

Animals transcribe at least half of the genome

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Abstract

One central goal of genome biology is to understand how the usage of the genome differs between organisms. Our knowledge of genome composition, needed for downstream inferences, is critically dependent on gene annotations, yet problems associated with gene annotation and assembly errors are usually ignored in comparative genomics. Here we analyze the genomes of 68 species across all animal groups and some single-cell eukaryotes for general trends in genome usage and composition, taking into account problems of gene annotation. We show that, regardless of genome size, the ratio of introns to intergenic sequence is comparable across essentially all animals, with nearly all deviations dominated by increased intergenic sequence. Genomes of model organisms have ratios much closer to 1:1, suggesting that the majority of published genomes of non-model organisms are underannotated and consequently omit substantial numbers of genes, with likely negative impact on evolutionary interpretations. Finally, our results also indicate that most animals transcribe half or more of their genomes arguing against differences in genome usage between animal groups, and also suggesting that the transcribed portion is more dependent on genome size than previously thought.

Author's Summary

Within our anthropocentric genomic framework, many analyses try to define humans, mammals, or vertebrates relative to the so-called "lower" animals. This implicitly posits that vertebrates are complex organisms with large genomes and invertebrates are simple organisms with small genomes. This has the problem that genome size is therefore presumed to correlate with complexity and ignores any unknown complexity of vast numbers of invertebrate groups, many with large genomes. Animals vary widely in genome size, by almost three orders of magnitude, but when sequencing new animal genomes preference is given to those with smaller genomes for reasons of cost. In trying to understand how genomes are used in general, there is an added layer of complication from quality of the assembly and annotation. We have examined genome usage across a wide range of animals and have described ways to account for errors of low-quality annotations. We also show that the genomes of invertebrates and vertebrates are not so different, and that when large-genome invertebrates are considered, the fraction of the genome that is genes appears to be strongly influenced by genome size.

Introduction

Understanding why genomes vary greatly in size and how organisms make different use their genomes have been central questions in biology for decades [1]. For many bacteria, the majority of the genome is composed

40 of relatively short genes, averaging around 1000bp, and coding for proteins. Indeed, the largest bacterial
41 genome (a myxobacterium) that has been sequenced is only 14 megabases, containing an estimated 11,500
42 genes [2]. However, in eukaryotic organisms genomes can be over a thousand-fold larger than bacterial
43 genomes, due to an increase in the number of genes (tens of thousands compared to a few thousand in most
44 bacteria), expansion of the genes themselves due to the addition of introns, and expansion of the sequence
45 between genes.

46
47 As the number of genome projects has grown, massive amounts of data have become available to study
48 how organisms organize and use their genomes. Genome projects vary substantially in quality of assembly
49 and annotation [3, 4]. Unfortunately, the predicted genes are often taken for granted as being correct when
50 these are only hypotheses of gene structure [5]. For example, one study found that almost half of the genes
51 in the *Rhesus* monkey genome had a predictable annotation error when compared to the closest human
52 homolog [6]. This has profound implications for all downstream analyses, such as studying evolution of
53 orthologous proteins [7] and phylogeny based on protein matrices or gene content [8, 9]. When considered
54 across all genes, systematic errors in genome assembly or annotation would severely skew bulk parameters
55 of a genome.

56
57 While issues of assembly are often thought to be technical problems that are resolved before continuing,
58 all subsequent analyses are dependent upon accurate genome assembly and annotation. The absence of a
59 protein family in a particular organism is only meaningful if it is certain that it is absent from the genome
60 and not merely the annotation, therefore it is of utmost importance that all genes are properly represented.
61 Yet for most genome projects of non-model organisms, there are limited methods to determine if the assem-
62 bly and annotation are sufficient for downstream comparative analyses. Internal metrics can be used, such
63 as the fraction of raw genomic reads or ESTs that map back to the assembly, though this does not tell us if a
64 gene is believable in the context of other animals. Alternatively, counts of “universal” single-copy orthologs
65 have been proposed as a metric of genome completeness [10, 11], though these genes only represent a small
66 subset of all genes (few hundred out of tens of thousands in most animals).

67
68 Identification of universal trends in genome organization and usage may enable better quantitative met-
69 rics of genome completeness. Mechanistic models relating to evolution of gene content or coding fractions
70 tended to focus on bacteria or archaea because of the relative ease of annotation. In regards to eukaryotes,
71 some patterns in genome size have been discussed [12–14]. Additionally, a handful of studies have analyzed
72 genome size in connection to other parameters such as indels [15], transposon content [16–19], average intron
73 length [20, 21] or total intron length [18]. Despite these advances, none of these studies have estimated the
74 amount of the genome that is genic, and none of them have described a way to account for technical problems
75 in assembly and annotation.

76
77 Here we examine basic trends of genome size and the relationship to annotation quality across animals and
78 some single-celled eukaryotes. We show that assembly and annotation errors are widespread and predictable
79 and that many genomes are likely to be missing many genes. We further show that re-annotation of select
80 species with publicly available tools and transcriptome data improves the annotation. Future users may
81 benefit if databases incorporate more recent data from transcriptome sequencing, and update annotation
82 genome versions more frequently. Finally, we show that many animals appear to transcribe almost half of
83 their genomes, suggesting this as a potential parameter to identify genome completeness across metazoans,
84 and potentially other eukaryotes.

86 **Methods**

87 **Genomic data sources**

88 Data sources and parameters are available in Supplemental Table 1.

89

90 Genomic scaffolds and annotations for *Ciona intestinalis* [22], *Branchiostoma floridae* [23], *Trichoplax ad-*
91 *herens* [24], *Capitella teleta* [25], *Lottia gigantea* [25], *Helobdella robusta* [25], *Saccoglossus kowalevskii* [26],
92 *Monosiga brevicollis* [27], *Emiliana huxleyi* [28], and *Volvox carteri* [29] were downloaded from the JGI
93 genome portal.

94
95 Genome assemblies and annotations for *Sphaeroforma arctica*, *Capsaspora owczarzaki* [30] and *Salpin-*
96 *goeca rosetta* [31] were downloaded from the Broad Institute.

97
98 GFF annotations v2.1 [32] for *Amphimedon queenslandica* were downloaded from the Amphimedon
99 Genome website (<http://amphimedon.qcloud.qcif.edu.au/downloads.html>), and v1 annotations [33] and as-
100 semblies were downloaded from Ensembl.

101
102 For *Nematostella vectensis*, Nemve1 assembly and annotations [34] were downloaded from JGI, and the
103 transcriptome for comparative reannotation was downloaded from <http://www.cnidariangenomes.org/> [35].

104
105 Genome assembly, transcriptome assemblies from Cufflinks and Trinity, and GFF annotations for *Mne-*
106 *miopsis leidy* [8] were downloaded from the Mnemiopsis Genome Portal (<http://research.nhgri.nih.gov/mnemiopsis/>).
107 Assembly and annotations for *Sycon ciliatum* [36] were downloaded from COMPAGEN. Assembly and
108 annotation for *Botryllus schlosseri* [37] were downloaded from the Botryllus Schlosseri genome project
109 (<http://botryllus.stanford.edu/botryllusgenome/>). Assembly and annotation for *Aiptasia sp.* [38] were down-
110 loaded from <http://reefgenomics.org>. Assembly and annotation for *Oikopleura dioica* [39] were downloaded
111 from Genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Oikopleura/>). Assembly and anno-
112 tation for *Tetrahymena thermophila* were downloaded from the Tetrahymena Genome Database (ciliate.org).
113 Assembly and annotation for *Symbiodinium kawagutii* [40] were downloaded from the Dinoflagellate Re-
114 sources page (web.malab.cn/symka_new/index.jsp).

115
116 Assemblies and annotations for *Symbiodinium minutum* [41], *Pinctada fucata* [42], *Acropora digitifera*
117 [43], *Lingula anatina* [44], *Ptychodera flava* [26], and *Octopus bimaculoides* [45] were downloaded from the
118 OIST Marine Genomics Browser (<http://marinegenomics.oist.jp/gallery/>).

119
120 Builds of *Homo sapiens*, *Pan troglodytes*, *Mus musculus*, *Canis lupus* [46], *Monodelphis domestica* [47],
121 *Ornithorhynchus anatinus* [48], *Xenopus tropicalis* [49], *Struthio camelus* [50], *Gallus gallus*, *Taeniopygia*
122 *guttata* [51], *Aptenodytes forsteri* [50], *Anas platyrhynchos* [52], *Melopsittacus undulatus* [53], *Alligator mis-*
123 *sissippiensis* [54], *Anolis carolinensis* [55], *Chrysemys picta bellii* [56], *Chelonia mydas* [57], *Pelodiscus*
124 *sinensis* [57], *Python bivittatus* [58], *Salmo salar*, *Danio rerio* [59], *Latimeria chalumnae* [60], *Petromy-*
125 *zon marinus* [61], *Callorhynchus milii* [62], *Crassostrea gigas* [63], *Dendroctonus ponderosae* [64], *Tribolium*
126 *castaneum* [65], *Bombyx mori* [66], *Limulus polyphemus* [67] were downloaded from the NCBI Genome server.

127
128 Genome assemblies and annotations of *Caenorhabditis elegans* [68], *Drosophila melanogaster*, *Strongy-*
129 *locentrotus purpuratus* [69], *Daphnia pulex* [70], *Apis mellifera* [71], *Ixodes scapularis* [72], *Strigamia mar-*
130 *itima* [73] were downloaded from Ensembl.

131 132 Calculation of exonic and genic sequence

133 For all analyses, we used the total number of bases in the assembly as the total genome size, bearing in
134 mind that this may result in a systematic underestimation of total genome size as repeated regions may be
135 omitted from assemblies. For example, the horseshoe crab *L. polyphemus* has a scaffold assembly of 1.8Gb
136 while the reported genome size is 2.7Gb [67], a difference of almost a gigabase.

137
138 If GFF format files were available for download with a genome project, or on databases (Ensembl or
139 NCBI), those were used preferentially. Total bases of exon, intron, intergenic, and gaps were counted from
140 each GFF file and genomic contigs (or scaffolds) with a custom Python script ([gtfstats.py](http://bitbucket.org/wrf/sequences), available at [bit-](http://bitbucket.org/wrf/sequences)
141 [bucket.org/wrf/sequences](http://bitbucket.org/wrf/sequences)). The script converts all gene and exon annotations to intervals and ignores the

142 strand. All overlapping exon intervals are merged, meaning that alternative splice sites, or exons on the
143 opposite strand, are treated as a single interval. The same is done for genes or transcripts, whichever is
144 available, and introns are calculated as the difference of the two sets. This means that any sequence that
145 is an exon on one strand and an intron on the other is treated for all purposes as an exon, meaning those
146 bases or their reverse compliment are transcribed and retained in some case. Intergenic sequence is defined
147 as the difference between total sequence bases and genic bases, and gaps are defined as any repeats of 'N's
148 longer than one base.

