

Supplementary material for: non-coding RNAs of bird genomes

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Supplementary results

In the following we explore in further detail the results that were not discussed in the main manuscript.

Classic RNAs: LUCA and LECA

Many RNA families constitute the most evolutionarily conserved genes across all life on this planet [1]. Examples of RNAs derived from the Last Universal Common Ancestor (LUCA) include, the transfer RNAs (tRNA), ribosomal RNAs (rRNA), RNA components of RNase P (RNase P RNA), RNase MRP (RNase MRP RNA) and the signal recognition particle (SRP RNA). Other classes of RNA are likely to have been components of the Last Eukaryotic Common Ancestor (LECA) include the telomerase RNA, major spliceosomal RNAs (U1, U2, U4, U5, and U6) and the minor spliceosomal RNAs (U11, U12, U4atac, and U6atac) [2].

Unsurprisingly, the bulk of these classes of RNAs are well represented across the bird genomes (See Figure 2). However, there appear to have been “losses” of a few of these RNAs in certain bird species. Some of these may be due to sequence divergence, of which there are several notable examples e.g. [3–7]. Other losses may be due to incomplete genome coverage.

A number of the classic RNAs are incorporated into RNA-protein complexes (RNPs) involved in core

cellular processes. An example of this are the spliceosomal RNAs. Based upon the presence/absence patterns of the major spliceosomal RNAs they are all well represented in these genome sequences. The exceptions to this observation are the U4 RNA in cormorant and the U5 RNA in the bee eater which are both missing. These two genomes are low coverage, suggesting these genes weren't captured in the current assembly. The minor spliceosomal RNAs are more interesting, the U4atac and U11 snRNAs show widespread patterns of loss, even in some of the high coverage genomes. These RNAs are frequently missed in bioinformatic screens. Indicating either frequent loss [8] or sequences that have diverged beyond the ability of detection by covariance models [9].

The telomerase RNA is also largely missing from the avian annotations. This RNA acts as a template for the telomerase enzyme that extends the telomeres found on chromosome ends. It is only found in the chicken, bald eagle, kea, budgerigar, crow and zebrafish. Homology searches with the telomerase reverse transcriptase (TERT) protein show that the protein component of the telomerase RNP is conserved across all the bird genomes (data not shown). This pattern of presumably divergent telomerase RNA and conserved telomerase protein has been noted previously, most notably in the fungi [3, 4].

The RNA components of RNase P and RNase MRP also appear to have undergone dramatic losses within the bird lineage. RNase P is required for the maturation of tRNA, the paralogous enzyme, RNase MRP is required for the maturation of rRNA. Each RNP cleaves smaller RNAs from larger transcripts [10]. It is unlikely that these genes have been lost in any of the birds. Homology searches with the RNase associated protein coding genes (POP1, POP4, POP5, POP7, RPP1, RPP14, RPP25, RPP38, RPP40 and RPR2), identified viable homologs of each in all of the bird genomes [11] (data not shown). This suggests that the bird RNase P and MRP RNAs may have diverged slightly from the canonical models.

The 5.8 S component of the ribosome in the turtle, turkey bustard, hoatzin, flamingo, tropicbird, seriema, owl, cuckoo roller, trogon, bee eater and falcon appears to have been lost (See Figure 2). The rRNA repeats are frequently not assembled, consequently it is not surprising to see "losses" in these [12]. Furthermore, the genomes for these species are also low-coverage.

Small nucleolar RNAs

Small nucleolar RNAs (snoRNAs) are important ncRNAs that participate in the maturation of other functional RNAs [13]. The bulk of the characterised snoRNAs guide either methylation or pseudouridylation modifications, primarily of rRNAs but also spliceosomal RNAs. The two types of modifications are guided by two different types of RNA, the box C/D and the H/ACA snoRNAs

respectively, each with a characteristic cohort of motifs and secondary structures [14].

