.

2.7 Exploration for overlaps between non-coding RNAs and SCCSs

I obtained coordinate information of non-coding RNAs in the human and mouse genome from the Functional RNA Database (Mituyama et al. 2009) (http://www.ncrna.org/). This database also provides a list of non-coding RNAs that overlaps with protein coding regions. Based on these information, I identified the types and numbers of non-coding RNAs that overlap with SCCSs or non-SCCS coding regions.

2.8 Analysis on gene expression

We referred to EGenetics (http://www.nhmrc.gov.au/your_health/egenetics/index.htm) to investigate gene expression of SCCS and non-SCCS genes. Human anatomical system data, which give information about in what organs a gene is expressed, were obtained from EGenetics database by way of Ensemble Biomart. For each organ we counted how many of SCCS genes or non-SCCS genes are expressed. Then we performed the Fisher's exact test to evaluate the difference. All p-values were corrected by FDR.

Chapter 3

Results and Discussion

3.1 Different characteristics of conserved sequences in the coding regions of seven taxa

3.1.1 Identification of significantly conserved coding sequences (SCCSs)

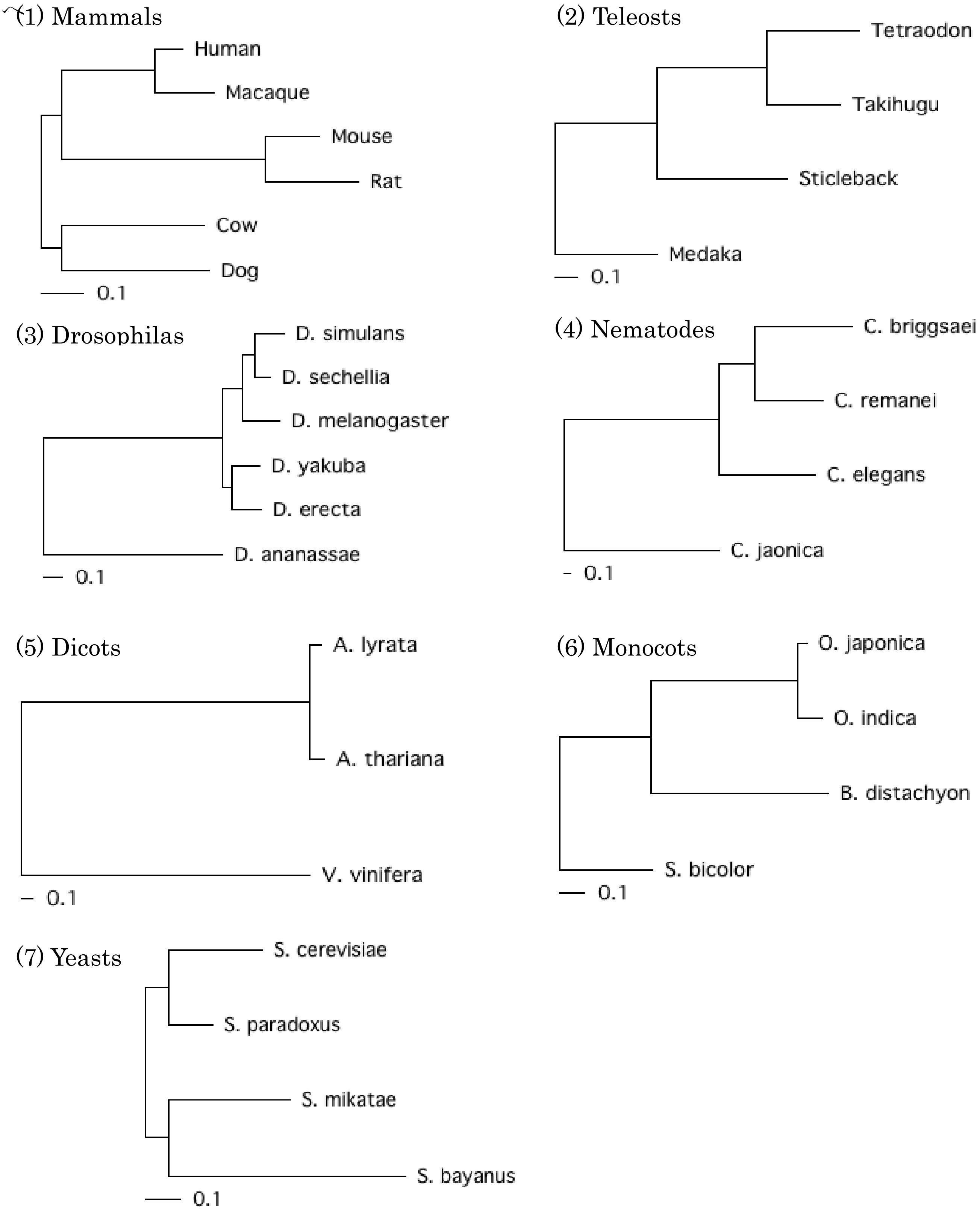
I selected single copy orthologs of the group of species (Figure 3-1) and constructed multiple alignments by ClustalW. Then I extracted nucleotide sequences invariant among the species. Nucleotide substitution ratio varies among the taxa reflecting the divergence of their member species. Difference of substitution ratio also exists among genes. This affects on the length and number of invariant sequences found in the alignments. For example, orthologous genes that are highly conserved in all the species would have more invariant sequences.

In consideration of these issues, I used permutation test to identify significantly conserved nucleotide sequences (SCCSs). First, I focused on invariant sequences longer than 30 nucleotides for mammals, 24, 27, 12, 21, 27 and 15 nucleotides for teleosts, Drosophilas, nematodes, dicots, monocots, and budding yeasts, respectively. These numbers were chosen to make permutation simulation in the next step to complete in a feasible computational time. Next, the distribution of the length and frequency of invariant sequences is constructed for each alignment by 500,000 runs of permutation for mammals, 300,000, 450,000, 300,000, 250,000, 400,000 and 300,000 runs for teleosts, Drosophilas, nematodes, dicots, monocots, and budding yeasts, respectively. The number of run is decided depending on the number of invariant sequences we focused on.

In mammals, teleosts, drosophilas, and monocots, the result of permutation show notably smaller number of invariant sequences compared with the invariant sequences in the original alignments (Figure 3-2). The difference between the permutation result and observed number increases as the conservation length elongates. In nematodes and dicots, the expected number is slightly lower than the observation. In yeasts, the observation and the permutation result correspond fairly well.

Figure 3-1. Phylogenic trees of the species used in this study

Concatenating gene trees constructed by codeML. The root is placed in the middle point of the outmost branch. The scales indices nucleotide distances.



(1) Mammals

(2) Teleosts

(3) Drosophilas

(4) Nematodes

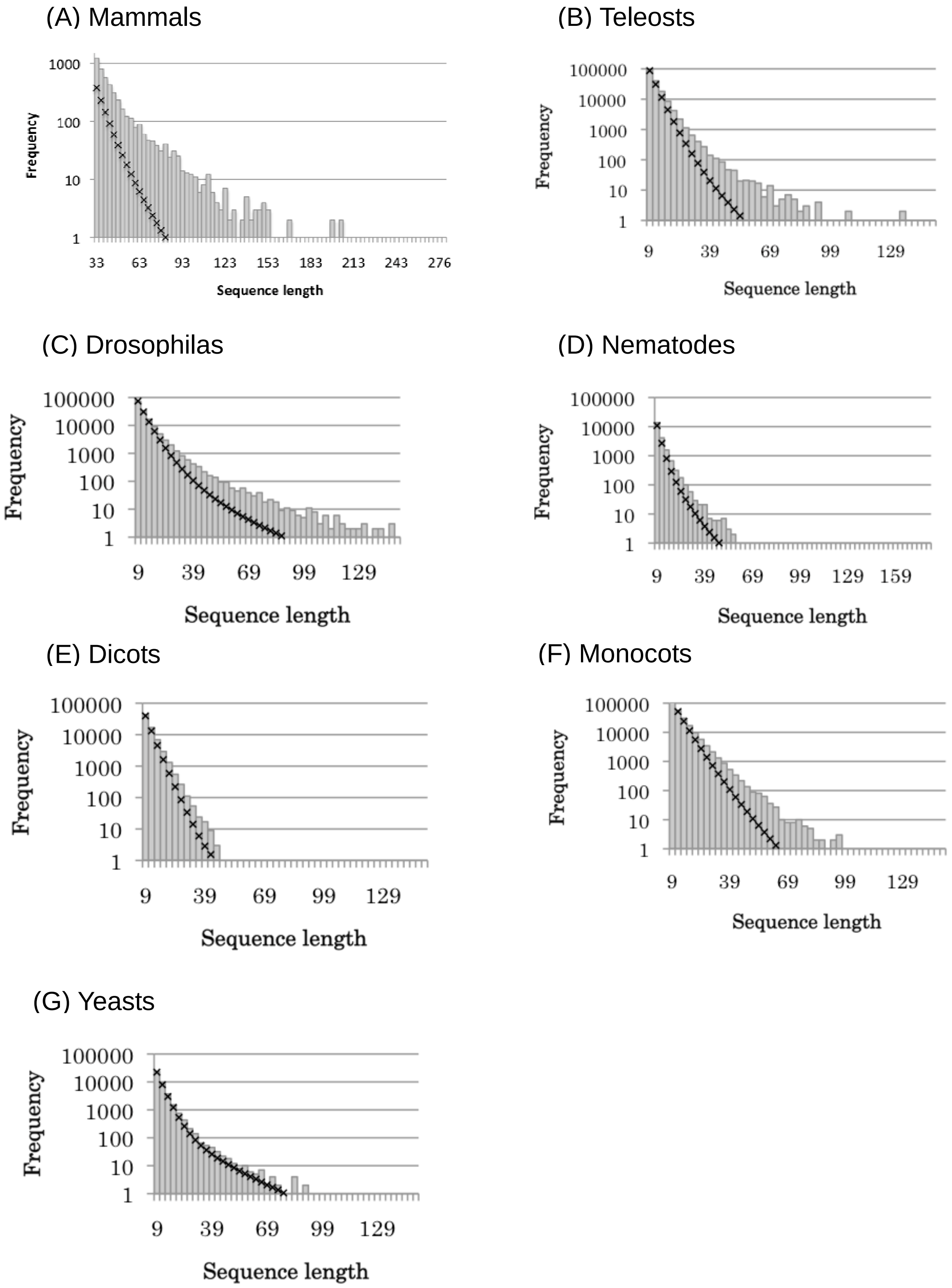
(5) Dicots

(6) Monocots

(7) Yeasts

Figure 3-2. Number of invariant sequences in original alignments and permuted alignments

X and Y-axes represent the length and frequency of invariant sequences, respectively. Gray bars are observed invariant sequences in the original alignment and black crosses are those obtained from the permutation simulation.



(A) Mammals

(B) Teleosts

(C) Drosophilas

(D) Nematodes

(E) Dicots

(F) Monocots

(G) Yeasts

3.1.2 Length and number of SCCSs

After the extraction of invariant sequences and permutation simulation, the p values of invariant sequences were determined based on the probability distributions constructed from the permutation results. All the p-values are adjusted by FDR. I extracted invariant sequences of $p < 0.01$ as significantly conserved coding sequences (SCCSs). Table 3-1 shows the numbers of SCCSs and number of genes that contain SCCSs (SCCS genes). The full list is shown in Appendix Table A2. The numbers of SCCSs in the yeast group is small because the difference between the observed number of invariant sequences and the result of permutation is small.

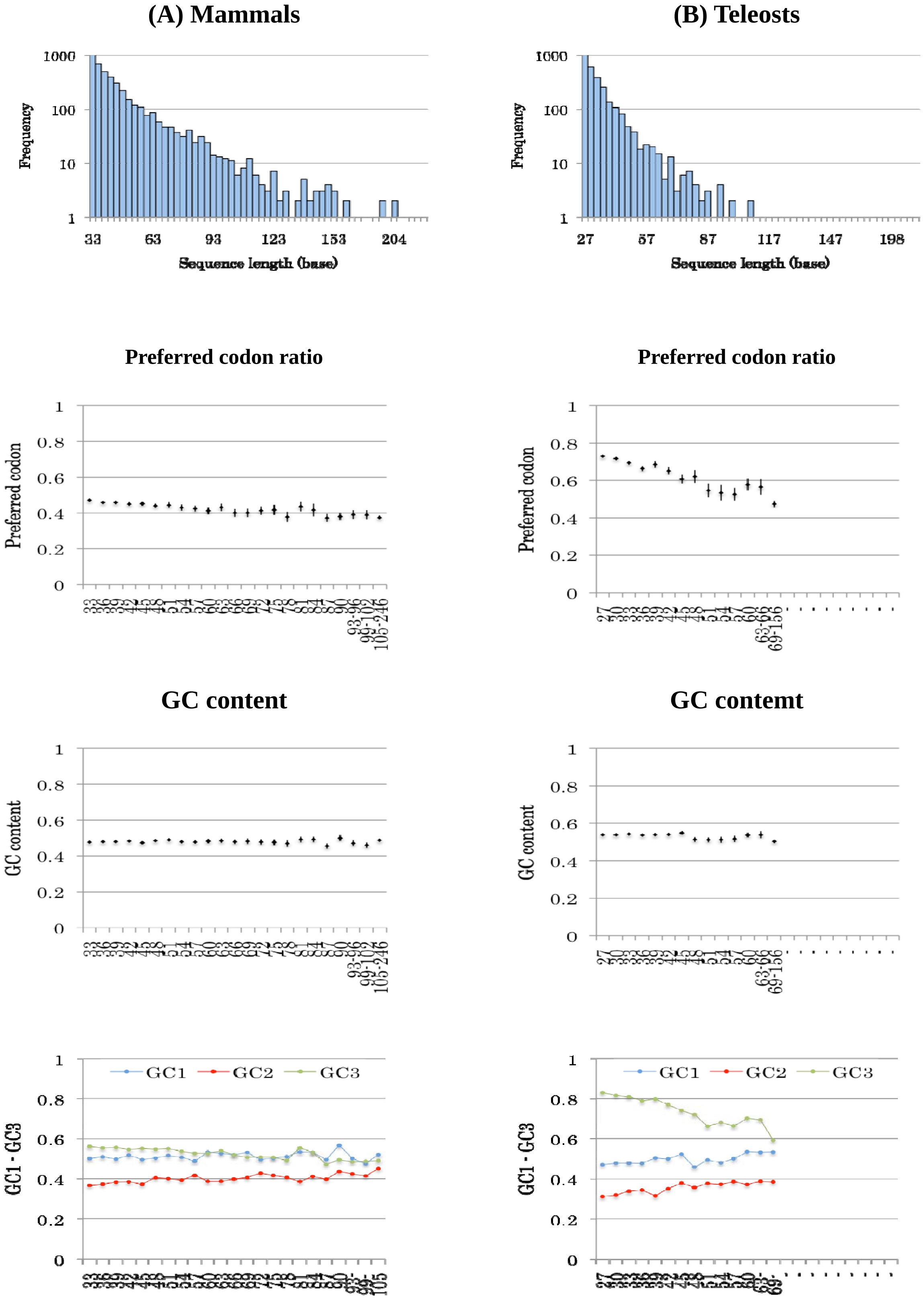
The bar charts of Figure 3-3 show lengths and numbers of SCCSs in each taxon. The length and number of SCCSs are influenced by divergence of the member species. The evolutionary distances among the four nematode species and the three dicot species are larger than the other taxa. Consequently, the number of SCCSs obtained from nematodes and dicots are smaller and the length is shorter than others.