149
150 If exons are not specified, then coding sequences (CDS) are used instead if they are available, such as
151 for AUGUSTUS predictions. Additional features such as “microRNA”, “tRNA”, “ncRNA” are included for
152 gene and exon calculations if they were in the standard GFF3 format. Some annotations had to determine
153 the gene ID from the exons. For example, most of the older GTF files from the earlier JGI genomes had only
154 exons annotated, without individual features for genes or mRNAs, so the gene was then defined as all of the
155 exons with the same feature ID. Exons defined as part of a “pseudogene”, or genes defined as pseudogenes,
156 were also excluded from all counts.

158 Calculation of average exon and intron length

159 The same script (`gtfstats.py`, available at bitbucket.org/wrf/sequences) also calculated the average exon and
160 intron length. All possible exons were taken into account for determination of averages. Identical exons of
161 splice variants were treated as one exon and counted once, however, alternative boundaries were treated as
162 a separate exons. Introns were calculated as the space between exons. To account for splice variants, the
163 maximum amount of exon is used, meaning that the most exons and largest exons are used in all cases;
164 retained introns are treated as exons, not introns.

166 Reannotation of select species

167 Due to unexpectedly high or low gene content, six genomes were selected for reannotation.

168
169 The original Triad1 scaffolds of *T. adherens* [24] were reannotated with AUGUSTUS v3.0.3 [74] with
170 the following options: `-strand=both -genemodel=atleastone -sample=100 -keep_viterbi=true -alternatives-`
171 `from-sampling=true -minexonintronprob=0.2 -minmeanexonintronprob=0.5 -maxtracks=2`. Species train-
172 ing was generated using the Triad1 ESTs with the webAugustus Training server [75].

173
174 The original Monbr1 scaffolds of *M. brevicollis* [27] were reannotated with AUGUSTUS as for *T. adherens*,
175 using the same parameters except trained using the Monbr1 ESTs with the webAugustus Training server [75].

176
177 For the hydrozoan *H. magnipapillata*, the original assembly was downloaded from JGI [76] and a new scaff-
178 fold assembly was downloaded from the FTP of Rob Steele at UC Irvine (at <https://webfiles.uci.edu/resteele/public>).
179 For both cases, the scaffolds were reannotated using TopHat2 v2.0.13 [77] and StringTie v1.0.4 [78] with
180 default options by mapping the reads from two paired-end RNAseq libraries, NCBI Short Read Archive
181 accessions SRR922615 and SRR1024340, derived from whole adult animals.

182
183 For the lancelet *B. floridae*, the Brafl1 scaffolds [23] were reannotated using TopHat2 v2.0.13 [77] and
184 StringTie v1.0.4 [78] with default options by mapping the reads from the paired-end RNAseq library, NCBI
185 SRA accession SRR923751, from the adult body.

186
187 For the lamprey *P. marinus*, we were unable to find any annotation as GFF or GTF, so we generated
188 one using TopHat2 v2.0.13 [77] and StringTie v1.0.4 [78] based on the Pmarinus-v7 scaffolds from NCBI and
189 the 16 single-end Illumina libraries from NCBI BioProject PRJNA50489.

190
191 For the octopus *O. bimaculoides*, scaffolds were downloaded from the OIST Marine Genomics plat-
192 form [45], and were reannotated using TopHat2 v2.0.13 [77] and StringTie v1.0.4 [78] with default options

193 by mapping 19 paired-end RNAseq libraries from NCBI BioProject PRJNA285380.

194

195 All reannotations are available for download as GTF or GFF files (see <https://bitbucket.org/wrf/genome-reannotations/downloads>).

197

198 Results

199 Overview and organization of data

200 A total of 68 genomes were analyzed, with 59 selected across all major metazoan groups and nine genomes
201 of single-celled eukaryotes. For each group, only select species were taken to avoid having a single group
202 dominate the analysis. For example, over 100 mammalian genomes are available though only six were used
203 including three model organisms (human, mouse, dog), opossum and platypus (for the non-eutherian clades,
204 marsupial and monotreme, respectively) and the chimp, to compare directly to the human annotation. In
205 general, parasites were excluded because they often have unusual biology, such as the single-celled eukaryote
206 *T. brucei*, which is known for its unusual RNA processing [79,80].

207

208 The smallest animal genome used in this study is that of the larvacean *Oikopleura dioica* (70Mb), while
209 the largest is that of the opossum *Monodelphis domestica* (3598Mb). It should be noted that some of the pub-
210 lic genome sequencing projects selected the animal of their clade based on their known small genomes. Two
211 examples of this are the shark *C. milii* and the pufferfish *T. rubripes*. Yet it must be considered that in terms
212 of genomes, they may not be representative of their clades; many other shark genomes are estimated to be
213 over 10Gb (haploid genome size) [81], such that a shark genome of only 1Gb may not be “normal” for sharks.

214

215 Additionally, not all of the species in the sample were sequenced or annotated with the same method,
216 making direct comparison more challenging. For instance, some of the earlier genomes (such as *Branchios-
217 toma floridae* and *Trichoplax adherens*) were annotated only with Sanger ESTs (order of tens of Mb), which
218 were used to train gene prediction algorithms. Because not all genes have features easily captured by the
219 EST training, several different results are expected: some genes are split because internal exons are not
220 properly found or may have misassemblies in the draft genomes; adjacent genes on the same strand are
221 fused; or genes are omitted entirely.

222

223 Connection between annotation and understanding of genomes

224 Genome projects generally seek to annotate protein coding regions of a genome. Broadly, there are two
225 methods of doing this, comparison to other proteins from other genomes and by aligning mRNA from ESTs
226 or RNAseq [3]. In practice, improvements in methods have made it relatively easy to directly predict proteins
227 from the genome sequence. However, untranslated regions (UTRs) are difficult to predict and often require
228 evidence from ESTs or transcriptome sequencing for accurate predictions, and this has implications for our
229 measurements of total exons in each genome. This means that even in a “perfect” genome where all coding
230 genes are correctly predicted by an annotation program (perhaps based on similarity to a related species)
231 that the precise positions and amount of UTR may still be unknown, resulting in an underestimation of
232 the amount of exonic sequence (Fig 1A and B). Because of this, the reliance on coding genes is likely to
233 underestimate the usable fraction of the genome.

234

235 To illustrate this, one may consider a hypothetical eukaryotic genome of 60Mb with 10,000 genes and
236 equal fractions of exons, introns, and intergenic sequence, at 20Mb each. For simplicity, all exons are the
237 same size (in this example, 200bp), so an average gene (with ten-exons) may contain one exon for the 5'-
238 UTR, and one for the 3'- UTR, and the remaining eight exons are coding. Based on the above annotation
239 scheme, 20% of the exonic fraction (those containing the 5' and 3'-UTRs) is missing in the final annotation.
240 Two introns per gene are also missing (the first and last introns), about 18% of the intronic fraction. This
241 would yield a final annotation where exons are predicted as 16Mb (26.6% of the genome) and introns as

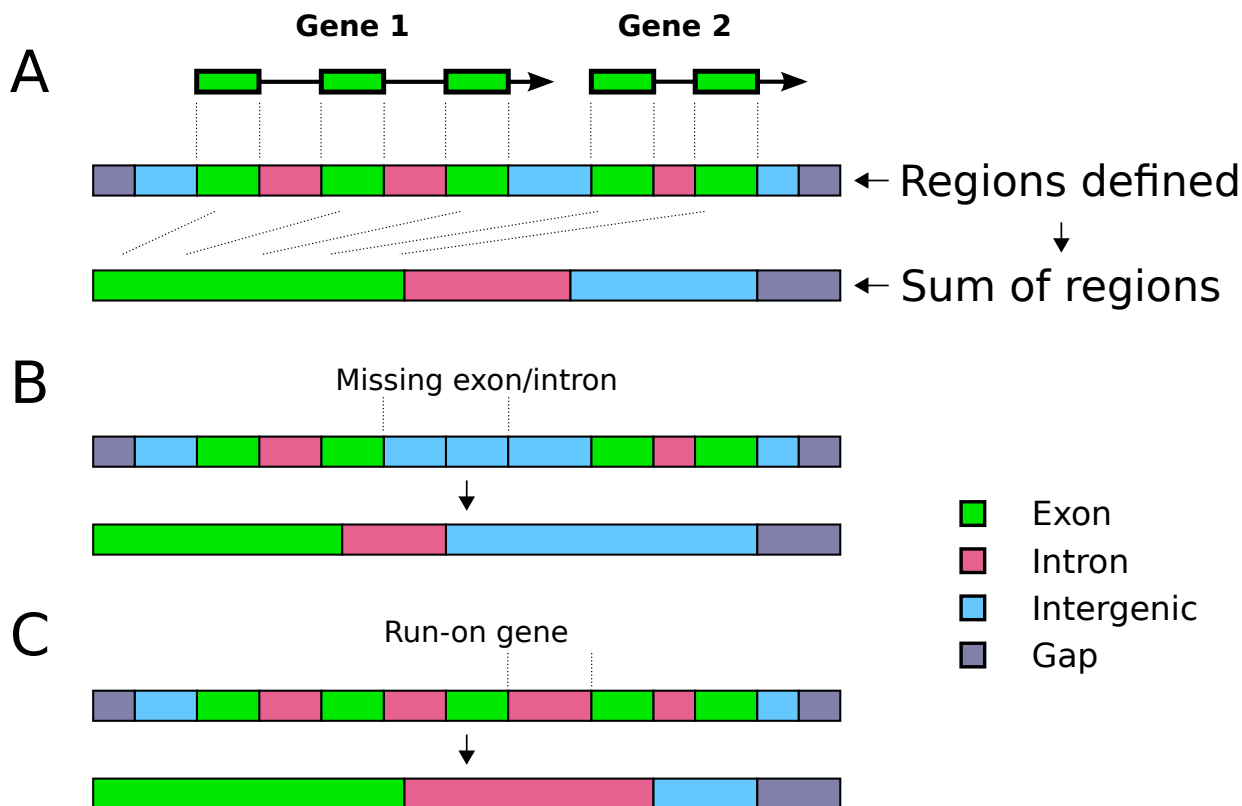


Figure 1: **Schematic of misannotations and the effects on coding fraction analyses** (A) In a normal case, two hypothetical genes on the same strand are identified. The exons and introns are defined, and the total lengths of those features are summed and displayed in the bars below. Because real genome assemblies can often contain gaps, sample gaps are also shown at the edges of the segment. (B) If individual exons (or potentially whole genes) were missing, then the measured total exons and introns would be smaller than the real values, and the ratio of intron:intergenic would decrease. (C) If neighboring genes were erroneously declared to be contiguous, the exonic fraction is mostly unchanged but the intron:intergenic ratio would increase.