There are 66 ribosomal modification sites, guided by 59 snoRNA families, that are preserved between *H. sapiens* and *S. cerevisiae* [15]. Of these, 45 snoRNA families are conserved in the bird dataset. Over a third of the apparent losses of the yeast-human conserved snoRNA families appear to cluster on 2 loci of the ancestral vertebrate genome. We investigated these losses further.

The first cluster is found at chr11:62620797-62622484 on the human genome (hg19) and contains SNORD27, SNORD29 and SNORD31 of the human-yeast conserved snoRNAs. These snoRNAs are located in the inside-out gene SNHG1 which hosts a total of eight C/D box snoRNAs: SNORD25, SNORD26, SNORD27, SNORD28, SNORD29, SNORD22, SNORD30 and SNORD31 [16]. Each of which are also found in the alligator and turtle genomes within a 3-4 KB locus, yet these have largely been lost in the birds. Notable exceptions are five of the eight are located in the tinamou genome, these are located on the same scaffold and are within 2 KB of each other. This implies that SNHG1 is conserved in the tinamou. Loci with four of the eight snoRNAs can be found in zebrafish, ground-finch, and bald eagle. Still, three of the eight are located in the ostrich, crow, and cockoo genomes, again within 2 KB of each other on the same scaffolds. This complex pattern of loss could be attributed to many different models, e.g. multiple losses in birds, poor homology modelling or incomplete genome sequences.

The second cluster is located at chr19:49993222-49994231 on the human genome (hg19) and contains two copies of SNORD33 and one SNORD34 all within a 1 KB genomic region. The turtle and alligator genomes retain the two copies of SNORD33 yet don't have an obvious SNORD34 gene on the same scaffold. Within the bird genomes, the crow and rifleman each retain a single SNORD33 and SNORD34 gene on the same scaffold. While the ground-finch and bald eagle retain a single SNORD33 and the zebrafish and seriema retain a single SNORD34 (See Figure 3). In human these snoRNAs are intronic to the host gene, ribosomal protein L13a (RPL13A). Based on BLASTP (version 2.2.18) homology searches for the RPL13A gene, the protein is conserved in the human and turtle genomes and in the bald eagle, crow, rifleman and zebrafish avian genomes (data not shown). Therefore the conservation patterns of the RPL13A host gene and corresponding intronic snoRNAs largely mimic each other, each independently supports a pattern of loss of the RPL13A gene and the intronic snoRNAs that it hosts in the bird genomes.

MicroRNAs

MicroRNAs are an important class of non-coding RNA. They have been found in the genomes of Chromalveolata [17,18], Metazoa [19–21], Mycetozoa [22,23], Viridiplantae [24–27] and Viruses [28–31].

The miRNAs have been shown to regulate the expression of large numbers of messenger RNAs [32]. The mature miRNA product is generally 22 nucleotides long which is usually processed from a larger RNA that is characterised by a stable hairpin-shaped secondary structure.

Chicken and zebrafish are the only birds with previously annotated microRNAs. We searched for homologs of these and other vertebrate microRNAs in the genomes of the 48 birds, American alligator and green turtle. Overall, we annotate a total of 16617 putative microRNA loci, homologous to 543 known microRNA genes, of which 487 are annotated in chicken and/or zebra finch, while 56 have been so far known only in non-avian vertebrates. The numbers of annotated loci in the individual species are approximately equal - 300-400 per species, except for the turkey (*Meleagris gallopavo*) where we identified 543 sequences homologous to known microRNAs.

In addition, we can confidently identify a further 3 microRNA families that are present in mammals, and turtle and/or crocodile, but not in any avian genome (mir-150, mir-208, mir-590). This suggests that these sequences were lost in the last common ancestor of archosaurs or birds. There are also a number of microRNAs that are predicted to be present in turtles and/or crocodiles, and only a small number of bird genomes. Indeed, there are many missing annotations, species-specific and otherwise, that are not consistent with the consensus phylogeny, and could be due to either incomplete genomes or widespread microRNA loss.