Table 3-1. Number of SCCSs and genes that contain SCCSs

Taxa	Species	Number of orthologous gene sets	Number of orthologous gene sets that contain SCCSs	Number of SCCSs
Mammals	Human, Macaque, Mouse, Rat, Cow, Dog	10,790	2,273	4,150
Teleosts	Tetraodon, Takifugu, Stickleback, Medaka	11,604	1,962	2,843
Drosophila	*Drosophila melanogaster, D. simulans*, *D. sechellia, D. yakuba, D. erecta, D. ananassae*	9,328	1,953	3,662
Caenorhabditis	*Caenorhabditis elegans, C. briggsae, C. remanei, C. japonica*	7,102	1,299	1,899
Dicots	*Arabidopsis thariana, A. lyrata, Vitis vinifera*	6,647	1,566	2,260
Monocots	*0ryza sativa japonica, O. s. indica, Sorghum bicolor, Brachypodium distachyon*	11,754	2,724	4,431
Saccharomyces	*Saccharomyces cerevisiae, S. paradoxus, S. bayanus, S. mikatae*	3,297	1,131	1,575

Figure 3-3. The preferred codon ratio and GC content of SCCSs

The blue bars in the top figures indicate the length and frequency of SCCSs. The second and the third rows show preferred codon ratio and GC content of SCCSs. The bottom figures show GC contents of the first position (GC1) to the third position of (GC3) codons. Classes whose sample size < 20 are integrated. Error bars represent 1 SE.



(A) Mammals

(B) Teleosts

Preferred codon ratio

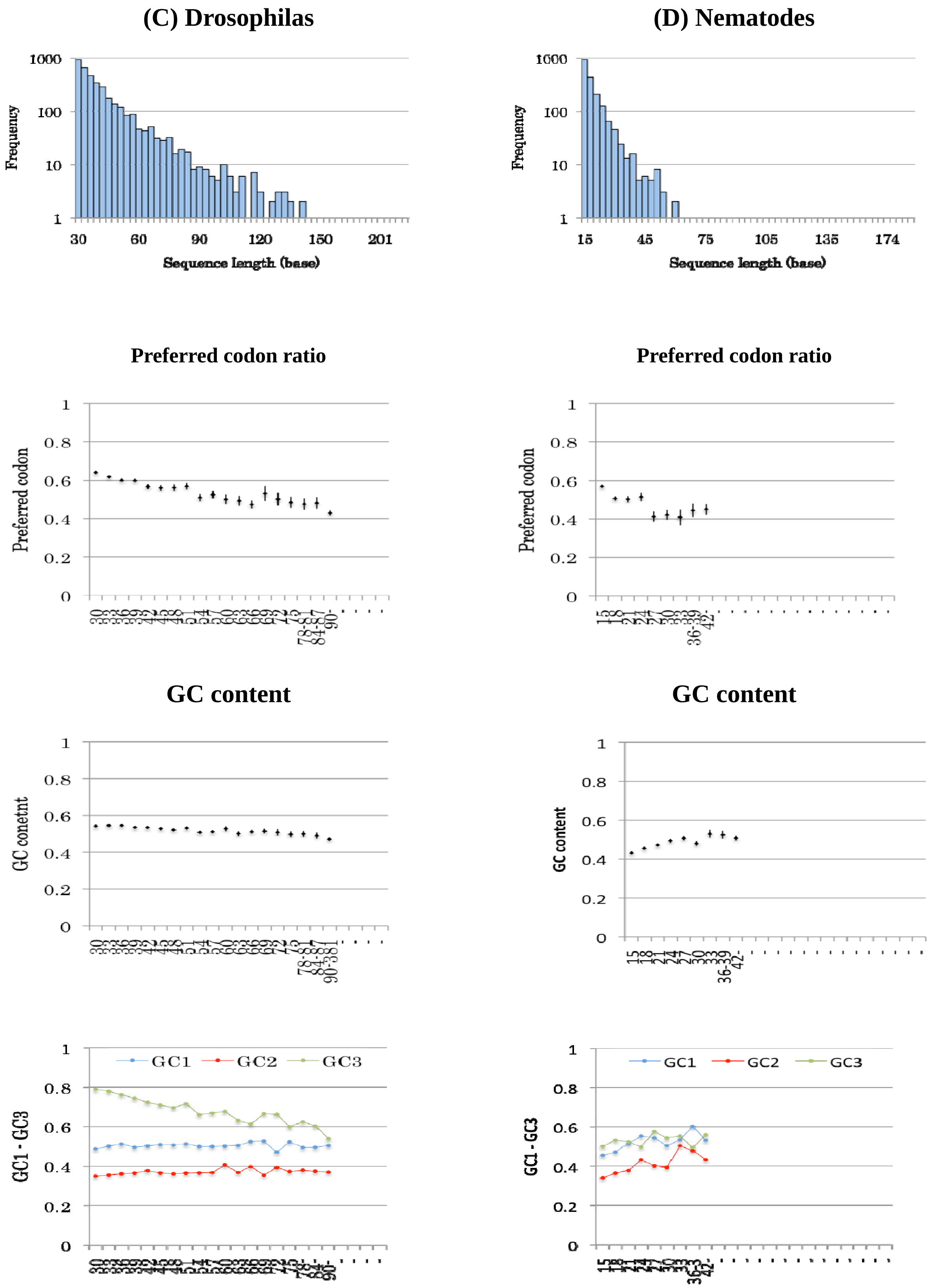
Preferred codon ratio

GC content

GC contemt

Figure 3-3. The preferred codon ratio and GC content of SCCSs (continued)

.



(C) Drosophilas

(D) Nematodes

Preferred codon ratio

Preferred codon ratio

GC content

GC content

Figure 3-3. The preferred codon ratio and GC content of SCCSs (continued)

.

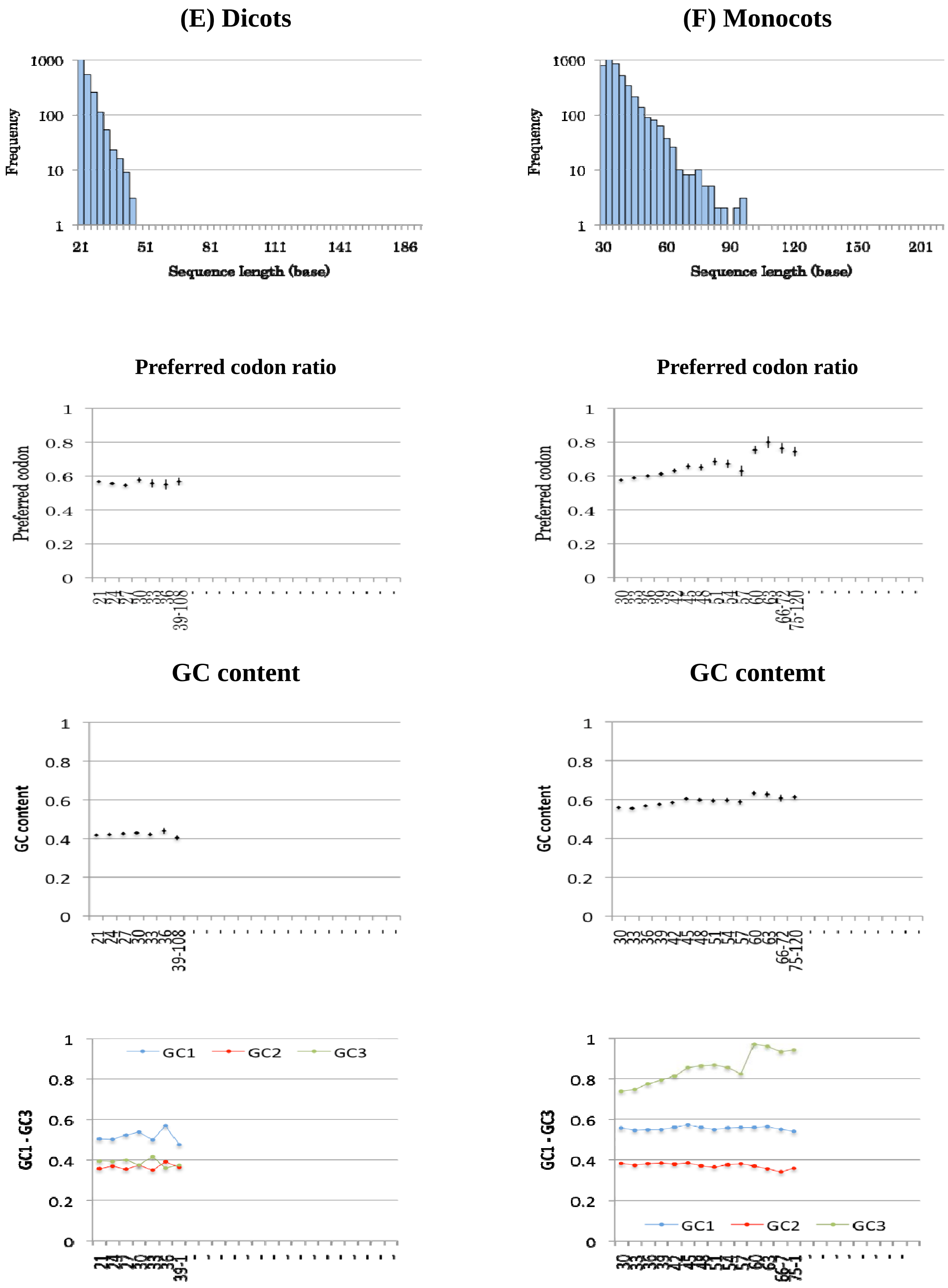
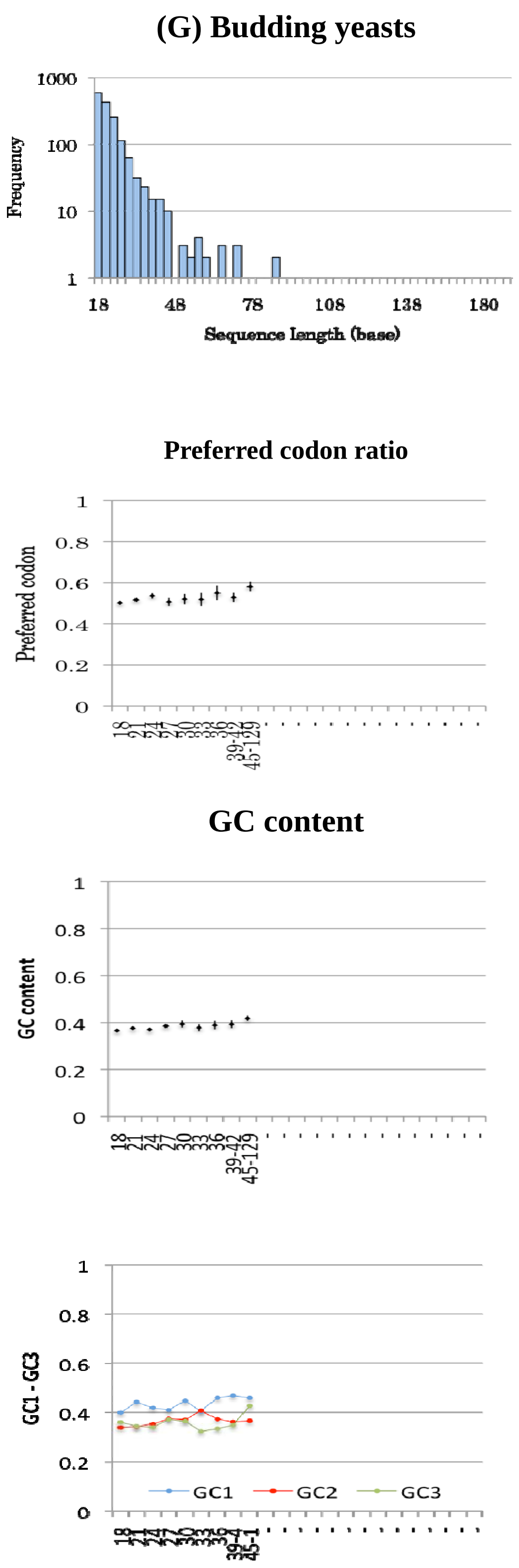


Figure 3-3. The preferred codon ratio and GC content of SCCSs (continued)

.