242 15.5Mb (25.9% of the genome). This would also indicate that 52.6% of the genome is genes, a substantial
 243 underestimation from the actual value of 66.6%.

244

245 However, other systematic errors can result in an overestimation of the genic fraction. If we consider mul-
 246 tiple genes on the same strand, in a head-to-tail arrangement, and recall that UTRs are often not predicted,
 247 then an exon containing the stop codon with a 3'-UTR may be omitted and the predicted gene may continue
 248 into the next gene (Fig 1C). If it is assumed that the majority of coding exons are correctly predicted, then
 249 if such predictions were made systematically one may expect that the measured amount of exons does not
 250 deviate much from the true exonic fraction. However, because introns are defined as the removed sequence
 251 between exons of the same gene, then the sequence between the two genes that should have been defined as
 252 intergenic will instead be defined as intronic, thus raising the intron:intergenic ratio above 1.

253

254 The above problems assume that the genomic assembly is nonetheless correct, yet the annotation is
 255 directly affected by assembly problems as well. Of the two main sources of problems, repeats [82] and het-
 256 erozygosity [26, 42, 63, 83], repeats often result in breaks in the assembly that could split genes (Fig 2A).
 257 Genes that are split at contig boundaries are likely to have exons missing (or on other scaffolds) and thus

258 the sequence that should be defined as introns would be instead defined as intergenic (Fig 2B).

259

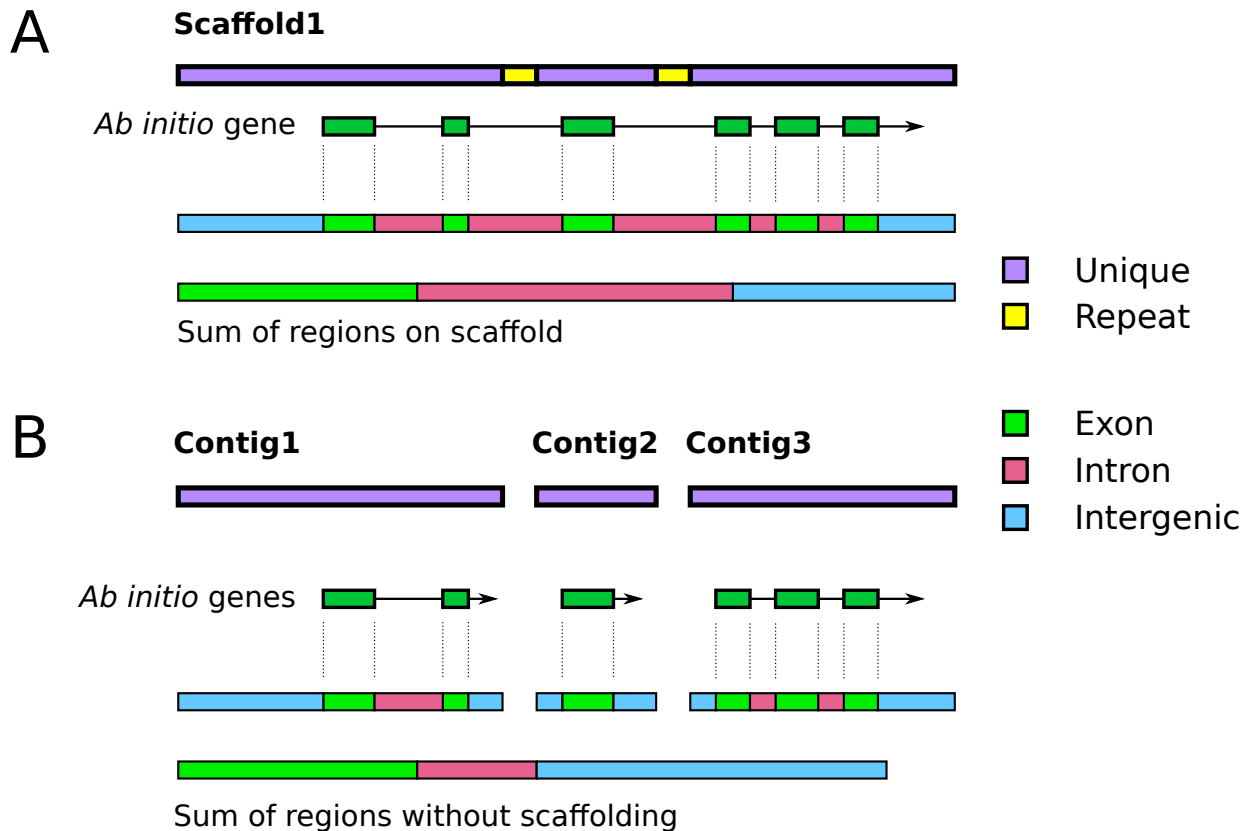


Figure 2: **Schematic of the effects of scaffolding and repeats on genic fraction analyses** (A) For a hypothetical scaffold in a genome assembly, two identical repeats are found within introns. The gene is correctly predicted to span the two repeats and the regions are defined below as in Fig 1. (B) For the case without scaffolding, or where the assembler breaks the assembly at repeats (or other high coverage regions), three contigs are generated. Note that the numbers are arbitrary, and in a real assembly they are unlikely to be in order. When annotated, all of the exons are correctly found, but the connections between them are missing for the single exon on Contig 2, resulting in a loss of intronic sequence. The final measured amount of exons is comparable, but the intron:intergenic ratio would decrease.

260 For normal diploid genomes (wild strains, not inbred lab strains), heterozygosity is not uniform across
 261 the genome. Some regions are identical between the two haplotypes (hence are homozygous alleles or loci),
 262 while others may vary by SNPs, short indels, or copy numbers of repeats, exons, or even genes. For sequences
 263 that are identical between both haplotypes, the contigs are generally kept as is, while a more complex deci-
 264 sion must be made for the heterozygous loci. During normal genome assembly, the assembler evaluates the
 265 coverage at each “bubble” (where the de Bruijn graph has two paths out of a node, and both paths merge
 266 again at the next node) and ultimately has to retain one of the paths at the exclusion of the other (Fig 3A)
 267 (also see schematics in [83] and [84]). This merging is the essential process that creates the reference genome,
 268 even though that reference is an arbitrary merge of the two haplotypes. Therefore, it must be kept in mind
 269 that predicted genes or proteins in reference genomes may not be identical to either haplotype.

270

271 Regions with relatively high heterozygosity may fail to be merged in this way, leaving contigs of both
 272 haplotypes in the assembly (Fig 3C). During subsequent scaffolding steps, contigs of separate haplotypes

273 can be fused head-to-tail if mate pairs are bridging the unique regions. Because this head-to-tail joining is
274 an artifact, no reads should map at the junction point, resulting in a region of zero coverage at the junction
275 and flanked by regions where coverage is half of the expected value (Fig 3D). One additional feature may
276 reveal this artifact: exons in the unmerged sections may be individually annotated but mapped ESTs or
277 *de novo* assembled transcripts may show a staggered exon pattern (Fig 3E) because transcripts can only
278 map to one of the two possible exons (2a or 2b, 3a or 3b). This may increase the ratio of intron:intergenic
279 sequence (Fig 3F), but also falsely indicate that splice variation is more prevalent for this gene.