The turkey genome contains a high number (190) of microRNAs so far found only in chicken, which account for the higher number of annotated sequences in this genome compared with other birds. This is consistent with its phylogenetic position as the closest chicken relative among the examined birds.

However, 101 chicken microRNAs have no homolog in the turkey or other bird genomes, suggesting that these genes are chicken-specific. This is consistent with previous reports of large number of species-specific microRNAs in all animals, and supports the view of fast microRNA turnover during animal evolution.

Cis-regulatory elements

The cis-regulatory RNAs are a group of RNA structures encoded on mRNAs. Generally they are involved in regulating the expression of the mRNA they are encoded within. Others may recode the translated protein product into an alternate sequence.

This group includes the iron response element (IRE) [33] and the histone 3' UTR (histone3) [34]. These are structured motifs bound by regulatory proteins. The selenocysteine insertion sequence (SECIS) is a structured motif that recodes UGA stop codons to selenocysteines [35] and the GABRA3 stem-loop is a

structure recognised by the ADAR enzyme family. This enzyme edits adenine nucleotides to inosine, in this case recoding an isoleucine codon to methionine in exon 9 of the GABRA3 gene [36].

These regulatory elements and others, including an internal ribosome entry site (IRES), potassium channel RNA editing signal (K chan RES), Antizyme RNA frameshifting stimulation element (Antizyme FSE), vimentin 3' UTR protein-binding region (Vimentin3) and a connective tissue growth factor (CTGF) 3' UTR element (CAESAR) are conserved across a diverse group of vertebrates, including the bird lineages explored here (See Figure 2).

Unexpectedly poorly conserved ncRNAs: testing divergence

In order to get an idea to what extent the absence of these RNAs from the **infernai**-based annotation is caused by sequence divergence beyond the thresholds of the Rfam CMs and/or missing or incomplete data, we complemented our analysis by dedicated searches for a few of these RNA groups.

The simplest case are the selenocysteine tRNAs. Here, tRNAscan is tuned for specificity and thus misses several occurrences that are easily found by **blastn** with $E \leq 10^{-30}$. In some cases the sequences appear degraded at the ends, which may be explained e.g. by low sequence quality at the very ends of contigs or scaffolds. A **blastn** search also readily retrieves additional RNase P and RNase MRP RNAs, capturing only the best conserved regions. In many cases these additional candidates are incomplete or contain undetermined sequence, explaining why they are missed by the CMs. Overall, we identify tRNA-Sec in most and RNase P and MRP RNAs in the majority of the genomes. An additional candidate could also be retrieved for telomerase RNA. Telomerase is well known to exhibit very poor sequence conservation and rapid variations in size that make it notoriously hard to identify by homology search [37]. The poor return thus does not come as a surprise. Vault RNA homology searches with **blastn** remained unsuccessful, therefore we constructed a sauropsid-specific CM. In addition to the hits identified by the Rfam model we obtained three additional homologs. Vault RNAs, with a size of about 100 nt, exhibit conserved sequence patterns only at their ends, with essentially unconstrained sequence in the central part. Their identification is one of the well-known and difficult problems for homology search [38, 39].

Our ability to find additional homologs for several RNA families that fill gaps in the abundance matrices (Figure 1) strongly suggests that conspicuous absences, in particular of LUCA and LECA RNAs, are caused by incomplete data in the current assemblies and sequence divergence rather than true losses.