(G) Budding yeasts

Preferred codon ratio

GC content

3.1.3 The preferred codon ratio, GC content, and codon degeneracy of SCCSs

Codon usage biases toward optimum codons are known to suppress synonymous substitution. Optimal codons reflect the composition of the genomic tRNA pool and advantageous for translation efficiency or accuracy. I used preferred codons, or most frequently used codon for an amino acid, as approximate index of optimum codons.

In the mammals, teleosts, Drosophilas and nematodes, there is tendency that the preferred codon ratio (PC ratio) decreases as the length of SCCS increases. The dicots show constant PC ratio independent of sequence lengths, while the Monocots and budding yeast show increase of PC ratio.

Because codon usage is associated with the genomic nucleotide composition, I also investigated GC content. The GC content shows slight decrease along SCCS length drosophilas, increase in nematodes, monocots and budding yeasts. Although the overall GC plot seems to be flat, GC content in the first (GC1), second (GC2) and third position (GC3) of codons varies greatly. Because most of preferred codons of mammals, teleosts, Drosophilas, and monocots are GC-ending, the decrease or increase of the preferred codons in these taxa are mainly attributed to the GC3. On the other hand, nematodes, dicots, and budding yeasts prefer AT-ending codons. The decrease of preferred codons in nematodes is therefore due to the increase of GC content, but in this case, not only the GC3 but also the GC1 and GC2. It’s notable that in mammals, teleosts, and Drosophilas, the decrease of GC3 seems to be partly cancelled out by the increase of GC1 and GC2 and they come closer as the conservation length elongates.

A precedent study investigated correlation between dS and fraction of optimal codons (F_{op}) and detected negative correlations between dS and F_{op} in rodents (*M. musculus* and *R. norvegicus*), Drosophilas (*D. melanogaster* and *D. yakuba*), nematodes (*C. elegans* and *C. briggsae*), budding yeasts (*S. cerevisiae* and *S. paradoxus*) and bacteria (*E. coli* and *S. typhimurium*) and positive correlation in human/dog (*H. sapiens* and *C. familiaris*), though the correlation in rodents and human/dog seems subtle (Drummond, Wilke 2008). If the SCCSs have the same trend as the dS of this study, preferred codon ratio of longer SCCSs would increase in mice, Drosophilas, nematodes, budding yeasts and decrease in human/dog. My results agree with this prediction in the budding yeast but not in other taxa. The methodological difference is that my research focused on local and complete conservation instead of the dS in the entire gene, and investigated conservation among three to six species instead of pair wise comparison. The difference of results may suggest that factors working on SCCSs differ from the factors working on the global conservation.

Judging from the decrease of preferred codons, the long conserved sequences of the four animal taxa (mammals, teleosts, Drosophilas, and nematodes) are not likely being retained by codon biases toward optimum codons.

3.1.4 Codon degeneracy of the invariant SCCSs

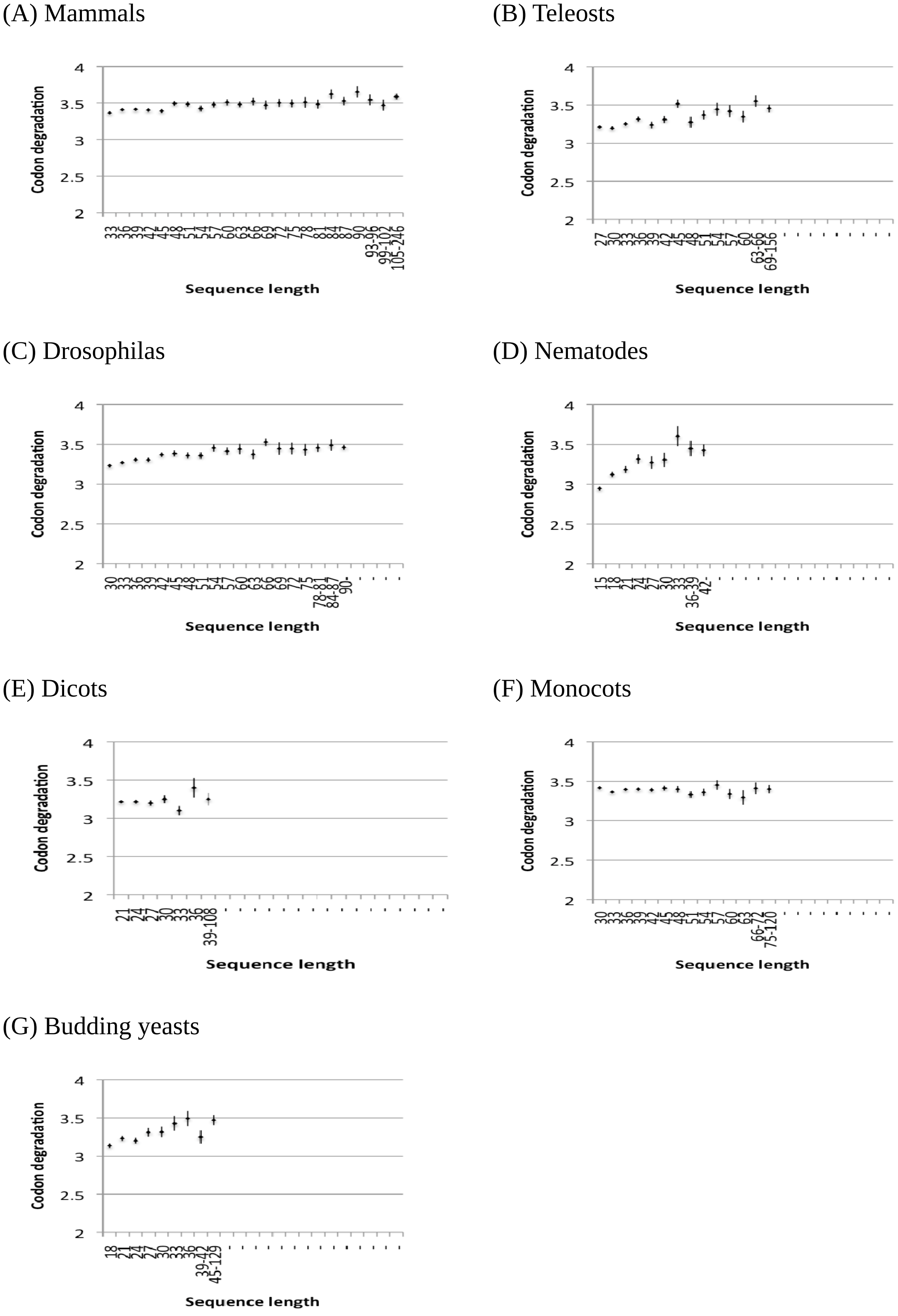
Though the codon bias toward optimum codon seems not to be the major factor for retaining long nucleotide conservation in the animal taxa, such conservation may occur by chance where amino acid constraint is strong and codon degeneracy is low.

To examine this possibility, I investigated codon degeneracy of SCCSs (Figure 3-4). The averaged degeneracy is constant or slightly increases along the sequence length. In most cases, the average codon degeneracy is between three and four. Judging from this degree of degeneracy, the probability is low for a long SCCS to be conserved due to amino acid constraint.

Makalowski and Boguski (1998) showed a correlation between synonymous substitution rate (dS) and non-synonymous substitution rate (dN). Such correlation may occur when the constraint on a certain nucleotide sequence is so strong that dN is also lowered.

Figure 3-4. Codon degeneracy of SCCSs

X-axis represents the length of SCCS and Y-axis represents the averaged codon degeneracy or the sequences. Error bars represent 1 SE.



(A) Mammals

(B) Teleosts

(C) Drosophilas

(D) Nematodes

(E) Dicots

(F) Monocots

(G) Budding yeasts

3.1.5 GO terms enriched with genes that contain SCCSs

I explored GO terms significantly ($p < 0.01$) enriched in SCCS genes compared with non-SCCS genes (Table 3-2). Terms with the lowest ten probabilities are shown where there are more than 10 significant terms (full list is shown in Appendix 1). There was no significant GO term for nematodes.

The terms DNA, nucleotide, or nucleoside binding are commonly observed in the all taxa. The terms related with transcription and protein kinase activity are notable in mammals, teleosts, Drosophilas, and monocts. In plants (dicots and monocots) and budding yeasts, ATP binding and ATPase activity are ranked high. SCCS genes of mammals show close association with neurons and dendrites compared to other taxa.

Preceding studies report that low dS genes or genes that reside near or overlap with ultraconserved elements of mammals and the chicken are enriched with these terms (Bejerano et al. 2004; Schattner, Diekhans 2006). As for the mammalian SCCSs, twenty-eight of them overlap with the ultraconserved elements (Appendix 2).

Table 3-2. GO terms significantly (P<0.01) enriched with genes that contain SCCSs

(A) Mammals

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
Biological process				
GO:0045941	Positive regulation of transcription	7.48	2.86	1.29E-17
GO:0010628	Positive regulation of gene expression	7.56	2.97	3.52E-17
GO:0048699	Generation of neurons	6.99	2.86	9.77E-15
GO:0009790	Embryo development	7.17	3.01	1.24E-14
GO:0022008	Neurogenesis	7.34	3.14	1.89E-14
GO:0030182	Neuron differentiation	6.38	2.54	2.59E-14
GO:0031328	Positive regulation of cellular biosynthetic process	8.09	3.7	3.38E-14
GO:0006325	Chromatin organization	4.57	1.56	3.88E-13
GO:0009952	Anterior/posterior pattern formation	2.51	0.5	6.11E-13
GO:0009792	Embryo development ending in birth or egg hatching	4.79	1.73	1.05E-12
Cellular components				
GO:0015629	Actin cytoskeleton	3.39	1.8	1.15E-03
GO:0043005	Neuron projection	4.09	2.32	1.15E-03
GO:0043025	Neuronal cell body	2.24	1.11	2.38E-03
GO:0030425	Dendrite	2.29	1.1	2.38E-03
GO:0043198	Dendritic shaft	0.48	0.06	2.38E-03
GO:0014704	Intercalated disc	0.35	0.04	8.43E-03
Molecular function				
GO:0043565	Sequence-specific DNA binding	7.26	2.84	9.44E-17
GO:0003723	RNA binding	6.07	2.69	6.50E-11
GO:0050825	Ice binding	2.86	0.76	6.50E-11
GO:0003702	RNA polymerase II transcription factor activity	3.03	0.94	1.55E-09
GO:0016563	Transcription activator activity	4.84	2.09	3.01E-09
GO:0008134	Transcription factor binding	5.41	2.48	3.68E-09
GO:0016564	Transcription repressor activity	3.65	1.37	5.36E-09
GO:0003682	Chromatin binding	2.15	0.59	4.08E-08
GO:0003712	Transcription cofactor activity	3.87	1.61	4.08E-08
GO:0004674	Protein serine/threonine kinase activity	6.02	3.24	5.51E-07

Note. The columns '% in SCCS containing genes' and '% in non-SCCS containing genes' represent percentages of genes that are labeled with the GO term in each gene group. P indicates probability for enrichment of the GO term in SCCS containing genes. Terms with the lowest ten probabilities are shown for Biological Process and Molecular function.

Table 3-2 (continued)

(B) Teleosts

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
Biological process				
GO:0006355	Regulation of transcription, DNA -dependent	12.24	5.42	2.13E-16
GO:0006811	Ion transport	3.70	1.68	4.50E-05
GO:0006468	Protein amino acid phosphorylation	7.92	4.72	9.20E-05
GO:0006816	Calcium ion transport	1.71	0.56	2.61E-04
GO:0051056	Regulation of small GTPase mediated signal transduction	1.13	0.30	9.94E-04
Cellular components				
GO:0005634	Nucleus	19.27	10.31	1.35E-14
GO:0005891	Voltage-gated calcium channel complex	0.72	0.06	4.50E-05
GO:0005622	Intracellular	16.23	11.35	2.74E-04
Molecular function				
GO:0003700	Transcription factor activity	9.43	3.20	4.74E-21
GO:0043565	Sequence-specific DNA binding	7.39	2.25	8.45E-20
GO:0003677	DNA binding	10.10	4.66	1.02E-12
GO:0008270	Zinc ion binding	16.16	8.95	1.55E-11
GO:0004672	Protein kinase activity	6.92	3.22	9.14E-09
GO:0004713	Protein tyrosine kinase activity	6.05	2.76	3.78E-08
GO:0005524	ATP binding	14.27	8.52	5.78E-08
GO:0003676	Nucleic acid binding	9.67	5.33	1.72E-07
GO:0005216	Ion channel activity	3.54	1.28	1.72E-07
GO:0004674	Protein serine/threonine kinase activity	6.28	3.04	1.99E-07

Note. Terms with the lowest ten probabilities are shown for Molecular function.