280

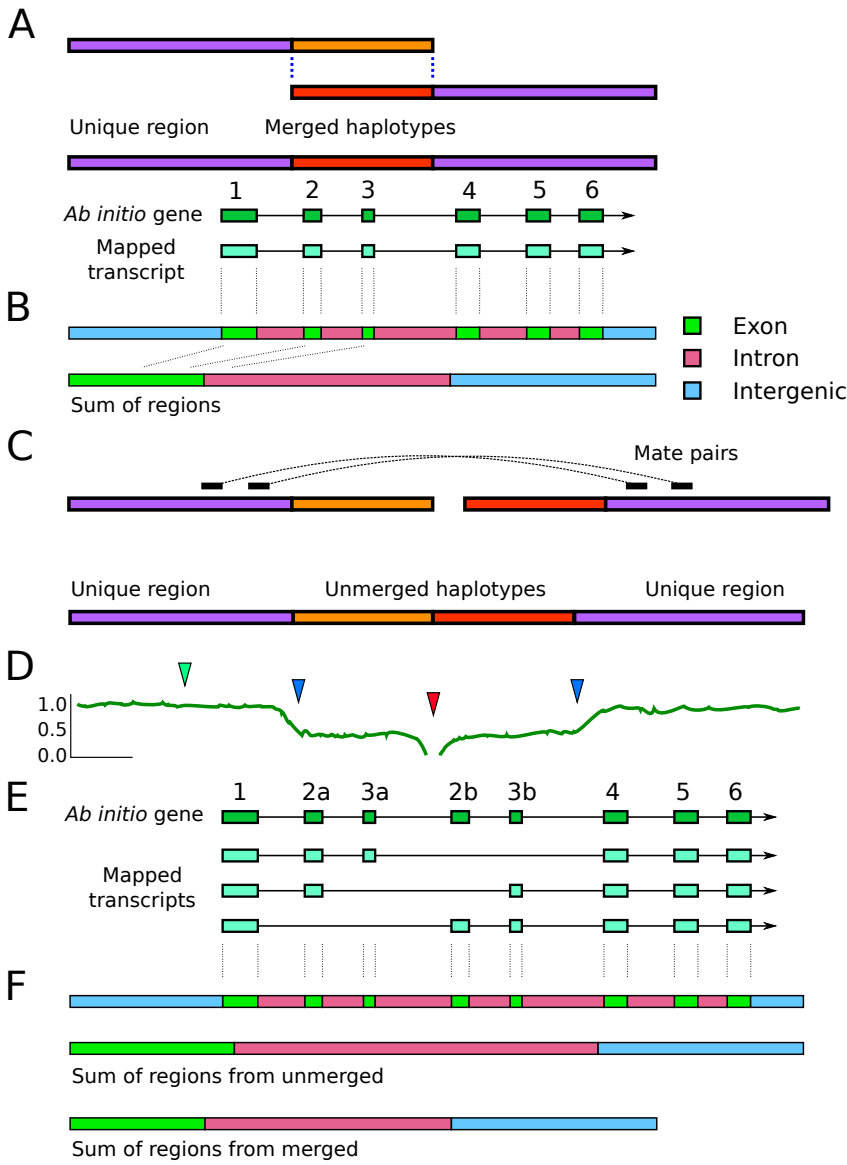


Figure 3: Schematic of misassembly and the effects on genic fraction analyses (A) During assembly, regions that are heterozygous (differing by SNPs or indels) are combined to make a single reference contig. When genes are predicting that this locus, or when assembled transcripts are aligned to the genome, the correct exon structure is found. (B) Regions are defined as exon, intron, or intergenic, as in Fig 1. (C) Reference genomes are a mix of the maternal and paternal haplotypes, but not uniformly. Rather than being merged into a single sequence, highly heterozygous regions may be assembled as different contigs that get erroneously fused during scaffolding steps. Mate pairs that bridge the two purple unique regions will instead result in a head-to-tail joining of the two unmerged haplotype sequences. (D) Hypothetical plot of read coverage across the contig. The green arrow shows a region of normal coverage (1x) while the blue arrows show sites where coverage is reduced because reads for each haplotype map separately. At the fusion point between the two haplotypes (red arrow), no reads will map since the sequence is an artifact, or is represented by a gap. (E) Mapped transcripts (or ESTs) or transcripts derived from mapped RNAseq reads (such as by Cufflinks or StringTie) may only be mapped to one of the two haplotypes, thereby producing a staggered exon structure. A mapped transcript can only align to either exon 2a or 2b, but not both, likewise for 3a or 3b, yet all other exons are unique and would align correctly. Genes predicted *ab initio* may annotate both sets of exons (2a/3a and 2b/3b), which may result in a duplication in some part of the protein, or a premature stop codon if 3a and 2b are out of phase. (F) For this hypothetical case, the sum of the regions would appear to have increased total exon size and the total intron size compared to the same genomic locus where the haplotypes were correctly merged.

281 Reannotation and changes following RNAseq reannotation

282 Keeping in mind the above error sources, some of the genomes used in our study had obvious problems of
 283 too much or too little genic content that would confound our analyses. For instance, the total amount of
 284 exons in the JGI annotation of *T. adherens* (Triad1) was only 14Mb, over twofold lower than the related
 285 species, the placozoan strain H13 (REF?), and thus it was expected to contain many more or longer genes
 286 than were present in the original Triad1 annotation. Because of this, we remade a gene annotation for
 287 five of the species (see Methods) and used two additional publicly available annotations for *N. vectensis*
 288 and *A. queenslandica*. For most species, the reannotation dramatically increased the total amount of exons
 289 as well as the total bases of genes (Fig 4). The only exception was *B. floridae*, where the original anno-
 290 tation had predicted 90% of the genome as genes, while the reannotation had annotated only 44.8% as genes.
 291

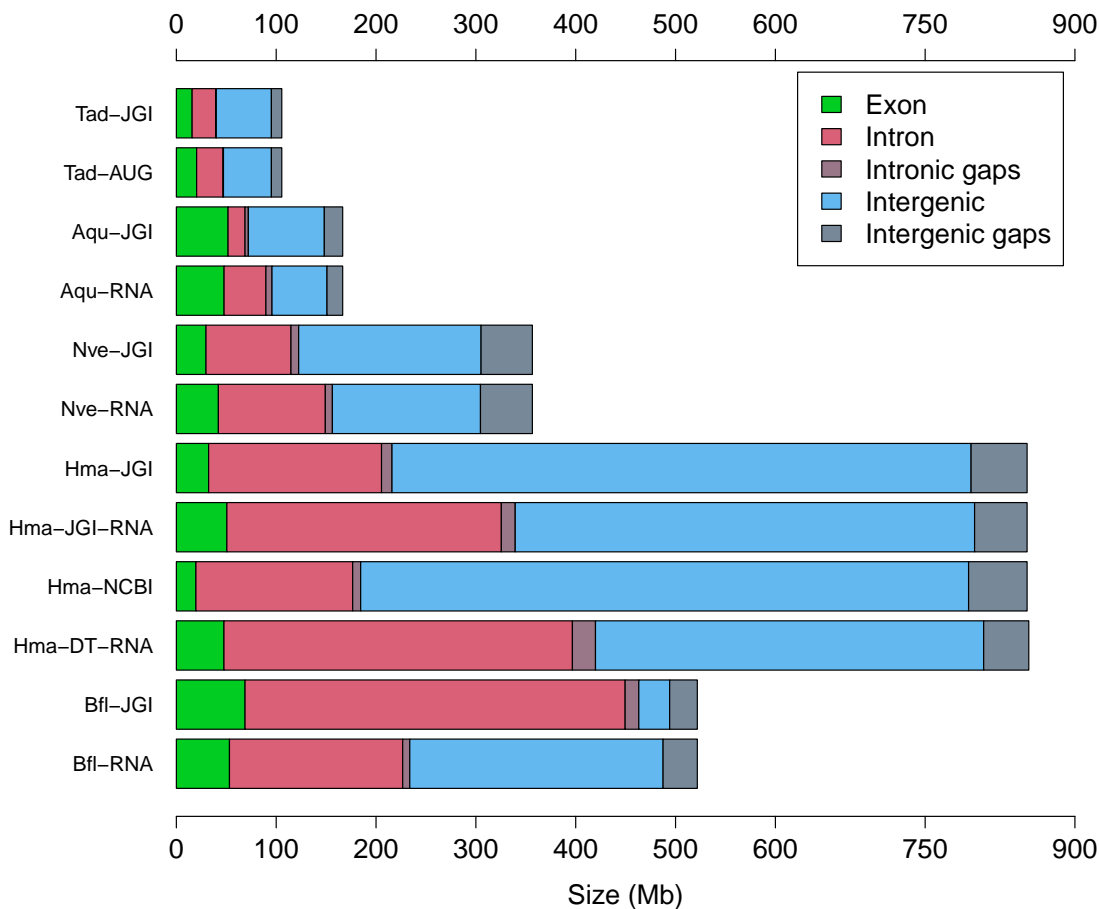


Figure 4: **Proportions of exons, introns, and intergenic sequences** Barplot showing the summed proportions of genomes composed of exons (green), introns (red) and intergenic sequences (blue). The reannotation for *O. bimaculoides* was not shown for clarity, as this genome is substantially larger than the others. Abbreviations are as follows: Tad:*T. adherens*, Aqu:*A. queenslandica*, Nve:*N. vectensis*, Hma:*H. magnipapillata*, Bfl:*B. floridae*. JGI refers to the original annotations for each species downloaded from the JGI Genome Portal. RNA refers to reannotation (see Methods) with RNAseq. Hma-NCBI is the NCBI GNOMON annotation of *H. magnipapillata*. Hma-DT-RNA is the Dovetail reassembly of *H. magnipapillata* annotated with RNAseq. AUG is the reannotation using AUGUSTUS for *T. adherens*.

292 We then compared the ratio of intron:intergenic sequence across seven of the reannotated species (Fig 5).
 293 Across these species, reannotation significantly shifted the ratio of intron:intergenic sequence, approaching a
 294 1:1 ratio (difference from 1:1 ratio, paired two-end t-test, p-value: 0.014). For *M. brevicollis*, the genome is

295 very small and the majority is exons, so the reannotation was likely to change gene boundaries (separating
 296 run-on genes) rather than defining many new genes; our reannotation contains 10,864 genes compared to
 297 the 9,196 genes in Monbr1 “best models”.
 298

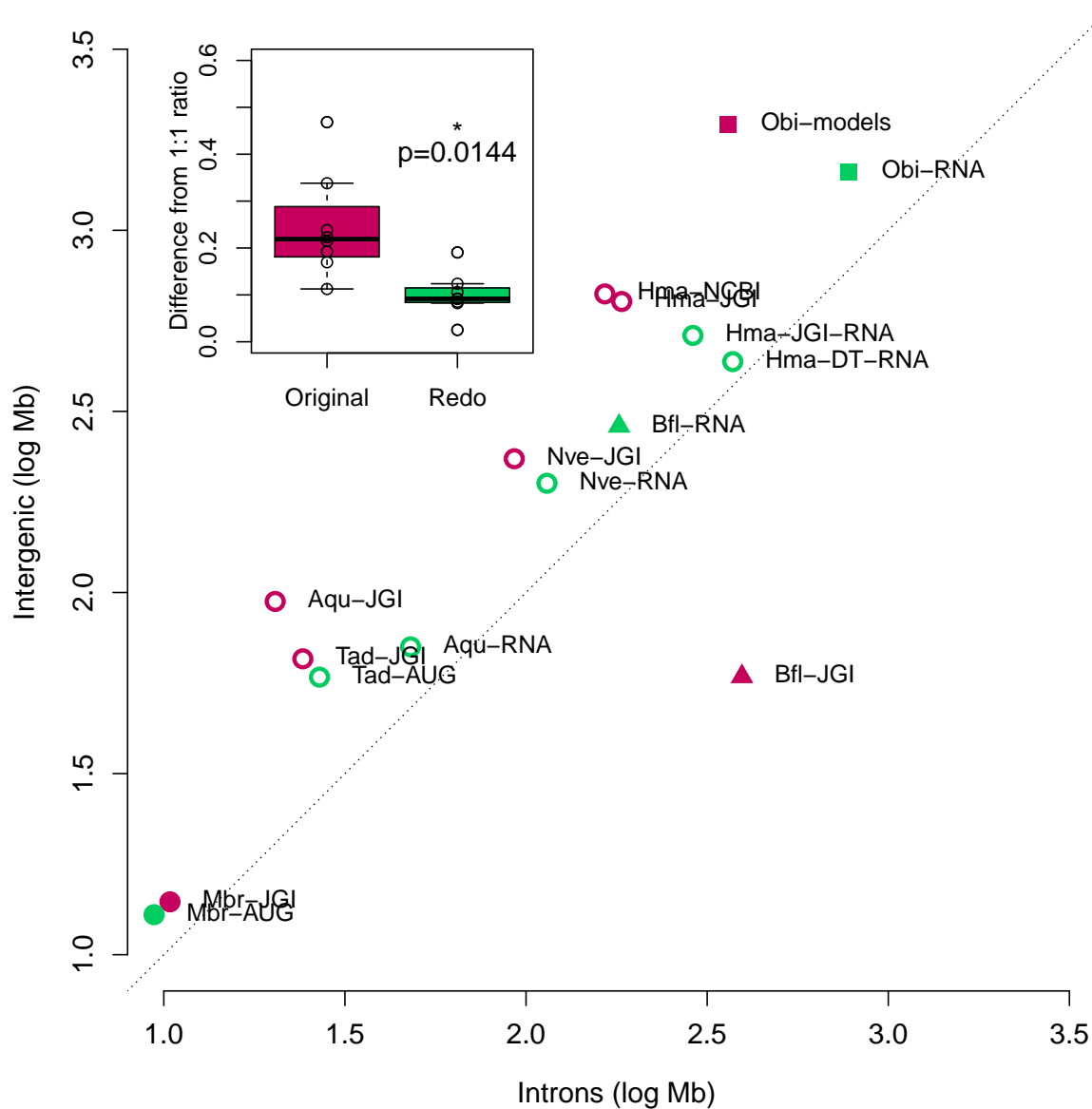


Figure 5: **Improvements from reannotation** Log-scale plot of total intronic size versus total intergenic size where original annotations from the published genomes are shown in red and reannotations are shown in green. The dotted line shows a ratio of 1:1 as a reference. Abbreviations are as in Fig 4, with the addition of Mbr: *M. brevicollis* from the original JGI annotation and the redo with AUGUSTUS, and Obi: *O. bimaculoides* from the published gene models and the reannotation with Tophat/StringTie. The inset graph shows box plot of difference of the intron:intergenic ratio to 1, showing the reannotated genomes (green) are significantly closer than the original version (paired two-end t-test, p-value: 0.0144).