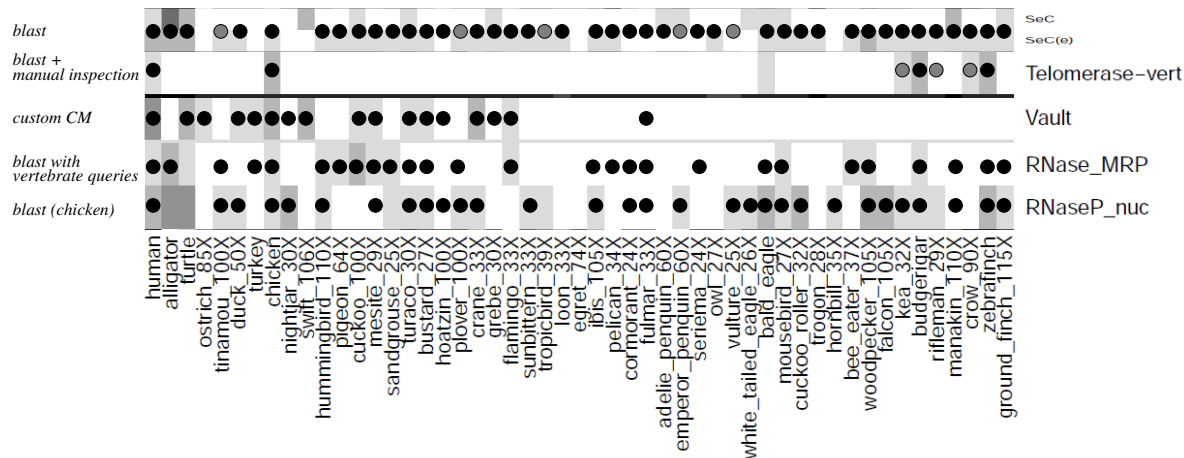


Figure 1: Additional homologs of some sparsely represented RNA families were discovered using dedicated search strategies combined with highly sensitive settings, synteny information, lineage-specific CMs and subsequent manual inspection.

Exceptional RNAs

A number of other ncRNAs can also be found in Eukaryotic genomes. These do not fit into the main classes of RNA but still perform vital roles in the function and evolution of Eukaryotes. Their functions are diverse and many have not yet been characterised.

An example of an uncharacterised RNA is the ultraconserved element, uc.338 (also known as TUC338) [40–42]. The uc.338 element is derived from a short interspersed element (SINE) called the lobe-finned fishes SINE (LF-SINE) as it is conserved between the coelacanth and mammals [41]. Analysis of the expression of uc.338 implies that it plays a role in the progression of hepatocellular carcinoma, possibly by influencing cell growth [42]. This RNA is conserved in the birds and appears to have been duplicated in several lineages.

The Y RNA is an enigmatic ncRNA where we know very little about the function. It was discovered in the 1980s in ribonucleoprotein complexes [43]. The function of the Y RNAs remain unknown, but evidence is emerging that they may be associated with DNA replication [44]. There are 4 functional Y RNAs encoded in the human genome, Y1, Y3, Y4 and Y5. However, there are hundreds of pseudogenised copies of the Y RNA scattered throughout the human genome [45]. In the birds and other lizards, we identify between two and seven Y RNA paralogs (See Figure 2).

The Vault RNA forms a major component of the vault ribonucleoprotein complex, this is one of the largest particles found in the vertebrate cell; In fact, it is larger than the ribosome [46]. As yet not much is known

about the function of Vault. The Vault RNA has been shown to be broadly conserved in metazoans [47]. However, in the bird lineages it appears to have either been lost or diversified.

Contamination

Bacterial families can be used to identify problematic sequences that are likely to be the result of contamination from non-avian sources. We identified a number of RNA families of bacterial origin in the avian genomes. These have been reported and will be dealt with in later updates to the avian genome sequences.