Table 3-2 (continued)

(C) Drosophilas

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
Biological process				
GO:0030154	Cell differentiation	8.85	8.85	7.89E-05
GO:0044267	Cellular protein metabolic process	11.98	11.98	7.89E-05
GO:0006464	Protein modification process	7.06	7.06	4.28E-04
GO:0016070	RNA metabolic process	10.64	10.64	4.28E-04
GO:0007275	Multicellular organismal development	13.61	13.61	4.53E-04
GO:0043687	Post-translational protein modification	6.04	6.04	4.53E-04
GO:0006355	Regulation of transcription, DNA-dependent	7.47	7.47	1.77E-03
GO:0009059	Macromolecule biosynthetic process	12.49	12.49	1.77E-03
GO:0006350	Transcription	8.65	8.65	1.79E-03
GO:0007166	Cell surface receptor linked signaling pathway	7.27	7.27	1.79E-03
Cellular components				
GO:0016021	Integral to membrane	12.49	8.55	3.37E-05
GO:0031224	Intrinsic to membrane	12.59	8.76	4.25E-05
Molecular function				
GO:0008270	Zinc ion binding	10.85	6.89	1.00E-05
GO:0046914	Transition metal ion binding	13.05	8.92	2.88E-05
GO:0017076	Purine nucleotide binding	9.72	6.76	2.70E-03
GO:0000166	Nucleotide binding	11.26	8.26	4.18E-03
GO:0004672	Protein kinase activity	3.38	1.8	4.18E-03
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	4.4	2.59	4.18E-03
GO:0004674	Protein serine/threonine kinase activity	2.87	1.47	5.32E-03
GO:0016772	Transferase activity, transferring phosphorus-containing groups	5.37	3.46	9.72E-03

Note. Terms with the lowest ten probabilities are shown for Biological process.

Table 3-2 (continued)

(D) Dicots

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
Biological process				
GO:0005975	Carbohydrate metabolic process	7.91	3.74	1.22E-07
GO:0044262	Cellular carbohydrate metabolic process	4.66	1.68	1.52E-07
GO:0007275	Multicellular organismal development	12.64	7.66	1.35E-06
GO:0006810	Transport	14.17	9.2	8.32E-06
GO:0009791	Post-embryonic development	7.91	4.52	7.15E-05
GO:0007017	Microtubule-based process	1.85	0.47	1.38E-04
GO:0048513	Organ development	5.49	2.81	1.38E-04
GO:0051641	Cellular localization	4.91	2.51	3.80E-04
GO:0009165	Nucleotide biosynthetic process	1.98	0.62	5.04E-04
GO:0007018	Microtubule-based movement	1.28	0.27	5.93E-04
Cellular components				
GO:0043234	Protein complex	11.17	6.28	7.93E-08
GO:0015630	Microtubule cytoskeleton	2.3	0.55	9.89E-07
GO:0005886	Plasma membrane	12.32	7.64	1.29E-06
GO:0005856	Cytoskeleton	3.19	1.11	3.84E-06
GO:0005794	Golgi apparatus	3.13	1.46	9.90E-04
GO:0016021	Integral to membrane	11.87	8.42	1.03E-03
GO:0000325	Plant-type vacuole	0.89	0.16	1.25E-03
GO:0005773	Vacuole	3.89	2.07	1.38E-03
GO:0031224	Intrinsic to membrane	12.38	9.34	6.81E-03
GO:0034707	Chloride channel complex	0.32	0	7.24E-03
Molecular function				
GO:0017076	Purine Nucleotide Binding	22.34	9.88	2.03E-31
GO:0001882	Nucleoside binding	20.49	9.02	6.13E-29
GO:0005524	ATP Binding	19.02	8.4	1.68E-26
GO:0017111	Nucleoside-triphosphatase Activity	9.7	3.12	3.22E-21
GO:0016462	Pyrophosphatase activity	9.89	3.45	2.36E-19
GO:0016818	Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	9.96	3.53	4.25E-19
GO:0016787	Hydrolase activity	21.57	13.21	4.53E-13
GO:0016887	ATPase activity	4.72	1.64	5.08E-09
GO:0016740	Transferase activity	18.83	12.63	1.17E-07
GO:0004386	Helicase activity	2.49	0.68	1.73E-06

Note. Terms with the lowest ten probabilities are shown for Biological Process and Molecular function.

(E) Monocots

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
Biological process				
GO:0006810	Transport	5.66	3.03	3.13E-06
GO:0008152	Metabolic process	9.35	6.00	3.15E-05
GO:0006812	Cation transport	0.89	0.20	3.46E-04
GO:0006468	Protein amino acid phosphorylation	7.24	4.72	6.45E-04
GO:0006350	Transcription	4.25	2.45	8.74E-04
GO:0045449	Regulation of transcription	7.16	4.72	1.04E-03
GO:0030244	Cellulose biosynthetic process	0.41	0.04	4.53E-03
GO:0006355	Regulation of transcription, DNA-dependent	5.99	4.04	6.18E-03
GO:0007047	Cellular cell wall organization	1.00	0.34	6.18E-03
Cellular component				
GO:0016020	Membrane	14.50	7.83	1.05E-14
GO:0016021	Integral to membrane	11.73	6.81	1.21E-09
GO:0005634	Nucleus	11.55	8.71	9.21E-03
Molecular function				
GO:0005524	ATP binding	18.08	9.98	4.84E-16
GO:0000166	Nucleotide binding	14.60	7.75	1.73E-15
GO:0003824	Catalytic activity	10.91	7.09	1.17E-05
GO:0017111	Nucleoside-triphosphatase activity	3.22	1.45	1.17E-05
GO:0004713	Protein tyrosine kinase activity	6.99	4.41	3.28E-04
GO:0004674	Protein serine/threonine kinase activity	7.20	4.59	3.37E-04
GO:0004672	Protein kinase activity	7.12	4.55	3.43E-04
GO:0016887	ATPase activity	1.60	0.59	3.43E-04
GO:0016757	Transferase activity, transferring glycosyl groups	2.21	1.01	9.54E-04
GO:0008237	Metallopeptidase activity	0.85	0.22	2.79E-03

Note. Terms with the lowest ten probabilities are shown for Molecular function.

(F) Budding yeasts

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
Cellular components				
GO:0000166	Nucleotide binding	18.46	10.52	1.46E-07
GO:0005524	ATP binding	12.54	6.58	2.92E-06
GO:0017076	Purine nucleotide binding	15.19	8.92	1.12E-05
GO:0042623	ATPase activity, coupled	4.86	2.11	1.88E-03
GO:0004713	Protein tyrosine kinase activity	3.09	1.03	2.82E-03
GO:0016887	ATPase activity	5.65	2.72	2.82E-03
GO:0017111	Nucleoside-triphosphatase activity	8.3	4.79	4.09E-03

3.1.7 SCCSs that form stable RNA secondary structures

There are cases that a secondary structure of mRNA conveys functions (Delgado et al. 1998; Bhalla et al. 2004). I examined secondary structures and free energy of the SCCSs using Vienna RNA package. There are 5 SCCSs whose local folding energy is significantly low (Table 3-3).

Cpt2 encodes a nuclear protein that is transported to the mitochondrial inner membrane. Together with carnitine palmitoyltransferase I, the encoded protein oxidizes long-chain fatty acids in the mitochondria. Gal3st3 encodes a member of the galactose-3-O-sulfotransferase protein family. This protein exists on the membrane of Golgi apparatus. Plod3 encodes a membrane-bound homodimeric enzyme that is localized to the cisternae of the rough endoplasmic reticulum. The enzyme (cofactors iron and ascorbate) catalyzes the hydroxylation of lysyl residues in collagen-like peptides. Polg encodes a catalytic subunit of mitochondrial DNA polymerase. POLG protein is the only polymerase known to be involved in replication of mtDNA. Smarcd3 encodes a protein of SWI/SNF family, whose members display helicase and ATPase activities. This protein is thought to regulate transcription of certain genes by altering the chromatin structure around those genes.

Table 3-3. Genes that contain an SCCS with significantly low free folding energy

(A) Mammals

Gene		Length	Free energy	P
cpt2	Carnitine O-palmitoyltransferase 2	24	-13.4	1E-5
polg	DNA polymerase subunit gamma-1	36	-19.9	3.1E-9
plod3	Lysyl hydroxylase 3	27	-20.9	0
gal3st3	Galactose-3-O-sulfotransferase 3	36	-22.6	0
smarcd3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member	39	-23.9	0

Note. The gene names are represented by those of human.

Figure 3-5 shows probability density of folding free energy constructed by randomly extracted sequences. Each line shows free energy of the sequences of the same length as the above five SCCSs. Gene names on the lines represent free energy of the SCCSs. Note that the lower the free energy, the more stable the structure is. This figure implies the five SCCSs may form stable secondary structures.

RNA secondary structures of the five SCCSs are shown in Figure 3-6. To explore how far in the mammalian lineage the structure is conserved, I examined secondary structures of the corresponding regions in platypus and opossum orthologs. The structure in *smarcd3* is conserved in opossum, though there are two nucleotide differences from the placental mammals. Because of the two-nucleotide difference, the structure of the placental mammals is more stable. This is similar for the structures in *cpt2* and *gal3st3*. Two nucleotide differences from platypus or opossum brought about the stretch of stem structures in the placental mammals.

Figure 3-5. Probability density of folding free energy.

Probability density was created from free energies of randomly extracted sequences (15 to 246 nt) using statistic package R. This graph shows probability density for 24 nt, 27 nt, 36 nt, and 39 nt sequences. The five SCCSs of significantly low energy are indicated on the graph.

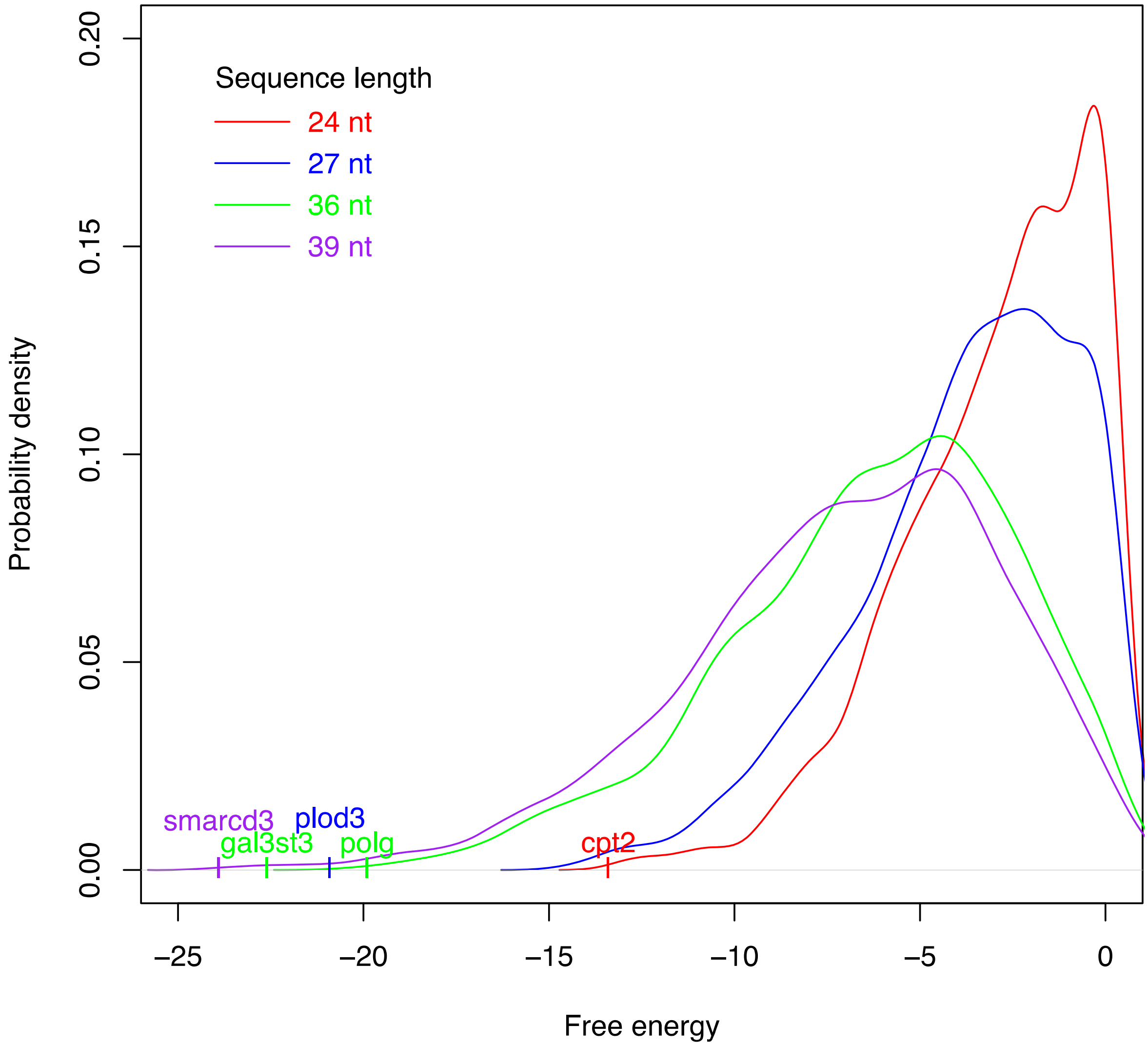


Figure 3-6. RNA secondary structures of SCCSs that have significantly low folding free energy

The red circles indicate the sites where the placental mammals can form more stable base pairs than the non-placental mammals.