299 Basic trends related to genome size

300 We observed linear correlations of total genome size to both total intronic size and intergenic size (Fig 6)
301 (p-value: $< 10^{-37}$ for both parameters). A much weaker correlation is observed for exons (R-squared:0.3856,
302 p-value: 10^{-8}). Because the the total amount of exons in the largest genomes can be several times greater
303 than the total size of the smallest genomes used in the study, a correlation is likely to be observed. Thus,
304 the total amount of exons is necessarily affected by total genome size, even if this is not strongly correlated.
305

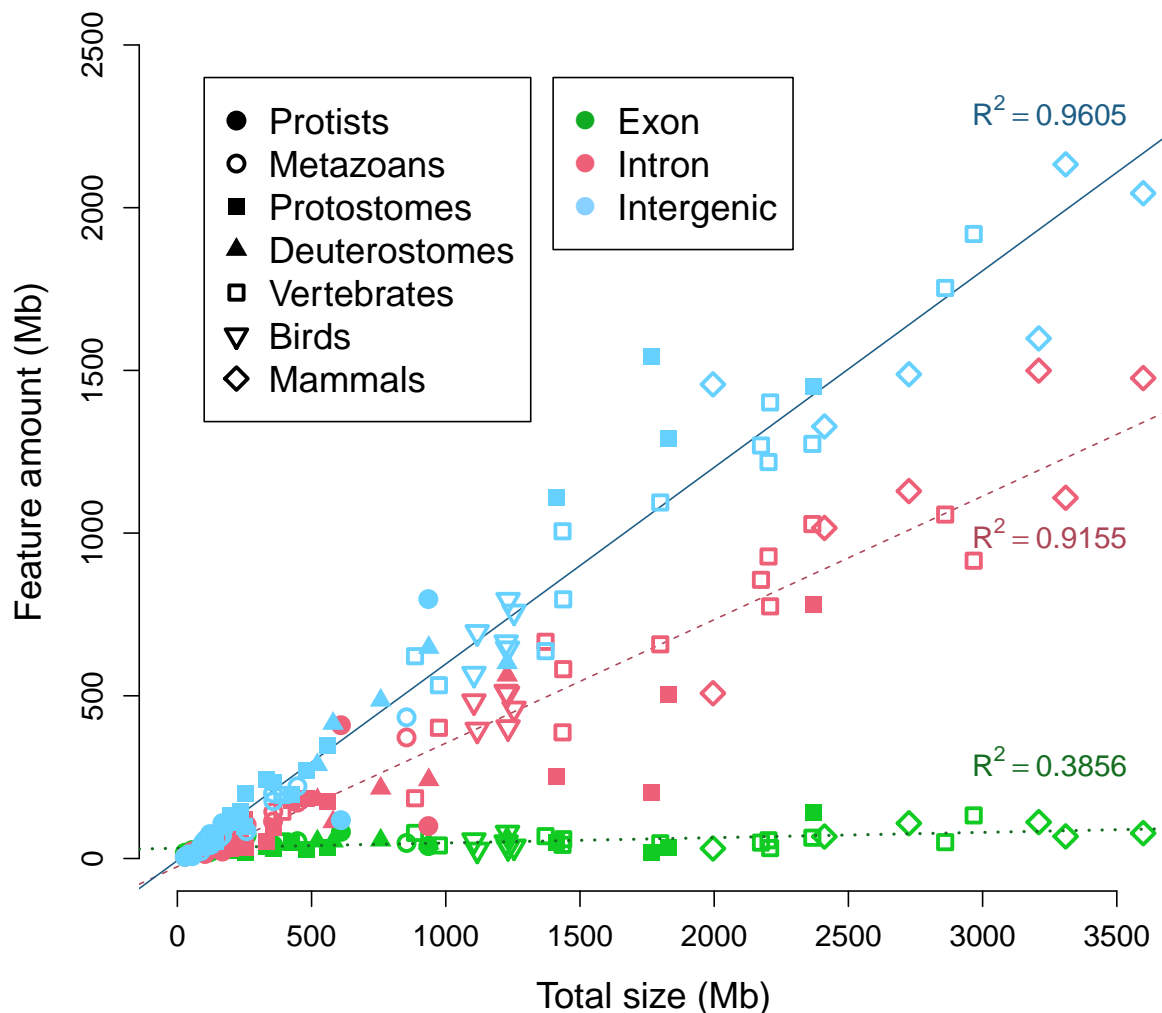


Figure 6: **Comparison of features to total genome size** The sums of exons, introns, and intergenic regions are plotted against total genome size. Linear correlation coefficients of the three features are displayed by their respective lines. For legend symbols, Deuterostomes refers to all invertebrate deuterostomes, Vertebrates excludes Reptiles, Birds and Mammals.

306 Average intron and exon length

307 The average length of introns linearly scales with the total genome size (Fig 7), in agreement with another
308 study [18]. However, the average exon length is clearly constrained across animals relative to total genome
309 size, and this may be related to interactions with nucleosomes [85]. Most species have an average exon

310 length between 200 and 300 bases (mean of 263bp), higher than values reported from previous surveys of
311 exon length [21,86]. It must be stated that the average values presented here should not be taken as final,
312 because variations in format of the annotations and quality of the genomes will affect the values. Since many
313 genomes are only annotated with *ab initio* gene predictions, UTR exons may be missing from the annotation
314 and all downstream calculations. Given that the first exon and intron tend to be longer than other exons
315 and introns [21], respectively, absence of five-prime UTRs may result in an underestimation of the average
316 exon length for that species.

317

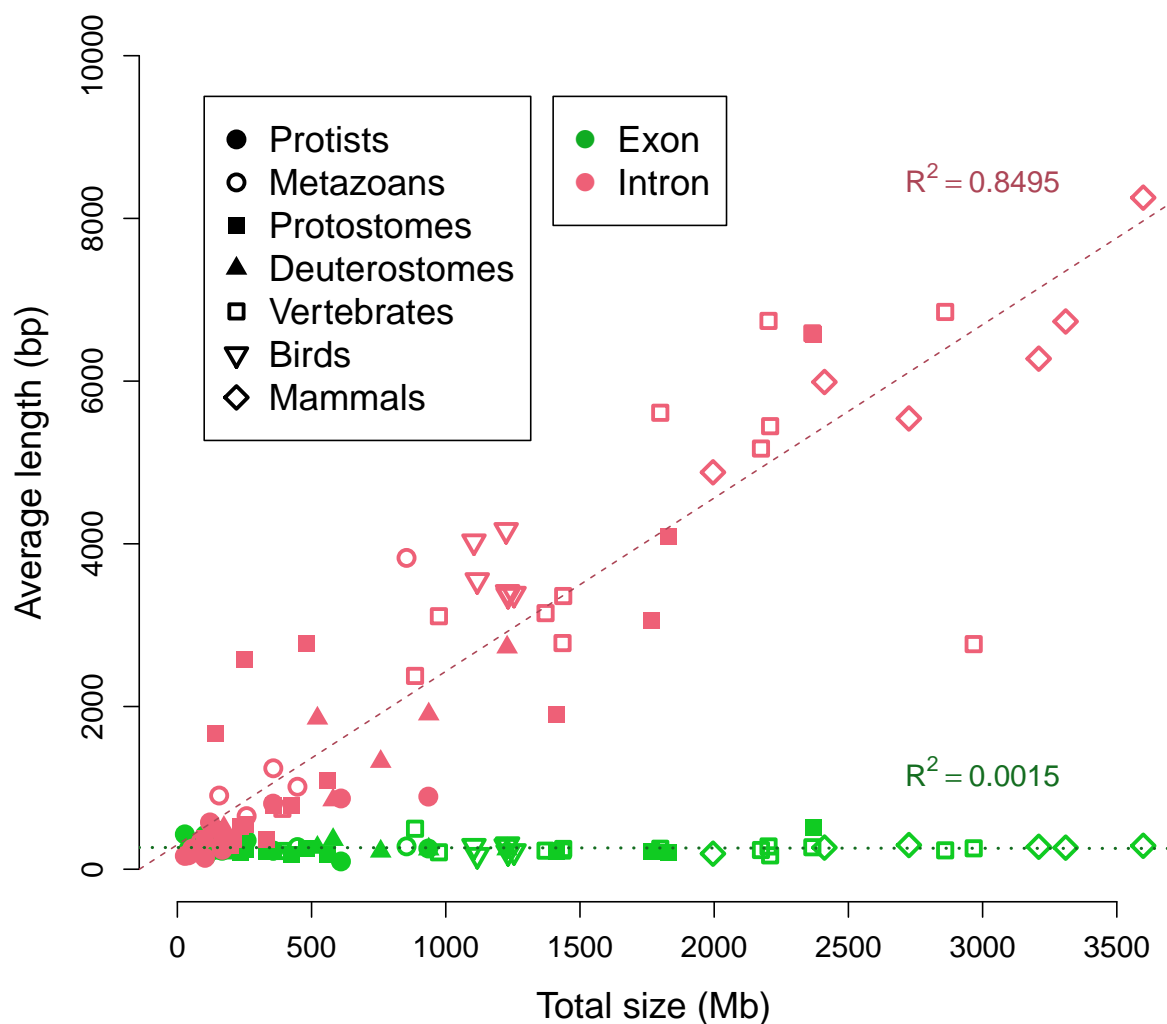


Figure 7: **Average length of exons and introns** Plot of the average length of exons (green) and introns (pink) as a function of total genome size across all species in this study. Linear correlation coefficients are displayed next to the green (dotted) and red (dashed) linear fit lines, for exons and introns, respectively.