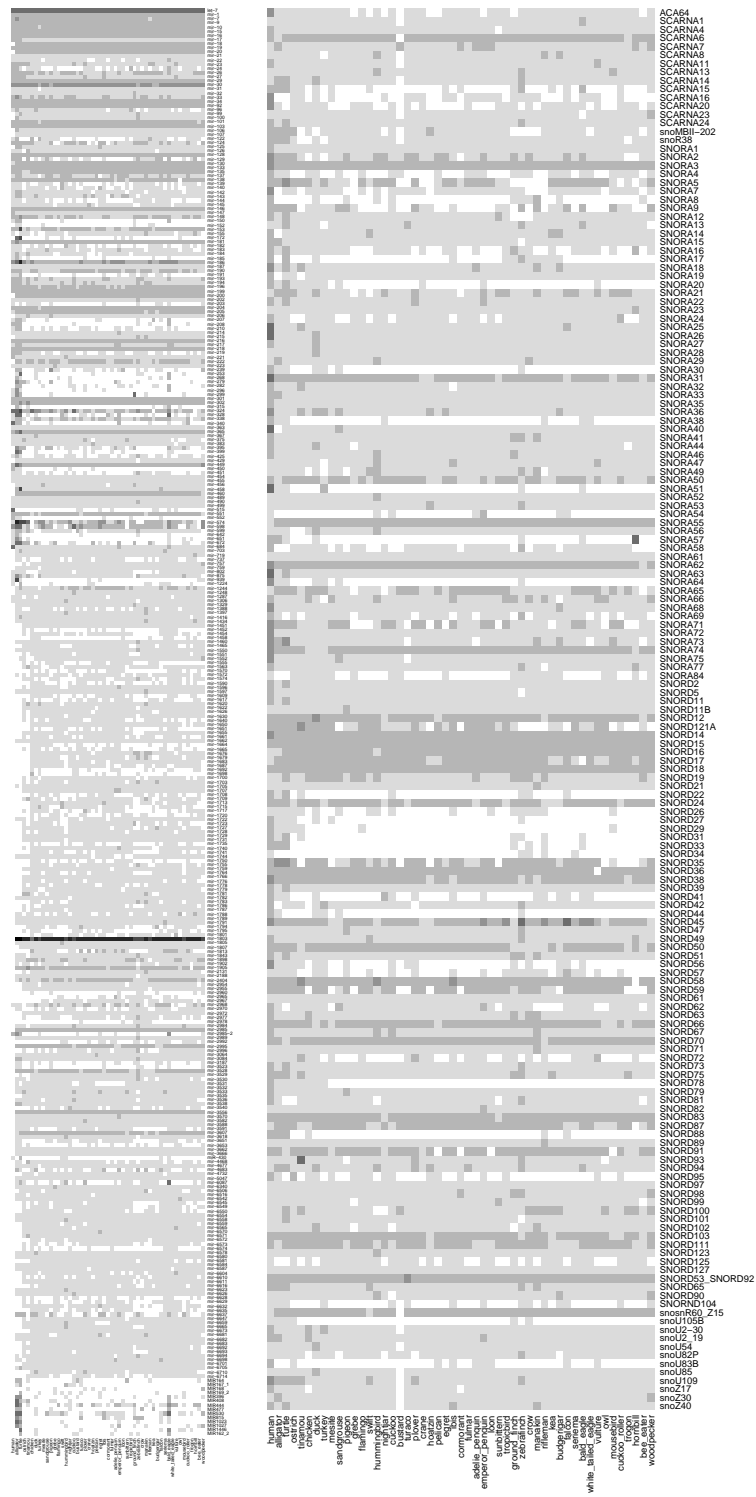


Figure 3: Heatmaps showing the presence/absence and approximate copy number of **miRNA** families on the right and **snoRNA** families on the left.