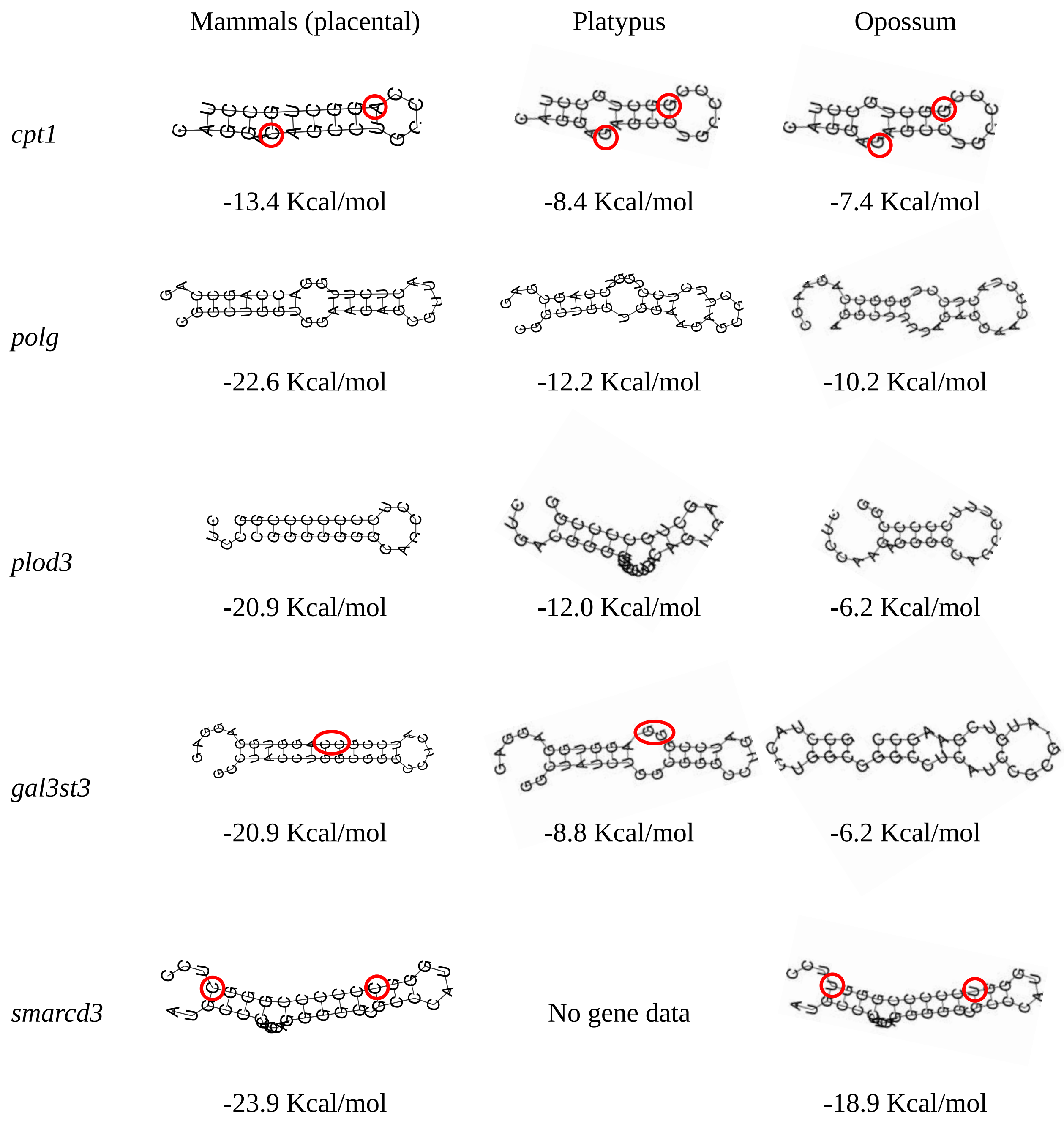


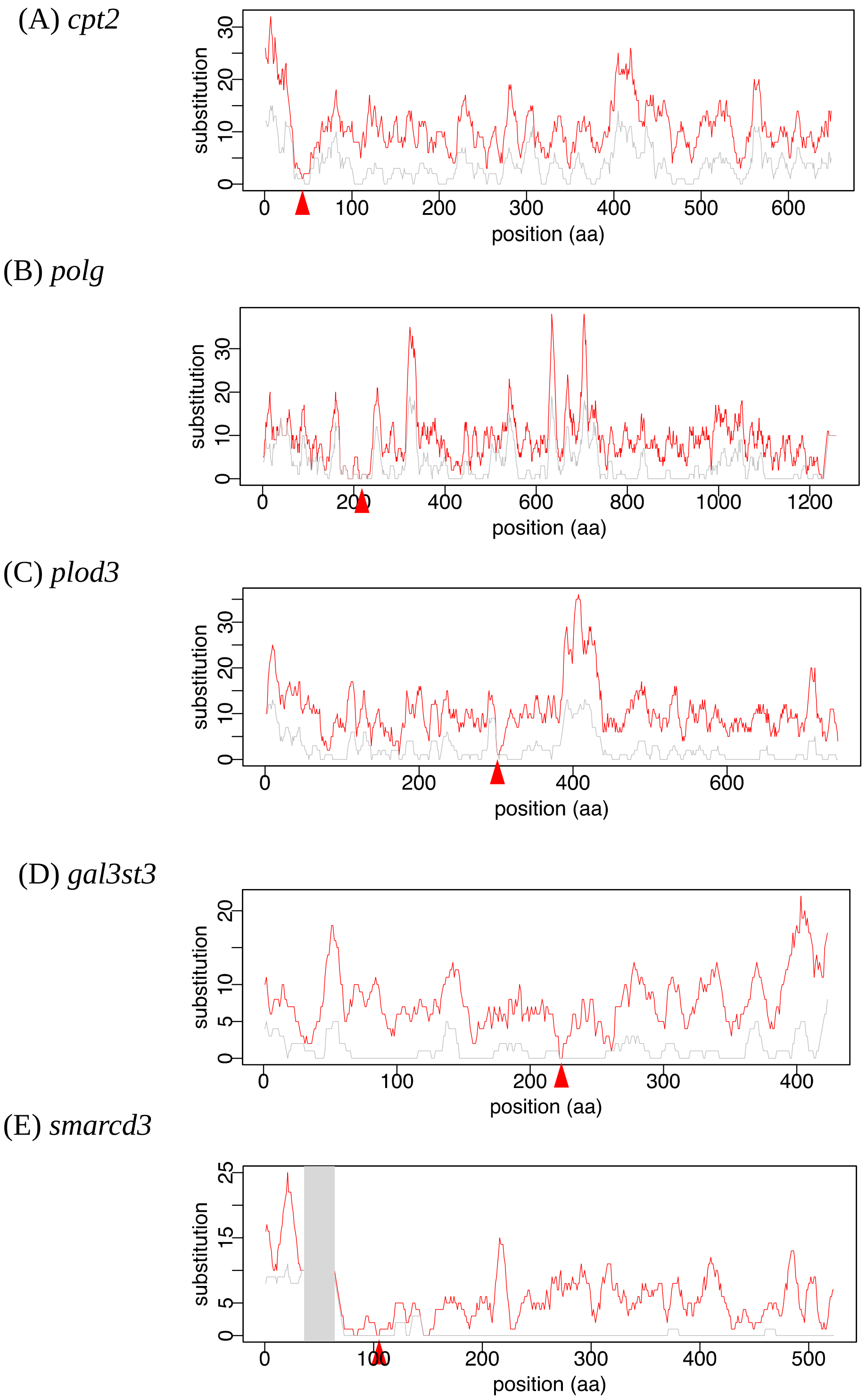
Figure 3-7 shows amino acid substitution and nucleotide substitution in the coding regions of the above five genes. The red triangles at the bottom of the boxes represent SCCSs. The number of substitution is counted parsimoniously in 30-nucleotide sliding windows. The red and gray line plots depict nucleotide and amino acid substitutions, respectively. Because the nucleotide substitution counts include both synonymous and non-synonymous changes, there is correlation between the amino acid substitution and nucleotide substitution, however, nucleotide substitution fluctuate in the regions where there’s no amino acid substitution.

These figures show that nucleotide substitution around the SCCSs is not necessarily low, which indicates that the conservation occurs in a limited area, rather than a part of a low mutation region.

Although there is no reported on the RNA secondary structure of these genes, there is possibility that these structures have some functions judging from the strong conservation and significantly low free energy.

Figure 3-7. Amino acid substitution and nucleotide substitution in the SCCSs

The box represents an alignment. The red and gray line plots indicate nucleotide and amino acid substitution, respectively. The red triangles on the bottom of the boxes represent SCCSs with significantly low free folding energy. The green triangles represent other SCCSs in the same alignment. Alignment gaps are shaded in gray.



(A) *cpt2*

(B) *polg*

(C) *plod3*

(D) *gal3st3*

(E) *smarcd3*

3.2 Additional analysis on conserved nucleotide sequences in the coding regions of mammalian species

3.2.1 The density of exonic splicing enhancers in SCCSs and non-SCCS coding regions

One of the well known functional nucleotide elements in the coding region is splicing signals. We obtained 238 hexamers from RESCUE-ESE Web server as candidates of exonic splicing enhancers, and counted the number of hexamers in SCCSs and the entire protein-coding regions of the human genome (Table 3-5). Then I calculated the density of hexamer and applied chi-square test. The density of hexamers is slightly lower in SCCSs than other regions and the difference is significant at 0.05 significance level (p=0.013). This result implies that splicing signals have little influence on SCCSs.

Table 3-5

	Region size	No. of hexamer (per nucleotide)
SCCS genes	192,314 nt	20,42 (0.106/nt)
Non-SCCS genes	73,367,573 nt	7,950,888 (0.108/nt)

3.2.2 Overlaps between SCCSs and non-protein coding RNAs

Recent advancement of RNA research revealed abundant non-protein coding RNAs in the cell. Most of them derive from intergenic or intronic regions but some of them overlap with coding regions. Such non-coding RNAs may affect on nucleotide substitution in coding regions. If a non-coding RNA overlapping with a coding sequence contains functional nucleotide element, nucleotide substitution in the coding region that corresponds with the functional element will be suppressed.

For this reason, I surveyed non-coding RNAs that overlap with SCCSs. As the result, I identified 962 ncRNAs overlapping with SCCSs (Table 3-6). Functions of antisense RNA, miRNA, piRNA, 5' UTR regulatory element are validated to some extent but functions of other categories are less clear. 'NcRNA' namely represents non-coding RNA, refers to uncategorized transcripts in general. 'Mature transcripts' have polyA and the 5' cap like regular messenger RNAs but seemingly do not produce proteins. 'Non coding conserved regions' are defined by Evofold (Pedersen et al. 2006) or RNAdb (Pang et al. 2005). These regions are predicted by the evolutionary conservation and the secondary structure but do not necessarily produce transcripts. Thought named 'non-coding', not a few of them overlap with coding regions.

The overlap of the SCCS in CHPF2 and micro RNA (miR-671) is shown in Figure 3-7A. The CHPF2 gene encodes chondroitin sulfate glucuronyltransferase. The bar pointed by the blue arrow represents the precursor of mir-671. The thicker part of the bar corresponds to the mature miRNA. The SCCS covers the mature miRNA region. Mir-671 was identified through extensive analysis of small RNAs but its target is not known. Figure 3-7B shows the overlap of the SCCS in the SPI1 gene and 5' UTR regulatory element. SPI1 is an ETS-domain transcription factor that activates gene expression during myeloid and B-lymphoid cell development. 5' UTR regulatory element of the SPI1 gene was identified from a highly conserved region between human and mouse. The 5’UTR regulatory element inhibits translation in vitro, however, the effect of this element is negligible in vivo. This regulatory element extends to the coding region and overlaps with the SCCS. The SCCS continues 12 nucleotides upstream of the 5’ UTR element. This excess region may also be involved in the regulatory element. These examples of known functional elements support the idea that functional nucleotide elements in the coding region may be detected by strong nucleotide conservation.

The component of overlapping non-coding RNAs is different between the SCCSs and the non-SCCS coding regions. The SCCSs have less overlap with piRNAs and more overlaps with ncRNAs. Although function of ncRNAs is largely unknown, they may have some association with SCCSs.

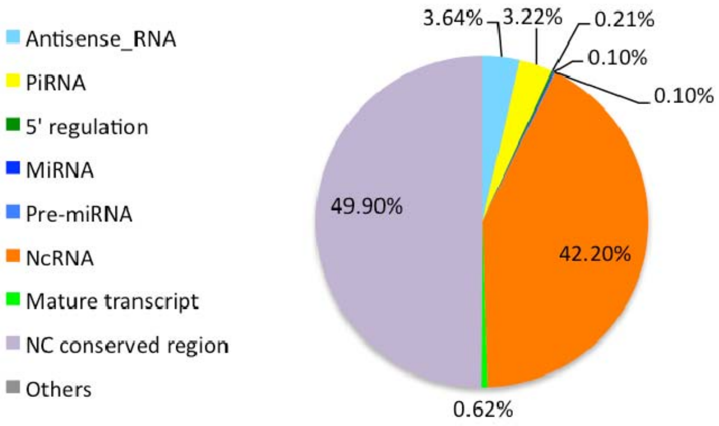
Table 3-6. Non-protein coding RNAs that overlap with SCCSs

Type of ncRNA	# in SCCSs	# in non-SCCS coding regions	# in both coding and non-coding
Function known			
Antisense RNA	35	1,297	2,771
piRNA	31	3,002	10,4243
5’ UTR regulatory element [*1]	2	8	16
(Pre) miRNA	2	78	1,695
Others	0	75	7,056
Function unknown			
ncRNA[*2]	406	7,492	34,156
Mature transcript [*3]	6	285	1,132
Non-coding conserved region [*4]	480	12617	84,964
Total	962	24853	236,032

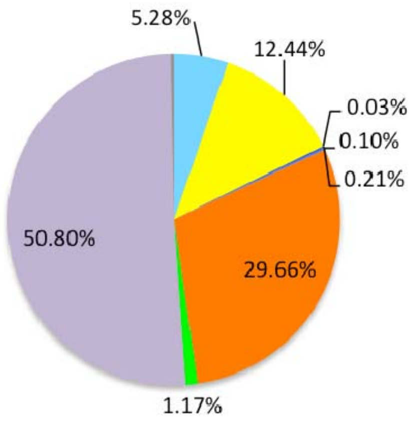
Note. NcRNAs corresponding to SCCSs are selected from exon-overlapping ncRNAs stored in the database and from those of blast hits with E <= 1e-4. The number in the parenthesis denotes ncRNAs detected by blast hits. The number of ncRNAs registration in the database is shown in the fight column. The type of ncRNA is following to Sequence Ontology database (http://www.sequenceontology.org/index.html). *1: 5' UTR regulatory element of Spi1 (spleen focus forming virus proviral integration oncogene). *2: RNA transcripts that do not encode proteins. *3: RNA transcripts that have undergone processing of splicing and modifications to the 5' and/or the 3', but are not necessarily translated. *4: Non-coding regions (may partially overlap with coding regions) that retain similarity by descent from the common ancestor. The number in the databases indicates the number of human’s ncRNAs.