318 Nature of the exonic fraction

319 The total amount of exons is not strongly correlated with total genome size (as seen in Fig 6). However, there
320 is a hyperbolic correlation of the relative fraction of exons (megabases of exons divided by total megabases)
321 compared to total genome size (Fig 8). The smallest genomes are dominated by exons, while the largest
322 genomes are dominated by introns and intergenic regions. This implies a relatively fixed pool of exons or

323 coding space that becomes spread over the genome as the total size increases. The hyperbolic trend resembled
 324 the observed hyperbolic relationship between total genome size and coding proportion [18]. As coding
 325 exons are a subset of total exons, measurements of total exons may be a reasonable approximation of coding
 326 sequence, but not necessarily vice versa.
 327

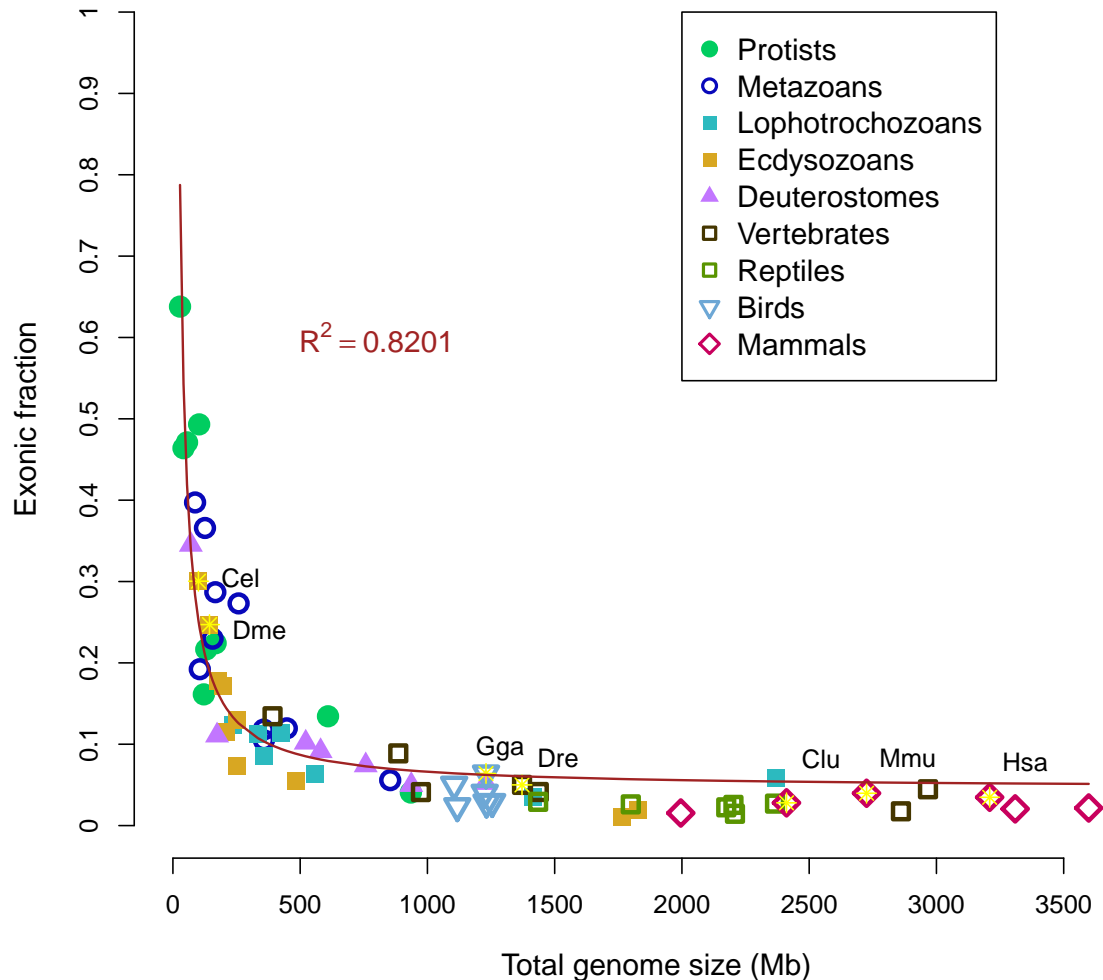


Figure 8: **Exonic fraction compared to total genome size** Relative fraction of the genome that is defined as exons compared as a function of total size. Correlation coefficient of a hyperbolic model is displayed. Seven model organisms (human, mouse, dog, chicken, zebrafish, fruit fly and nematode) are indicated by the yellow stars.

328 Ratio of introns to intergenic

329 Because both intronic and intergenic fractions displayed a linear correlation to total genome size (Fig 6),
 330 we next examined the connection between the two fractions. While many species have a ratio of in-
 331 trons:intergenic approaching 1:1 (R-squared: 0.8286, p-value: 5.6×10^{-27}), the majority of genomes are
 332 composed of sequence annotated as intergenic regions (Fig 9).
 333

334 Because of the potential issue of gene annotation accuracy, we tested the linear correlation of in-

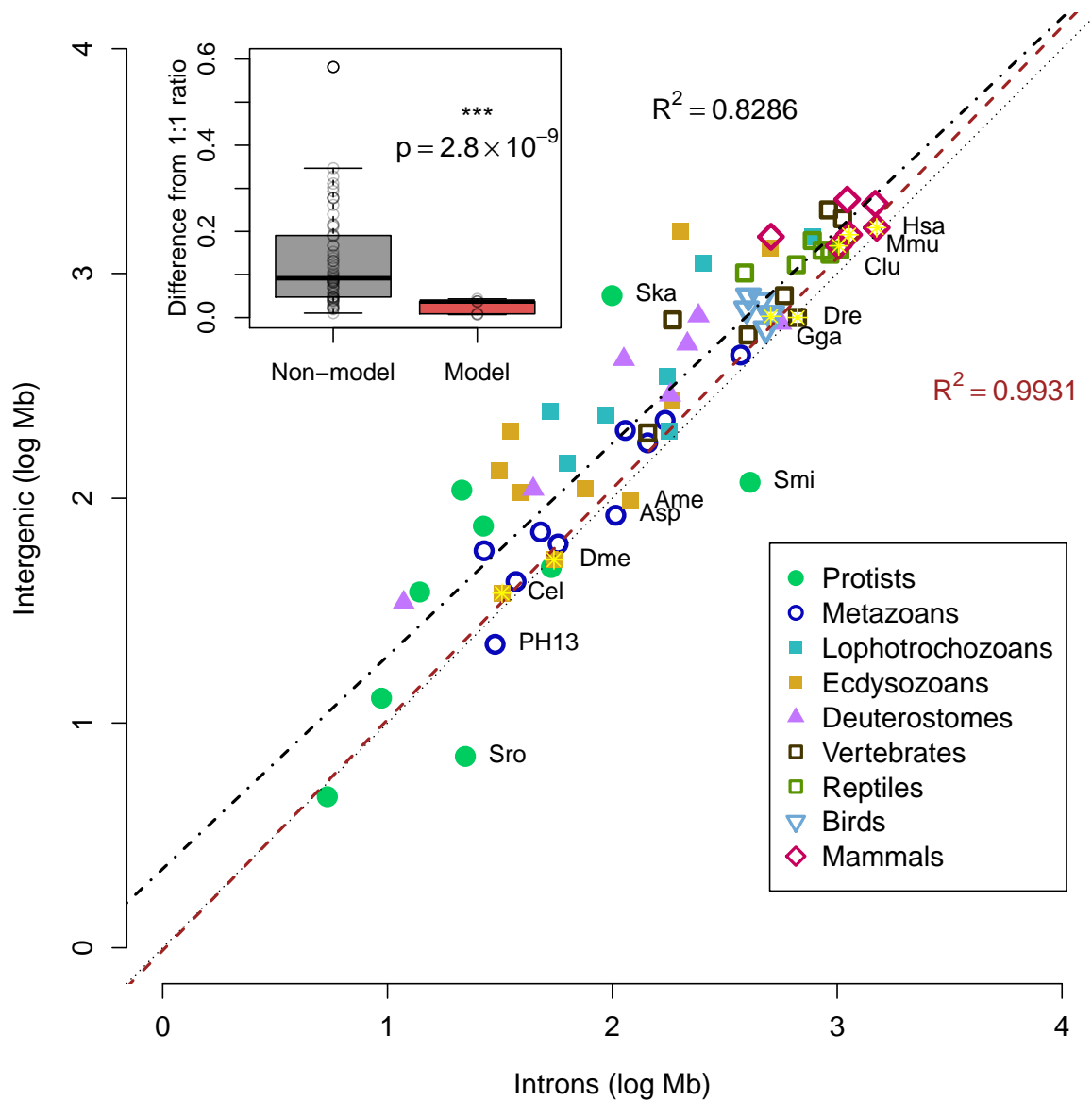


Figure 9: **Comparing intronic and intergenic fractions** Log-scale plot of total intronic size versus total intergenic size. The dotted line shows a ratio of 1:1 as a reference, although most genomes are above this line. Seven model organisms (as in Fig 8) are indicated by the yellow stars. Black dashed line displays the linear fit of all species in the study (R-squared: 0.8286, p-value: 5.6×10^{-27}), while the red line displays the linear fit for only the seven model organisms (R-squared: 0.9931, p-value: 1.3×10^{-6}). Names are displayed for model species, two dinoflagellates (Ska: *S. kawagutii*, Smi: *S. minutum*) and select species with ratios of intron:intergenic greater than 1, choanoflagellate *S. rosetta* (Sro), honeybee *A. mellifera* (Ame), anemone *Aiptasia sp.* (Asp), and Undescribed placozoan H13 (PH13). All other species names are omitted for clarity. The inset graph shows box plot of difference of the intron:intergenic ratio to 1, showing the model organisms (red) have significantly different ratios compared to the rest of the genomes (paired two-end t-test, p-value: 2.8×10^{-9}).