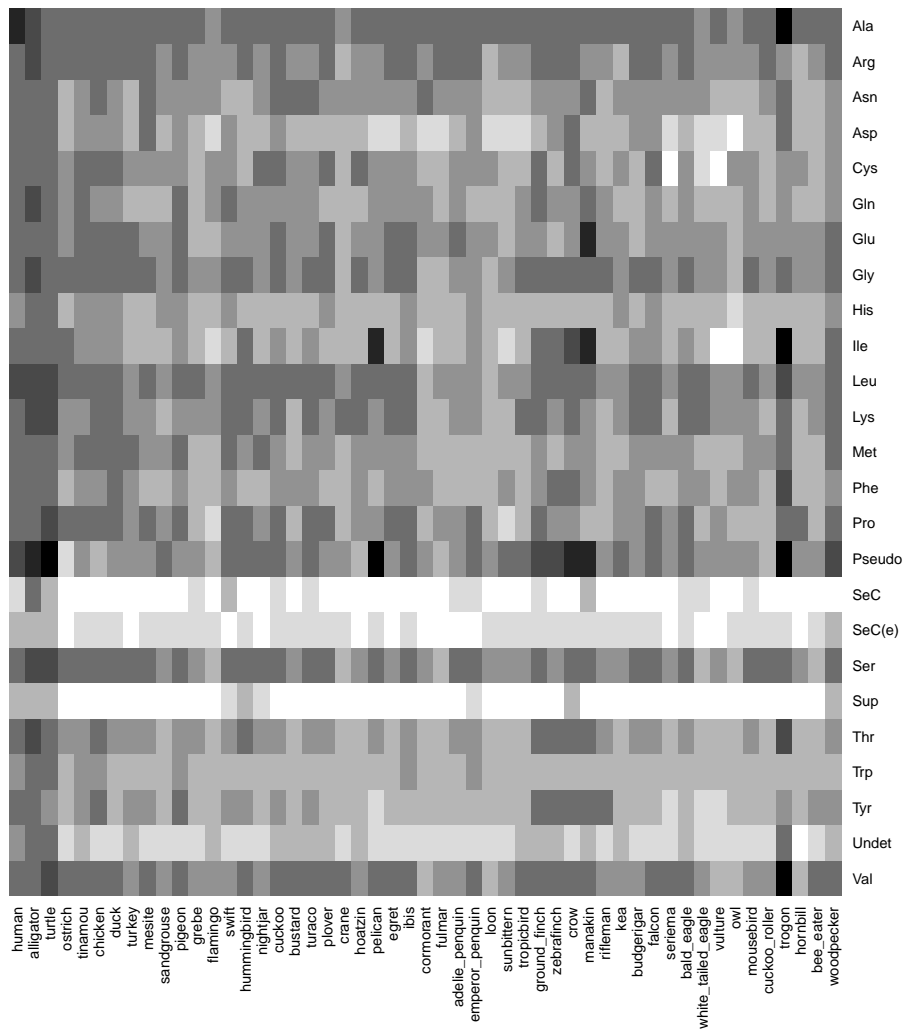


Figure 4: Heatmaps showing the presence/absence and approximate copy number of **tRNA** families.