Figure 3-8. The components of non-protein coding RNAs that overlap with SCCSs, non-SCCS coding regions, and both coding and non-coding regions.

(A) SCCS



(B) Non-SCCS coding region



(C) Both coding and non-coding regions

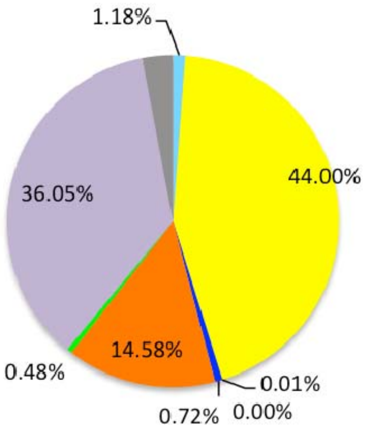
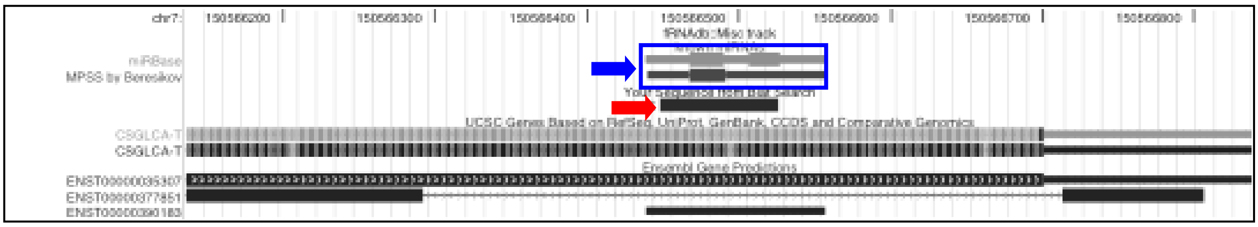


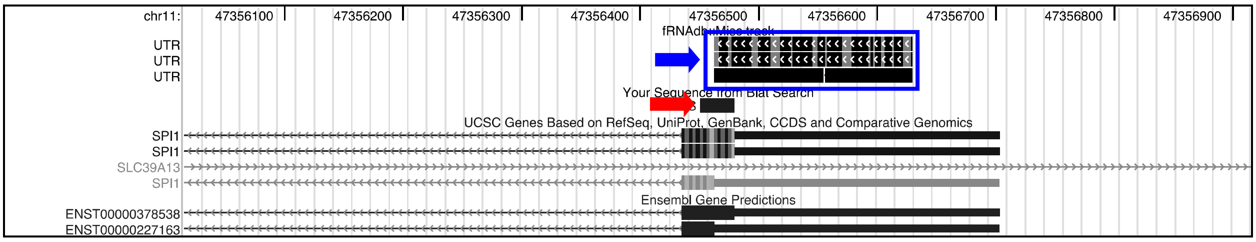
Figure 3-9. Overlap of SCCSs with non-protein coding RNAs of known function.

The red arrow represents an SCCS and the blue arrow represents an ncRNA.

(A) The SCCS in CHPF2 and miR-671



(B) The SCCS in SPI1 and 5' untranslated region (UTR) regulatory element



3.2.3 Gene expression

I investigated difference of gene expression between SCCS genes and non-SCCS genes referring to anatomical system data of EGenetics, which give qualitative information about in what organs a gene is expressed. I counted the number of SCCS genes and non-SCCS genes expressed in the organs and performed the Fisher’s exact test as described in Materials and Method section.

In general, higher percentage of SCCS genes is expressed in the organs than non-SCCS genes. Table 3-7 shows organs with the lowest 20 p-values where the percentage of expressed genes is higher in SCCS genes than non-SCCS genes. The difference is significant in all the twenty organs. Table 3-8 shows organs with the lowest 20 p-values where the percentage of expressed genes is lower in SCCS genes than non-SCCS genes. The difference is significant only in medulla oblongata and trophoblast.

It is notable that the significantly higher ratio of SCCS genes is expressed in the new brain as frontal lobe, while the significantly lower ratio of SCCS genes is expressed in the old brain as medulla oblongata. SCCS genes also show high expression in organs involved with mammalian specific reproduction specific to mammals such as breast, uterus, and endometrium.

Table 3-7. Organs with the lowest 20 p-values where the percentage of expressed genes is higher in SCCS genes than non-SCCS genes

Organ	SCCS			Non-SCCS			*P*
	#Expressed	#Not-expressed	%expressed	#Expressed	#Not-expressed	%expressed	
Breast	1133	905	55.59%	2430	3491	41.04%	7.68E-28
Frontal lobe	899	1139	44.11%	1803	4118	30.45%	1.05E-26
Thyroid	1076	962	52.80%	2375	3546	40.11%	1.44E-21
Cochlea	436	1602	21.39%	723	5198	12.21%	4.00E-21
Head and neck	1089	949	53.43%	2437	3484	41.16%	2.38E-20
Brain	1798	240	88.22%	4686	1235	79.14%	8.73E-20
Germinal center	1154	884	56.62%	2653	3268	44.81%	6.90E-19
Skeletal muscle	1252	786	61.43%	3003	2918	50.72%	7.57E-16
Retina	1360	678	66.73%	3372	2549	56.95%	7.68E-14
Parathyroid	1166	872	57.21%	2796	3125	47.22%	9.43E-14
Visual apparatus	1261	777	61.87%	3079	2842	52.00%	1.02E-13
Skin	1571	467	77.09%	4057	1864	68.52%	1.05E-12
Heart	1423	615	69.82%	3601	2320	60.82%	2.15E-12
Larynx	642	1396	31.50%	1385	4536	23.39%	7.78E-12
Amygdala	201	1837	9.86%	318	5603	5.37%	1.06E-10
Uterus	1553	485	76.20%	4056	1865	68.50%	2.20E-10
Bone marrow	939	1099	46.07%	2257	3664	38.12%	2.26E-09
Endometrium	1177	861	57.75%	2961	2960	50.01%	1.02E-08
Pituitary gland	421	1617	20.66%	877	5044	14.81%	1.08E-08
Blood	1204	834	59.08%	3051	2870	51.53%	2.14E-08

Table 3-8. Organs with the lowest 20 p-values where the percentage of expressed genes is lower in SCCS genes than non-SCCS genes

Organ	SCCS			Non-SCCS			*P*
	#Expressed	#Not-expressed	%expressed	#Expressed	#Not-expressed	%expressed	
Medulla oblongata	200	1838	9.81%	790	5131	13.34%	7.53E-05
Trophoblast	26	2012	1.28%	158	5763	2.67%	3.82E-04
Synovium	81	1957	3.97%	277	5644	4.68%	0.28
Lymph	0	2038	0.00%	5	5916	0.08%	0.46
Tonsil	192	1846	9.42%	600	5321	10.13%	0.49
Cerebrum	2	2036	0.10%	13	5908	0.22%	0.50
Adrenal medulla	7	2031	0.34%	29	5892	0.49%	0.56
Middle ear	0	2038	0.00%	3	5918	0.05%	0.70
Temporal lobe	0	2038	0.00%	3	5918	0.05%	0.70
Myocardium	147	1891	7.21%	449	5472	7.58%	0.75
Lymphoreticular	254	1784	12.46%	755	5166	12.75%	0.88
Arterial adventitia	0	2038	0.00%	1	5920	0.02%	1.00
Bronchus	0	2038	0.00%	1	5920	0.02%	1.00
Ileum	0	2038	0.00%	1	5920	0.02%	1.00
Motor	0	2038	0.00%	1	5920	0.02%	1.00
Parietal lobe	0	2038	0.00%	1	5920	0.02%	1.00
Submandibular gland	0	2038	0.00%	1	5920	0.02%	1.00
Vestibule	0	2038	0.00%	1	5920	0.02%	1.00
Ciliary body	12	2026	0.59%	36	5885	0.61%	1.00
Nose	2	2036	0.10%	7	5914	0.12%	1.00
Parotid gland	0	2038	0.00%	2	5919	0.03%	1.00
Peritoneum	0	2038	0.00%	2	5919	0.03%	1.00
Seminal vesicle	0	2038	0.00%	2	5919	0.03%	1.00
Foreskin	3	2035	0.15%	11	5910	0.19%	1.00
Vein	143	1895	7.02%	419	5502	7.08%	1.00

Chapter 4

Conclusion

In this study, I investigated significantly conserved coding sequences in the coding regions of seven taxa, 31 eukaryote species. Analyses on preferred codon ratio, GC content, and codon degeneracy revealed different characteristics of the invariant sequences among the taxa. The preferred codon ratio decreases as the conservation length elongates in the four animal taxa (mammals, teleosts, *Drosophilas*, and nematodes), while GC content and codon degeneracy do not show notable fluctuation. This result implies that selection toward optimum codons may not be the dominant factor in the above taxa. Judging from this result and moderate codon degeneracy, it’s not likely that long SCCSs has been retained solely by amino acid constraint in the mammals and teleosts.

Next I extracted significantly conserved coding sequences (SCCSs) from invariant sequences by conducting permutation simulation. This approach helped identify invariant sequences whose length is significantly rare to appear in each alignment. The difference between the number of invariant sequences in the original alignment and permutation result suggests that the distribution of conserved sites is skewed in the mammals and teleosts, while it is rather homogeneous in the budding yeasts.

Use of relatively rare codons might be beneficial for sequence recognition. The skewed conservation suggests local and strong constraint in the area. Considering these points, the traits of SCCSs of mammals and teleosts may be preferable as functional nucleotide elements.

Five mammalian SCCSs are identified to have significantly low folding free folding energy. The threshold p-value (p<0.05, corrected by FDR) is rather strict that false negatives like miR-671 or 5’ UTR regulatory element may exist. Although the number of identified elements is small, strong conservation and significantly low free energy suggest that these regions may have some functions.

Additional investigation on mammalian SCCSs give hints about the functionality of SCCSs. Exonic splicing signals does not show significant difference of density between SCCSs and non-SCCS regions. On the other hand, about 16% of SCCSs are overlapped with non-protein coding RNAs and the components of non-protein coding RNAs in SCCSs and non-SCCS coding regions are different. This implies that non-protein coding RNAs may have some association with SCCSs.

Expression pattern of SCCS genes of mammals shows involvement with nervous system. This corresponds to GO terms enriched with SCCS genes. Association with reproductive system is also notable. SCCS genes are involved with organs such as breast, frontal lobe, uterus, and endometrium, which are highly developed in the mammalian lineage.

The fraction of SCCSs to the number of aligned sites is small that they would not influence on evolutionary analysis. Even the fraction is small, or because of the fraction is small, they have potential as functional elements.

Acknowledgements

I am profoundly grateful to Dr. Naruya Saitou for his persistent supports on this work and my doctoral education for five years. Grateful acknowledgement is extended to Drs. Hiroshi Akashi, Tetsuji Kakutani, Yoshiyuki Suzuki, Aya Takahashi, and Toshimichi Ikemura. I thank Saitou laboratory members, Dr. Kenta Sumiyama, Ms. Tatsuko Kawamoto, Ms. Masako Mizuguchi, Ms. Yoshimi Noaki, Dr. Kirill Kryukov, Dr. Yukuto Sato, Ms. Mahoko Takahashi, Mr. Masatoshi Matsunami, Mr. Tim Jinam, Mr. Toshiaki Kanzawa, Mr. Jeff Ngai, and ex-laboratory member Dr. Kiyoshi Ezawa for their advice and encouragements during this work. I also express my appreciation to three cats in NIG who soothed my stresses.

Literature Cited

Akashi, H. 1994. Synonymous codon usage in Drosophila melanogaster: natural selection and translational accuracy. Genetics 136:927-935.

Akashi, H. 2003. Translational selection and yeast proteome evolution. Genetics 164:1291-1303.

Bejerano, G, M Pheasant, I Makunin, S Stephen, WJ Kent, JS Mattick, D Haussler. 2004. Ultraconserved elements in the human genome. Science 304:1321-1325.

Benjamini, Y, D Drai, G Elmer, N Kafkafi, I Golani. 2001. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 125:279-284.

Bhalla, T, JJ Rosenthal, M Holmgren, R Reenan. 2004. Control of human potassium channel inactivation by editing of a small mRNA hairpin. Nat Struct Mol Biol 11:950-956.

Delgado, MD, P Gutierrez, C Richard, MA Cuadrado, F Moreau-Gachelin, J Leon. 1998. Spi-1/PU.1 proto-oncogene induces opposite effects on monocytic and erythroid differentiation of K562 cells. Biochem Biophys Res Commun 252:383-391.