335 trons:intergenic sequence for seven model organisms likely to have accurate annotations. A better linear
 336 fit was observed when restricted to the model organisms (R-squared: 0.9931, p-value= 1.3×10^{-6}), sug-

337 gesting that deviations from the 1:1 ratio of intron:intergenic sequence are due to missing annotations,
338 rather than biological differences. Genomes of model organisms are significantly closer to the reference line
339 (two-tailed t-test, p-value: $< 10^{-7}$ for both absolute distance from 1:1 reference or absolute difference of
340 intron:intergenic ratio to 1), suggesting that the better annotations of model organisms predict a ratio of
341 1:1 of intron:intergenic sequence. Overall, the comparison of genomes of model to non-model organisms is
342 compatible with the hypothesis that the predicted amount of the genome that is transcribed varies more by
343 annotation quality than biological differences.

344
345 We then examined if there is a difference between genomes of vertebrates and invertebrates. No sig-
346 nificance difference is observed between the two model invertebrates and five vertebrates (two-tailed t-test,
347 p-value:0.99). Among all species in the study, significant differences are tenuous and highly dependent on
348 the species selected. For example, chordates against non-chordates is not significant (p-value:0.128) while
349 vertebrates against invertebrates is significant (p-value:0.008). However, the observed significance appears
350 to be an artifact of the abundance of low-quality genomes of protostomes, since comparison of vertebrates
351 against non-bilaterians is not significant (p-value:0.83). This difference is most simply explained by the
352 similarity between vertebrate groups. That is to say, annotation of a new mammalian genome is facilitated
353 by existing knowledge of gene structures in other mammals.

354
355 Several genomes are below the 1:1 reference line, indicating slightly more introns than intergenic, such
356 as the choanoflagellate *S. rosetta*, the honeybee *A. mellifera*, the anemone *Aiptasia sp.*, and Undescribed
357 placozoan H13. For *A. mellifera*, it was noted that improvements in versions of the genome also included
358 better placement of repetitive intergenic sequences [71], suggesting that the relative surplus of introns is
359 merely due to the absence of some intergenic sequences in the final assembly. As for *Aiptasia sp.* and Unde-
360 scribed placozoan H13, these species stand out as having relatively high heterozygosity, 0.4% [87] and 1.8%
361 (manuscript in preparation), respectively. Although these values are lower than the observed heterozygosity
362 in many other invertebrates [88], some highly heterozygous sequences may have caused assembly problems
363 during scaffolding (as proposed in Fig 3).

365 Evolution of the genic fraction

366 The amount of the genome that is composed of genes was highly variable across the genomes in our study,
367 ranging from 12.5% up to 87.1% of the genome. Unlike the exonic fraction, the relationship of the fraction
368 of the genome that is genes to the total size is less obvious (Fig 10), in part because this parameter is most
369 subject to gene annotation accuracy. The fraction of the genome that is exons (and perhaps coding) ap-
370 peared relatively fixed (Fig 8), yet the fraction that is intron was linearly correlated to the total size (Fig 6),
371 therefore the fraction that is genes (exons and introns combined) was expected be a combination of the two
372 modes. Three correlation models were tested: hyperbolic (double-log), exponential (single-log), and linear.
373 Of these, the hyperbolic model fit best (R-square: 0.3649, p-value: $< 10^{-8}$), and no correlation was found
374 for the other models. Restricting the linear model to only genomes larger than 500Mb found essentially no
375 correlation (R-squared: $2.5 * 10^{-4}$), suggesting that the genic fraction is unrelated to total genome size in
376 large genomes but not small genomes.

377
378 Again, the importance of gene annotation accuracy cannot be ignored and needs to be emphasized. When
379 restricting to the seven model organisms, the range of values is narrower, from 44.9% to 62.9%. The same
380 three correlation models were applied to the genomes of model organisms, again finding that the hyperbolic
381 model best explained the variation in the genic fraction of model organisms (hyperbolic R-squared: 0.8091,
382 p-value=0.0058; exponential R-squared=0.6709; linear R-squared=0.6835). Rather than simply having no
383 correlation to total size, these results suggest that the genic fraction is fixed at around 50% in large genomes.

384

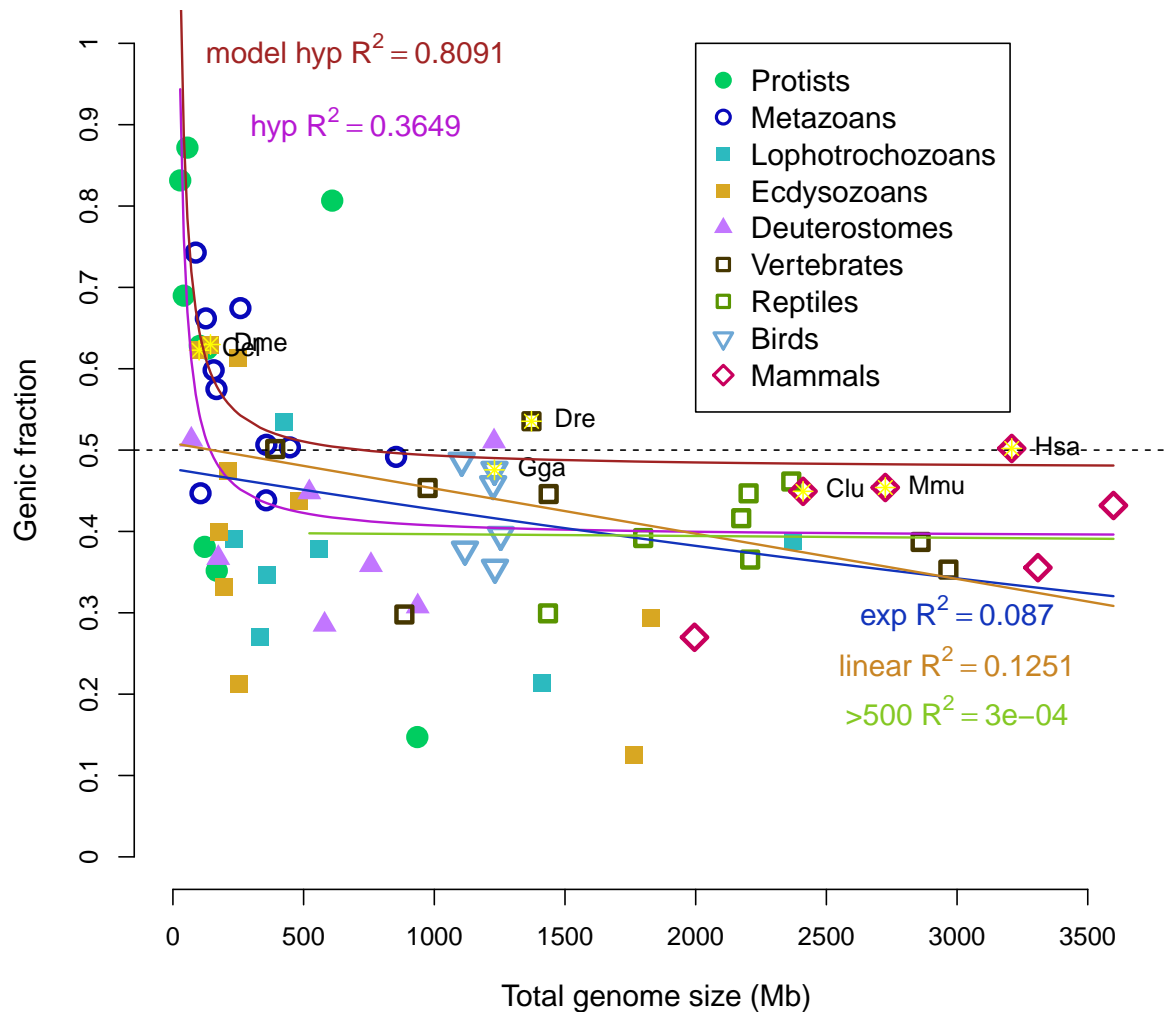


Figure 10: **Genic fraction compared to total genome size** Relative fraction of the genome that is defined as genes compared as a function of total size. A number of correlative models (hyperbolic in purple, exponential in blue, linear in orange) were tested and coefficients are displayed. Linear correlation is expected to be zero if genic and intergenic fractions “expand” indifferently after a certain size, which appears to be around 500Mb. Linear correlation including only genomes larger than 500Mb is also displayed as the green line. Seven model organisms (as in Fig 8) are indicated by the yellow stars. The hyperbolic correlation model for the seven model organisms is shown in red.

385 Discussion

386 Diagnostic relationship of introns to intergenic sequence

387 An increasing number of genomes of any non-model organisms are sequenced to answer evolutionary ques-
388 tions. For example, genomes of taxa from all four non-bilaterian groups were recently sequenced to under-
389 stand how similar these genomes are to humans [8,24,33,34], and found that we share much more in terms of
390 genes with these groups than had been previously thought. Yet, one of the main challenges in studying the
391 genomes of non-model organisms is that there is little *a priori* information about gene structure or content.
392 It would be expected that finding orthologs of human genes is relatively easy, but does not inform us about
393 other genes that differ from humans. How should we know when we have found all of the genes? Our results
394 provide some guidance here and suggest that there is a constant ratio of introns to intergenic sequence in
395 all animals. This relationship holds even for animals with small genomes, such as the model organisms *D.*
396 *melanogaster* and *C. elegans*, suggesting that organisms with small genomes and many currently sequenced
397 invertebrates are subject to the same forces as organisms with large genomes.

399 Unusual cases of genomes

400 Based on our model, the majority of genomes appear to be underannotated, in that substantial portions of
401 the genome are not predicted to be transcribed when in fact many probably are. However, only two species,
402 the lancelet *B. floridae* and the dinoflagellate *S. minutum*, display a dramatic trend in the opposite way,
403 that is, the majority of the genome is annotated as genic (being primarily introns).

404
405 For the lancelet *B. floridae*, the original JGI gene models had annotated almost 90% of the genome as
406 genes [23], the majority (85%) of that sequence being introns. Our reannotation of this genome displays the
407 opposite trend, where more of the genome is intergenic than intronic. The original JGI annotations did not
408 include any validation of the predicted genes, as predictions were made using mapped ESTs only as inputs
409 for the gene model training. From this, we consider it more likely that the RNAseq-based transcripts more
410 accurately resemble the true gene structures, albeit missing some genes. However, other evidence suggests
411 that the *B. floridae* annotations may have been unusual or erroneous [89]. A study of domain combinations
412 found that *B. floridae* had by far more fusions than any other species (across all eukaryotes) and had to be
413 excluded from the analysis [90], precisely the expected result if the majority of genes were erroneously fused.