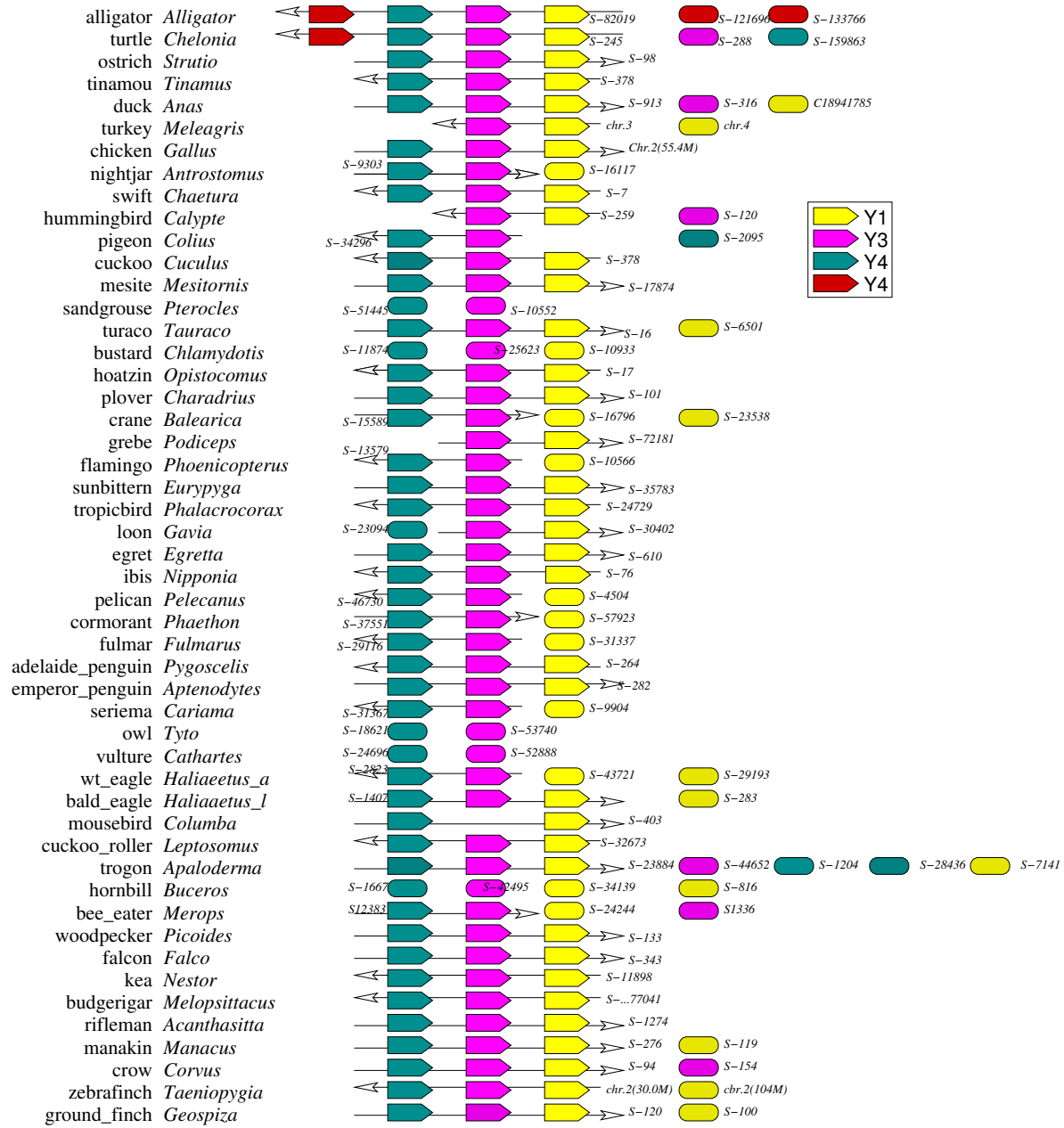


Figure 5: Syntenic conservation of the Y RNA cluster in avian and reptile genomes.