Drummond, DA, CO Wilke. 2008. Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. Cell 134:341-352.

Fairbrother, WG, RF Yeh, PA Sharp, CB Burge. 2002. Predictive identification of exonic splicing enhancers in human genes. Science 297:1007-1013.

Hofacker, IL. 2009. RNA secondary structure analysis using the Vienna RNA package. Curr Protoc Bioinformatics Chapter 12:Unit12 12.

Hurst, LD, EJ Williams. 2000. Covariation of GC content and the silent site substitution rate in rodents: implications for methodology and for the evolution of isochores. Gene 261:107-114.

Ihaka, R, and R. Gentleman. 1996. R: a language for data analysis and graphics. J. Comp. Graph. Stat 5:299-314.

Ikemura, T. 1985. Codon usage and tRNA content in unicellular and multicellular organisms. Mol Biol Evol 2:13-34.

Kanaya, S, Y Yamada, M Kinouchi, Y Kudo, T Ikemura. 2001. Codon usage and tRNA genes in eukaryotes: correlation of codon usage diversity with translation efficiency and with CG-dinucleotide usage as assessed by multivariate analysis. J Mol Evol 53:290-298.

Kimura, M. 1983. The neutral theory of molecular evolution. Cambridge: Cambridge University Press.

Lareau, LF, M Inada, RE Green, JC Wengrod, SE Brenner. 2007. Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements. Nature 446:926-929.

Licatalosi, DD, RB Darnell. 2010. RNA processing and its regulation: global insights into biological networks. Nat Rev Genet 11:75-87.

Lin, Z, H Ma, M Nei. 2008. Ultraconserved coding regions outside the homeobox of mammalian Hox genes. BMC Evol Biol 8:260.

Makalowski, W, MS Boguski. 1998. Synonymous and nonsynonymous substitution distances are correlated in mouse and rat genes. J Mol Evol 47:119-121.

Mituyama, T, K Yamada, E Hattori, H Okida, Y Ono, G Terai, A Yoshizawa, T Komori, K Asai. 2009. The Functional RNA Database 3.0: databases to support mining and annotation of functional RNAs. Nucleic Acids Res 37:D89-92.

Nei, M. 1987. Molecular evolutionary genetics. New York: Columbia Univ. Press.

Pang, KC, S Stephen, PG Engstrom, K Tajul-Arifin, W Chen, C Wahlestedt, B Lenhard, Y Hayashizaki, JS Mattick. 2005. RNAdb--a comprehensive mammalian noncoding RNA database. Nucleic Acids Res 33:D125-130.

Parmley, JL, LD Hurst. 2007. Exonic splicing regulatory elements skew synonymous codon usage near intron-exon boundaries in mammals. Mol Biol Evol 24:1600-1603.

Pedersen, JS, G Bejerano, A Siepel, K Rosenbloom, K Lindblad-Toh, ES Lander, J Kent, W Miller, D Haussler. 2006. Identification and classification of conserved RNA secondary structures in the human genome. PLoS Comput Biol 2:e33.

Schattner, P, M Diekhans. 2006. Regions of extreme synonymous codon selection in mammalian genes. Nucleic Acids Res 34:1700-1710.

Serganov, A, DJ Patel. 2007. Ribozymes, riboswitches and beyond: regulation of gene expression without proteins. Nat Rev Genet 8:776-790.

Sharp, PM, WH Li. 1987. The rate of synonymous substitution in enterobacterial genes is inversely related to codon usage bias. Mol Biol Evol 4:222-230.

Takahashi, A. 2009. Effect of exonic splicing regulation on synonymous codon usage in alternatively spliced exons of Dscam. BMC Evol Biol 9:214.

Thompson, JD, DG Higgins, TJ Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680.

Ticher, A, D Graur. 1989. Nucleic acid composition, codon usage, and the rate of synonymous substitution in protein-coding genes. J Mol Evol 28:286-298.

Appendix 1. The full list of GO terms enriched with SCCS genes.

(A) Mammals

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
GO Biological process				
GO:0045941	Positive regulation of transcription	7.48	2.86	1.29E-17
GO:0010628	Positive regulation of gene expression	7.56	2.97	3.52E-17
GO:0048699	Generation of neurons	6.99	2.86	9.77E-15
GO:0009790	Embryo development	7.17	3.01	1.24E-14
GO:0022008	Neurogenesis	7.34	3.14	1.89E-14
GO:0030182	Neuron differentiation	6.38	2.54	2.59E-14
GO:0031328	Positive regulation of cellular biosynthetic process	8.09	3.7	3.38E-14
GO:0006325	Chromatin organization	4.57	1.56	3.88E-13
GO:0009952	Anterior/posterior pattern formation	2.51	0.5	6.11E-13
GO:0009792	Embryo development ending in birth or egg hatching	4.79	1.73	1.05E-12
GO:0043009	Chordate embryonic development	4.75	1.7	1.17E-12
GO:0016568	Chromatin modification	3.74	1.15	1.90E-12
GO:0048598	Embryonic morphogenesis	4.35	1.5	1.90E-12
GO:0007389	Pattern specification process	3.91	1.27	4.40E-12
GO:0048666	Neuron development	4.93	1.91	1.17E-11
GO:0051276	Chromosome organization	5.32	2.19	1.79E-11
GO:0010629	Negative regulation of gene expression	5.58	2.37	2.20E-11
GO:0000904	Cell morphogenesis involved in differentiation	4.13	1.46	2.28E-11
GO:0050826	Response to freezing	2.86	0.76	2.57E-11
GO:0001659	Temperature homeostasis	3.08	0.88	2.97E-11
GO Cellular components				
GO:0015629	Actin cytoskeleton	3.39	1.8	1.15E-03
GO:0043005	Neuron projection	4.09	2.32	1.15E-03
GO:0043025	Neuronal cell body	2.24	1.11	2.38E-03
GO:0030425	Dendrite	2.29	1.1	2.38E-03
GO:0043198	Dendritic shaft	0.48	0.06	2.38E-03
GO:0014704	Intercalated disc	0.35	0.04	8.43E-03

Appendix 1 (continued)

(A) Mammals (continued)

GO Molecular function				
GO:0043565	Sequence-specific DNA binding	7.26	2.84	9.44E-17
GO:0003723	RNA binding	6.07	2.69	6.50E-11
GO:0050825	Ice binding	2.86	0.76	6.50E-11
GO:0003702	RNA polymerase II transcription factor activity	3.03	0.94	1.55E-09
GO:0016563	Transcription activator activity	4.84	2.09	3.01E-09
GO:0008134	Transcription factor binding	5.41	2.48	3.68E-09
GO:0016564	Transcription repressor activity	3.65	1.37	5.36E-09
GO:0003682	Chromatin binding	2.15	0.59	4.08E-08
GO:0003712	Transcription cofactor activity	3.87	1.61	4.08E-08
GO:0004674	Protein serine/threonine kinase activity	6.02	3.24	5.51E-07
GO:0004672	Protein kinase activity	6.77	4.07	1.28E-05
GO:0004713	Protein tyrosine kinase activity	5.8	3.32	1.28E-05
GO:0003713	Transcription coactivator activity	2.37	0.92	1.43E-05
GO:0051020	GTPase binding	1.58	0.5	4.74E-05
GO:0016881	Acid-amino acid ligase activity	2.33	0.98	1.07E-04
GO:0019899	Enzyme binding	4.97	2.91	1.72E-04
GO:0019904	Protein domain specific binding	3.47	1.79	1.72E-04
GO:0017016	Ras GTPase binding	1.28	0.39	2.45E-04
GO:0031267	Small GTPase binding	1.41	0.46	2.45E-04
GO:0019787	Small conjugating protein ligase activity	1.98	0.8	2.47E-04

Appendix 1 (continued)

(B) Teleosts

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
GO Biological process				
GO:0006355	Regulation of transcription, DNA-dependent	12.24	5.42	2.13E-16
GO:0006811	Ion transport	3.70	1.68	4.50E-05
GO:0006468	Protein amino acid phosphorylation	7.92	4.72	9.20E-05
GO:0006816	Calcium ion transport	1.71	0.56	2.61E-04
GO:0051056	Regulation of small GTPase mediated signal transduction	1.13	0.30	9.94E-04
GO Cellular components				
GO:0005634	Nucleus	19.27	10.31	1.35E-14
GO:0005891	Voltage-gated calcium channel complex	0.72	0.06	4.50E-05
GO:0005622	Intracellular	16.23	11.35	2.74E-04
GO Molecular function				
GO:0003700	Transcription factor activity	9.43	3.20	4.74E-21
GO:0043565	Sequence-specific DNA binding	7.39	2.25	8.45E-20
GO:0003677	DNA binding	10.10	4.66	1.02E-12
GO:0008270	Zinc ion binding	16.16	8.95	1.55E-11
GO:0004672	Protein kinase activity	6.92	3.22	9.14E-09
GO:0004713	Protein tyrosine kinase activity	6.05	2.76	3.78E-08
GO:0005524	ATP binding	14.27	8.52	5.78E-08
GO:0003676	Nucleic acid binding	9.67	5.33	1.72E-07
GO:0005216	Ion channel activity	3.54	1.28	1.72E-07
GO:0004674	Protein serine/threonine kinase activity	6.28	3.04	1.99E-07
GO:0005515	Protein binding	17.20	11.72	4.29E-05
GO:0005096	GTPase activator activity	0.98	0.17	1.05E-04
GO:0005249	Voltage-gated potassium channel activity	1.92	0.63	1.09E-04
GO:0005245	Voltage-gated calcium channel activity	0.82	0.14	4.84E-04
GO:0004879	Ligand-dependent nuclear receptor activity	1.50	0.46	4.88E-04

Appendix 1 (continued)

(C) Drosophilas

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
GO Biological process				
GO:0030154	Cell differentiation	8.85	8.85	7.89E-05
GO:0044267	Cellular protein metabolic process	11.98	11.98	7.89E-05
GO:0006464	Protein modification process	7.06	7.06	4.28E-04
GO:0016070	RNA metabolic process	10.64	10.64	4.28E-04
GO:0007275	Multicellular organismal development	13.61	13.61	4.53E-04
GO:0043687	Post-translational protein modification	6.04	6.04	4.53E-04
GO:0006355	Regulation of transcription, DNA-dependent	7.47	7.47	1.77E-03
GO:0009059	Macromolecule biosynthetic process	12.49	12.49	1.77E-03
GO:0006350	Transcription	8.65	8.65	1.79E-03
GO:0007166	Cell surface receptor linked signaling pathway	7.27	7.27	1.79E-03
GO:0007186	G-protein coupled receptor protein signaling pathway	4.71	4.71	2.04E-03
GO:0045449	Regulation of transcription	7.98	7.98	2.04E-03
GO:0019222	Regulation of metabolic process	10.54	10.54	2.06E-03
GO:0006351	Transcription, DNA-dependent	7.83	7.83	2.44E-03
GO:0007154	Cell communication	11.98	11.98	7.66E-03
GO:0006796	Phosphate metabolic process	5.63	5.63	8.45E-03
GO:0009653	Anatomical structure morphogenesis	8.8	8.8	8.45E-03
GO:0045165	Cell fate commitment	2.46	2.46	9.00E-03
GO:0006461	Protein complex assembly	2.81	2.81	9.70E-03
GO Cellular components				
GO:0016021	Integral to membrane	12.49	8.55	3.37E-05
GO:0031224	Intrinsic to membrane	12.59	8.76	4.25E-05
GO Molecular function				
GO:0008270	Zinc ion binding	10.85	6.89	1.00E-05
GO:0046914	Transition metal ion binding	13.05	8.92	2.88E-05
GO:0017076	Purine nucleotide binding	9.72	6.76	2.70E-03
GO:0000166	Nucleotide binding	11.26	8.26	4.18E-03
GO:0004672	Protein kinase activity	3.38	1.8	4.18E-03
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	4.4	2.59	4.18E-03
GO:0004674	Protein serine/threonine kinase activity	2.87	1.47	5.32E-03
GO:0016772	Transferase activity, transferring phosphorus-containing groups	5.37	3.46	9.72E-03

Appendix 1 (continued)

(D) Dicots

Terms		%in SCCS containi ng genes	%in non-SCCS containing genes	P
GO Biological process				
GO:0005975	Carbohydrate metabolic process	7.91	3.74	1.22E-07
GO:0044262	Cellular carbohydrate metabolic process	4.66	1.68	1.52E-07
GO:0007275	Multicellular organismal development	12.64	7.66	1.35E-06
GO:0006810	Transport	14.17	9.2	8.32E-06
GO:0009791	Post-embryonic development	7.91	4.52	7.15E-05
GO:0007017	Microtubule-based process	1.85	0.47	1.38E-04
GO:0048513	Organ development	5.49	2.81	1.38E-04
GO:0051641	Cellular localization	4.91	2.51	3.80E-04
GO:0009165	Nucleotide biosynthetic process	1.98	0.62	5.04E-04
GO:0007018	Microtubule-based movement	1.28	0.27	5.93E-04
GO:0016310	Phosphorylation	7.47	4.52	6.40E-04
GO:0044267	Cellular protein metabolic process	17.23	12.88	1.15E-03
GO:0006793	Phosphorus metabolic process	8.17	5.2	1.19E-03
GO:0006796	Phosphate metabolic process	8.17	5.2	1.19E-03
GO:0009117	Nucleotide metabolic process	2.43	0.97	1.36E-03
GO:0046907	Intracellular transport	4.15	2.12	1.36E-03
GO:0048366	Leaf development	1.91	0.66	1.38E-03
GO:0016051	Carbohydrate biosynthetic process	2.36	0.92	1.40E-03
GO:0016192	Vesicle-mediated transport	2.62	1.11	1.74E-03
GO:0006464	Protein modification process	9.7	6.57	1.80E-03
GO Cellular components				
GO:0043234	Protein complex	11.17	6.28	7.93E-08
GO:0015630	Microtubule cytoskeleton	2.3	0.55	9.89E-07
GO:0005886	Plasma membrane	12.32	7.64	1.29E-06
GO:0005856	Cytoskeleton	3.19	1.11	3.84E-06
GO:0005794	Golgi apparatus	3.13	1.46	9.90E-04
GO:0016021	Integral to membrane	11.87	8.42	1.03E-03
GO:0000325	Plant-type vacuole	0.89	0.16	1.25E-03
GO:0005773	Vacuole	3.89	2.07	1.38E-03
GO:0031224	Intrinsic to membrane	12.38	9.34	6.81E-03
GO:0034707	Chloride channel complex	0.32	0	7.24E-03
GO:0048046	Apoplast	1.98	0.9	8.12E-03