414
415 The only other species have a much larger ratio of intron to intergenic was the dinoflagellate *S. minutum*.
416 It was described that its genome contained many long stretches of genes on the same strand, sometimes
417 continuing for hundreds of kilobases [41]. The authors also note that the *de novo* assembled transcriptome
418 appears to contain transcripts spanning multiple genes and containing multiple open reading frames, indi-
419 cating the possibility that dinoflagellate symbionts can make cistronic transcripts. This species is not an
420 animal, so it should not be assumed that animal modes of transcription are conserved across all eukaryotes.
421 However, it should be noted that a recently published genome of another symbiotic dinoflagellate species *S.*
422 *kawagutii* [40] does not display the same pattern, and instead appears to have a much greater fraction of
423 intergenic regions than introns.

425 Genome composition across metazoa

426 Previous studies have discussed problems with trying to relate the number of genes to the size of the
427 genome [91–93]. One study [18] found a weak positive correlation between genome size and number of genes.
428 This parallels our finding that total exonic sequence is weakly correlated to total genome size (Fig 6). How-
429 ever, this measurement can be problematic if the genome assembly is highly fragmented, containing a large
430 number of short contigs or scaffolds. In such cases, gene number is unlikely to a relationship to genome
431 size for the same reason as the difficulties in predicting the genic fraction, that is, it is strongly affected by
432 gene annotation errors. In our schematic (Fig 2), a gene that is split up onto three contigs would therefore
433 be counted as three genes, albeit short ones. If this occurs on a genome-wide scale, the count of genes will

434 be inaccurate. Parts of genes would be individually annotated as genes, increasing the total gene number
435 without much change to the total number of exonic bases.

436
437 Rather than relying on counts of genes or determining coding sequence, we instead examined sequence
438 that is annotated as exons. We found that the exonic fraction is largely unchanged with the total size of
439 the genome, showing that most of the difference in size is related to introns and intergenic sequence. The
440 amount of the genome that is composed of introns is linearly related to the total genome size (Fig 6). Also
441 considering the measured linear correlation of intergenic sequence to total size, it is not surprising that most
442 species have roughly a 1:1 ratio of introns:intergenic sequence (Fig 9). This appears to be the case regardless
443 of the size of the genome or the total exon sequence. For instance, the genome of the choanoflagellate *M. bre-*
444 *vicollis* has 9.3Mb of introns and 10.1Mb of intergenic sequence (a ratio of 0.92) compared to 19.3Mb of exons.

445
446 Therefore, model animals (and probably all animals) transcribe at least half of the genome, where species
447 with smaller genomes transcribe more than half. There does not appear to be a significant difference in the
448 genic fraction based on animal group, that is, all animals appear to follow this rule. One study had shown
449 that some larger metazoan genomes were depleted in genes [94], yet this study made use of a small number
450 of species for comparison and included several chordates known for their very small genomes, the tunicate *C.*
451 *intestinalis* and the pufferfish *T. rubripes*. The authors examined windows of 50kb and found that 80% of the
452 human genome was lacking any gene [94], though it is unclear if this analysis was restricted to protein cod-
453 ing genes. However, we found that 50.2% of the human genome is composed of genes (93% of that is introns).

454
455 A large number of the genomes in this study appear to be composed of less than 50% genes. We propose
456 that the observed data are compatible with the hypothesis that most genomes are missing genes, which may
457 be coding (perhaps lineage-specific proteins) or not. Because annotation of the genome by RNAseq per se
458 cannot distinguish coding genes from non-coding ones, the coding fraction may still contribute heavily to the
459 total amount of exons. Even for putative non-coding transcripts, some may be coding [95–97], thus protein
460 sequencing may reveal the true nature of these transcripts.

462 Evolution of genomes

463 The genic fraction has a hyperbolic relationship to the total genome size. The modeled curve flattens around
464 500Mb, after that point, introns and intergenic regions are expected to expand, on average, equally across
465 the genome resulting in approximately 50% of the genome as genes (the majority of that being introns) and
466 the other 50% as intergenic sequence.

467
468 It has been theorized that changes in genome size are a balance between short deletions and long in-
469 sertions [98]. If the last common ancestor of all metazoans had a relatively small genome (under 100Mb,
470 resembling some single-cell eukaryotes in our study), then the majority of modern animals have undergone
471 dramatic expansion of their genomes, meaning dominated by insertions or duplications. How does this ex-
472 pansion occur and does it favor a novel origin of introns or expansion of intergenic sequences? Following
473 the trend in Fig 9 and Fig 10, it appears that small genomes are dominated by genes, and both genes and
474 intergenic sequences are expanded in equally as the genomes enlarge. Mechanistically, these insertions are
475 likely to be mediated by transposable elements. As small genomes become invaded by transposable ele-
476 ments, introns appear and expand at roughly the same rate as intergenic sequences producing a 1:1 ratio of
477 intron:intergenic across all species (Fig 9).

478
479 Above a certain size (around 500Mb), genic and intergenic sequences expand equally, where 50% of the
480 genome is genic; exons comprise an almost negligible fraction of the genome, which is otherwise composed
481 of approximately equal fractions of introns and intergenic sequences. This might be explained by changes
482 in diversity of transposable elements, as the highest diversity was found in genomes ranging from 500Mb to
483 1.5Gb [17]. Larger genomes appeared to be flooded by transposable elements of a single type. Thus, above
484 500Mb, it can be predicted that select transposable elements become prevalent and multiply throughout the
485 genome, but on average end up expanding introns and intergenic sequences equally.

486

487 Relationship to phenotypic complexity

488 The size of the genome can vary greatly even for closely related organisms. This has been called the “c-value
489 paradox” [1,99], based on the observation that although the many organisms have larger genomes relative to
490 similar species (bigger “c-value”), this measurement does not relate to complexity in a straightforward way.
491 A classic example of this is frog genus *Xenopus*, where the genome of the species *X. laevis* is almost twice
492 as large as the species *X. tropicalis* [100], though the animal is not twice as “complex”. Similar observations
493 have been made that the number of genes appears unrelated to the size of the genome and the complexity
494 (sometimes called the “g-value paradox” [91,101]).

495

496 If neither genome size nor gene number are clearly related to complexity, then what is? Another relation-
497 ship has been proposed between the usage of alternative splice variants and organismic complexity because
498 variation in splicing can increase the number of potential proteins from an overall fixed pool of exons [102].
499 Vertebrates and specifically mammals tend to splice transcripts more than invertebrates (meaning models
500 fruit fly and nematode) [103,104]. One study reported a good correlation (R-squared of 0.80) of splicing to
501 organismic complexity measured by cell types [105], but also reported that this trend effectively disappeared
502 when correcting for sequencing depth, using the number of ESTs available as a proxy for annotation quality.
503 The largest invertebrate genome used in that study was the deer tick *I. scapularis*, which did have a mea-
504 sured number of cell types but unfortunately could not be analyzed further, leaving the bulk of the analysis
505 weighted heavily by mammals and small-genome insects.

506

507 However, other studies report that alternative splicing is more frequent when the surrounding introns
508 are long [106,107], suggesting that organisms with large genomes (and therefore larger introns) might be
509 predisposed to splice. This could suggest that some of the invertebrates in our study may have more complex
510 splicing patterns than are annotated in the current genome versions. For the largest invertebrate genome in
511 our study, the octopus *O. bimaculoides*, only 14.8% of loci appeared to have alternative splice variants [45].
512 Indeed, in our reannotation we found only 16.9% of loci have any type of splice variant. However, the ma-
513 jority of predicted loci are single exon (75%), possibly many genes are fragmented across multiple contigs.
514 When restricted to loci with multiple exons, 68% have more than one variant. These data from *O. bimac-*
515 *uloides* seem to contradict the role of splicing in complexity, or more specifically, that overall patterns in
516 splicing do not display a reliable connection to organismic complexity when complexity is generalized across
517 animal groups. However, without proper measurements of cell types from the octopus, it cannot be assumed
518 that the number of cell types resembles the value for the fruit fly, which was implicit in other studies given
519 that protostomes were effectively represented by insects. Thus, it could be the case that the octopus has a
520 large genome, a large number of cell types, and many genes are spliced, all in agreement with the splicing
521 complexity hypothesis.

522

523 It is a challenge to separate these observations from biases in sequencing depth (of transcripts or ESTs)
524 and data availability. In our study, we could only make use of five invertebrates with relatively large genomes,
525 the cnidarian *H. magnipapillata*, the pearl oyster *P. fucata*, the horseshoe crab *L. polyphemus*, the deer tick
526 *I. scapularis*, and the octopus *O. bimaculoides*. On the other hand, NCBI has over 100 genomes of mammals
527 available for download. Alternatively, the repertoire of splice factors or the genes that are most spliced may
528 be of greater importance than just splicing in general. Our understanding is likely to be improved with more
529 deeply-sequenced transcriptomes from large-genome invertebrates.

530 Limitations

531 Because we were making use of mostly public data, our analyses were subject to both technical and biologi-
532 cal limitations. There are a small number of taxa with sequenced genomes from many invertebrate groups.
533 Because the majority of sequenced vertebrate genomes are large and the majority of sequenced invertebrate
534 genomes are small [92], the axis of simple invertebrate to complex vertebrate is synonymous with small to
535 large genomes, and thus the prevalence of splicing in large-genome animals may be a consequence of the size
536 of the genome and complexity may be only correlated. This issue is not simple to resolve, as there may not

537 be members in all animal groups with both small and large genomes. For instance, a survey of genome sizes
538 across Porifera stated that the largest genome out of the 70 species sampled was around 600Mb [108]. Thus,
539 there may not be any “large” genomes in this phylum, and likewise for other invertebrate groups. Compared
540 to birds, however, where the smallest genome identified to date is from the black-chinned hummingbird
541 (estimated 910Mb) [109], perhaps no bird will be found that has a “small” genome.

542

543 Conclusion

544 We have shown that all animals transcribe at least half of their genomes in a size-dependent fashion. For large
545 genomes, the amount of exons is almost negligible, where introns account for most of the genic sequence. In
546 such cases, genic sequence is almost equal to the amount of intergenic sequence. Whereas for small genomes,
547 exons can be a major fraction of the genome, resulting in the appearance of gene-dense genomes. This
548 parity between introns and intergenic sequence is a universal feature of animal genomes, and indicates that
549 most genomes could benefit from new annotations. Previous findings of genomic differences between animal
550 groups are likely to result from a sampling bias, rather than biological differences. Future sequencing of more
551 high-quality genomes from animals may reveal unanticipated sources of complexity and gene regulation with
552 implications for the evolution of animals.

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