Appendix 1 (continued)

(D) Dicots (continued)

GO Molecular function				
GO:0017076	Purine nucleotide binding	22.34	9.88	2.03E-31
GO:0001882	Nucleoside binding	20.49	9.02	6.13E-29
GO:0005524	ATP binding	19.02	8.4	1.68E-26
GO:0017111	Nucleoside-triphosphatase activity	9.7	3.12	3.22E-21
GO:0016462	Pyrophosphatase activity	9.89	3.45	2.36E-19
GO:0016818	Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	9.96	3.53	4.25E-19
GO:0016787	Hydrolase activity	21.57	13.21	4.53E-13
GO:0016887	ATPase activity	4.72	1.64	5.08E-09
GO:0016740	Transferase activity	18.83	12.63	1.17E-07
GO:0004386	Helicase activity	2.49	0.68	1.73E-06
GO:0003924	GTPase activity	1.72	0.37	1.16E-05
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	8.49	5.16	8.80E-05
GO:0016772	Transferase activity, transferring phosphorus-containing groups	10.4	6.74	1.13E-04
GO:0016820	Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	1.66	0.43	1.77E-04
GO:0016301	Kinase activity	8.93	5.67	2.77E-04
GO:0005525	GTP binding	2.49	0.94	3.89E-04
GO:0000287	Magnesium ion binding	2.94	1.25	4.28E-04
GO:0003774	Motor activity	1.53	0.43	5.91E-04
GO:0019001	Guanyl nucleotide binding	2.62	1.11	1.03E-03

Appendix 1 (continued)

(E) Monocots

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
GO Biological process				
GO:0006810	transport	5.66	3.03	3.13E-06
GO:0008152	metabolic process	9.35	6.00	3.15E-05
GO:0006812	cation transport	0.89	0.20	3.46E-04
GO:0006468	protein amino acid phosphorylation	7.24	4.72	6.45E-04
GO:0006350	transcription	4.25	2.45	8.74E-04
GO:0045449	regulation of transcription	7.16	4.72	1.04E-03
GO:0030244	cellulose biosynthetic process	0.41	0.04	4.53E-03
GO:0006355	regulation of transcription, DNA-dependent	5.99	4.04	6.18E-03
GO:0007047	cellular cell wall organization	1.00	0.34	6.18E-03
GO Cellular component				
GO:0016020	membrane	14.50	7.83	1.05E-14
GO:0016021	integral to membrane	11.73	6.81	1.21E-09
GO:0005634	nucleus	11.55	8.71	9.21E-03
GO Molecular function				
GO:0005524	ATP binding	18.08	9.98	4.84E-16
GO:0000166	nucleotide binding	14.60	7.75	1.73E-15
GO:0003824	catalytic activity	10.91	7.09	1.17E-05
GO:0017111	nucleoside-triphosphatase activity	3.22	1.45	1.17E-05
GO:0004713	protein tyrosine kinase activity	6.99	4.41	3.28E-04
GO:0004674	protein serine/threonine kinase activity	7.20	4.59	3.37E-04
GO:0004672	protein kinase activity	7.12	4.55	3.43E-04
GO:0016887	ATPase activity	1.60	0.59	3.43E-04
GO:0016757	transferase activity, transferring glycosyl groups	2.21	1.01	9.54E-04
GO:0008237	metallopeptidase activity	0.85	0.22	2.79E-03
GO:0005525	GTP binding	2.06	0.98	3.08E-03
GO:0016301	kinase activity	6.49	4.32	3.08E-03
GO:0016787	hydrolase activity	6.82	4.59	3.08E-03
GO:0042626	ATPase activity, coupled to transmembrane movement of substances	0.55	0.10	3.84E-03
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.67	0.16	4.54E-03
GO:0003677	DNA binding	11.14	8.21	4.60E-03
GO:0016760	cellulose synthase (UDP-forming) activity	0.37	0.03	4.60E-03
GO:0003924	GTPase activity	1.30	0.52	6.13E-03
GO:0008233	peptidase activity	2.14	1.10	9.36E-03

Appendix 1 (continued)

(F) Budding yeasts

Terms		%in SCCS containing genes	%in non-SCCS containing genes	P
GO Cellular components				
GO:0000166	nucleotide binding	18.46	10.52	1.46E-07
GO:0005524	ATP binding	12.54	6.58	2.92E-06
GO:0017076	purine nucleotide binding	15.19	8.92	1.12E-05
GO:0042623	ATPase activity, coupled	4.86	2.11	1.88E-03
GO:0004713	protein tyrosine kinase activity	3.09	1.03	2.82E-03
GO:0016887	ATPase activity	5.65	2.72	2.82E-03
GO:0017111	nucleoside-triphosphatase activity	8.3	4.79	4.09E-03

Appendix 2. SCCSs that overlap UCRs

Ensembl protein ID	Gene name	UCR	UCR region (build34)	SCCS
ENSP00000328452	CLK3	uc.393	chr15:72630059-72630333	AGAAGCCAACAGAGCAGTAAGCGCAGCAGCCGGAGTGTGGAAGATGACAAGGAG
ENSP00000316948	CLK4	uc.185	chr5:178157908-178158318	AAGAGCCACCGAAGGAAAAGATCCAGGAGTATAGAGGATGATGAGGAGGGTCACCTGATCTGTCAAAGTGGAGACGTTCTAAGAGCAAGATATGAAATCGTG
ENSP00000225792	DDX5	uc.420	chr17:63046872-63047104	GATGTGGCCTCCAGAGGGCTAGATGTGGAAGATGTGAAATTTGTCATCAATTATGACTACCCTAACTCCTCAGAGGATTATATTCATCGAATTGGAAGAACTGCTCGCAGTACCAAAACAGGCACAGCATACACTTTCTTT
ENSP00000362300	EIF2C1	uc.13	chr1:35786852-35787088	GTGGGCCGCTCCTTCTTCTCACCGCCTGAGGGCTACTACCACCCGCTGGGGGGTGGGCGCGAGGTCTGGTTCGGCTTTCACCAGTCTGTGCGCCCTGCCATGTGGAAGATGATGCTCAACATTGATGTCTCAGCCACTGCC
ENSP00000264108	HAT1	uc.97	chr2:173025175-173025616	TATCATGAAAGGCTTCAGACCTTTTTGATGTGGTTTATTGAAACTGCTAGCTTTATTGACGTGGATGATGAAAGATGGCACTACTTTCTAGTATTTGAGAAGTATAATAAGGATGGAGCT
ENSP00000264108	HAT1	uc.97	chr2:173025175-173025616	CTCTTTGCGACCGTAGGCTACATGACAGTCTATAATTACTATGTGTACCCAGACAAAACCCGGCCACGTGTAAGT
ENSP00000222726	HOXA5	uc.213	chr7:26925404-26925604	ATGAGCTCTTATTTTGTAAACTCATTTTGCGGTCGCTATCCAAATGGCCCGGACTACCAGTTGCATAATTATGGAGATCATAGTTCCGTGAGC

Appendix 2. (continued)

ENSP00000305973	HOXC4	uc.345	chr12:52733867-52734167	ATGATCATGAGCTCGTATTTGATGGACTCTAACTACATCGATCCGAAATTTCCTCCATGCGAAGAATATTCGCAAAATAGCTACATCCCTGAACACAGTCCGGAATATTACGGCCGGACCAGGGAATCGGGATTCCAGCATCACCACCAGGAGCTGTACCCACCACCGCCTCCGCGCCCTAGCTAC
ENSP00000343867	MRRF	uc.267	chr9:120429935-120430137	GTGAATATGGCCAGCTTCCCAGAGTGTACAGCTGCAGCTATCAAGGCTATAAGAGAAAGTGGAATGAAT
ENSP00000346420	NFAT5	uc.407	chr16:69457343-69457668	GAGCAGAGCTGCAGTATGTGGATGGAGGATTCCCCCTCCAACTTCAGTAACATGAGCACCAGTTCCTACAATGATAACACTGAGGTACCTCGTAAATCACGAAAACGAAATCCAAAGCAGAGGCCGGGGGTCAAACGACGAGATTGTGAAGAATCTAATATGGATATATTTGATGCCGACAGTGCCAAAGCACCTCACTATGTGCTTTCTCAGCTTACCACGGACAACAAAGGC
ENSP00000346420	NFAT5	uc.407	chr16:69457343-69457668	TTGTACATCTCACCACCACCTGAGGACTTGCTGGATAACAGTCGGATGTCCTGCCAGGATGAGGGGTGTGGATTGGAA
ENSP00000325819	NR2F1	uc.169	chr5:92995090-92995293	CACTACGGCCAATTCACCTGCGAGGGCTGCAAAAGTTTCTTCAAGAGGAGCGTCCGCAGGAACTTAACTTACACATGCCGTGCCAACAGGAACTGTCCCATCGACCAGCACCACCGCAACCAGTGCCAATACTGCCGCCTCAAGAAGTGC
ENSP00000362586	PBX3	uc.280	chr9:124054051-124054270	CCCCAGCTAATGAGACTGGACAATATGCTTTTGGCAGAAGGGGTTTCAGGTCCTGAGAAAGGTGGGGGATCGGCGGCAGCAGCTGCAGCCGCGGCAGCCTCTGGAGGTTCTTCAGATAACTCTATTGAACACTCAGATTACAGAGCCAAATTGACCCAGATCAGACAAATCTATCACACAGAACTGGAGAAATATGAACAGGCATGTAATGAA
ENSP00000300651	PPARBP	uc.413	chr17:37941482-37941753	ACACCAACCAACACCTTTCCGGGGGGTCCCATTACCACCTTGTTTAATATGAGCATGAGCATCAAAGATCGGCATGAGTCGGTGGGCCATGGGGAGGACTTCAGCAAG

Appendix 2. (continued)

ENSP00000300651	PPARBP	uc.413	chr17:37941482-37941753	TCTCAGAACCCAATTCTTACCAGTTTGTTGCAAATCACAGGGAACGGGGGGTCTACCATTGGCTCGAGTCCGACCCCTCCTCATCACACGCCGCCACCTGTCTCTTCGATGGCCGGCAACACCAAGAACCACCCGATGCTCATGAACCTTCTTAAAGAT
ENSP00000354370	PUM2	uc.48	chr2:20462845-20463142	TCATCAGTTGGCAGTTCTGCAAGTAGTAGTGCCACAAGGAGAGAGTCTCTATCTACTAGCTCTGACTTGTACAAAAGATCTAGTAGCAGCCTAGCACCCATAGGGCAACCATTTTACAATAGTCTGGGATTTTCCTCCTCTCCAAGTCCAATAGGCATGCCTCTGCCAAGCCAAACTCCAGGACATTCACTTACGCCACCGCCATCACTTTCATCACATGGATCCTCATCCAGTTTGCATTTAGGA
ENSP00000317872	RBBP6	uc.395	chr16:24545554-24545802	GAAGAGGAAAAGAAAAAGTCCAAGCTAGATGAGTTTACAAATGATTTTGCTAAGGAATTGATGGAATACAAAAAGATTCAAAAGGAGCGTAGGCGCTCATTTTCCAGG
ENSP00000350071	STRN3	uc.366	chr14:29372746-29372947	GCAGAGGAAGCTGAACCAATAACGTTTCCATCTGGAGGAGGCAAGTCATTTATTATGGGTTCTGATGATGTTTTGTTAAGTGTACTGGGCCTTGGAGACCTTGCAGACTTGACGGTAACAAATGATGCAGACTATAGTTATGATTTGCCT
ENSP00000336712	TNPO1	uc.153	chr5:72279759-72279998	ACAGCAATAACAATTGGTCGTCTTGGTTACGTTTGTCCTCAAGAGGTGGCCCCCATGCTACAGCAGTTTATAAGACCCTGG
ENSP00000265069	ZFR	uc.151	chr5:32425638-32425851	CTGACATCTCCAATTATTCGAGAAGAGAACATGAGGGAAGGAGATGTAACCTCGGGTATGGTGAAAGACCCACCGGACGTCTTGGACAGGCAAAAATGCCTTGACGCTCTGGCTGCTCTACGCCACGCTAAGTGGTTCCAGGCTAGAGCTAAT
ENSP00000351539	ZNF238	uc.44	chr1:241164431-241164660	ACAGAGTCTTTGTCCCAGAGGTCTGTCACCTCCGTGAGGGATTCGGCAGATGTTGACTGTGTGCTGGACCTGTCTGTCAAGTCCAGCCTT
ENSP00000351539	ZNF238	uc.44	chr1:241164431-241164660	GGAGTTGAAAATCTGAACAGCTCTTATTTCTCTTCACAGGACGTGCTGAGAAGCAACCTGGTGCAGGTGAAGGTGGAGAAAGAG