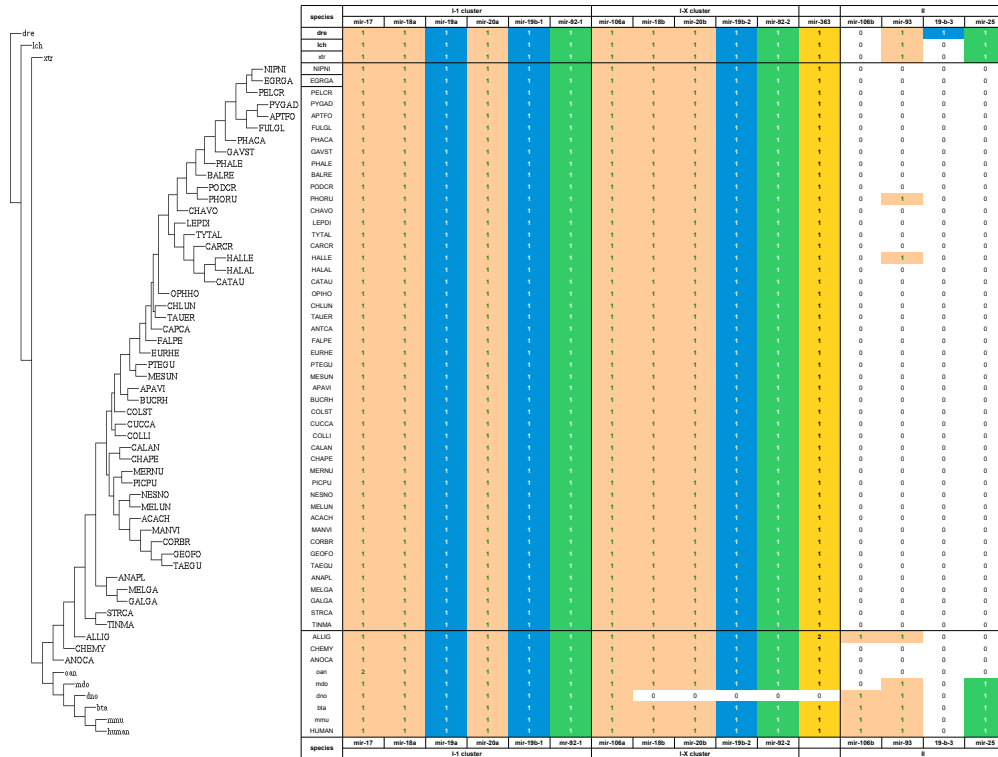


Figure 6: This figure illustrates the distribution of all members of the mir-17 clusters across birds, compared to fish and mammals. Columns correspond to single miRNA sequences, grouped by clusters and ordered by their position within the corresponding cluster. Rows correspond to the species and the cells contain the number of copies of the miRNA in the respective species. Colors correspond to orthologous miRNA families: mir-17 (orange), mir-19 (blue) and mir-92/25 (green). There are 3 clusters, I-1 and I-X, and cluster II. While the two copies of cluster I are completely conserved, cluster II has been lost as a whole in birds.

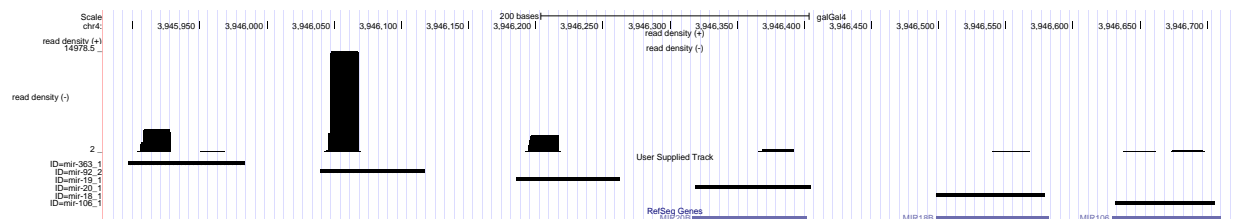


Figure 9: microRNA 17 I-X cluster: mir-363.1, mir-92.2, mir-19.1, mir-20.1, mir-18.1, mir-106.1. The figure indicates the genomic location and the RNA-seq read-depths for these 6 microRNAs.

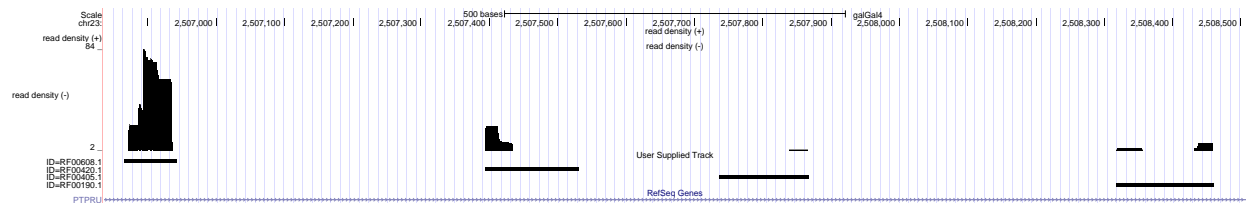


Figure 10: H/ACA box snoRNA cluster: SNORA61, SNORA44, SNORA16. The figure indicates the genomic location and the RNA-seq read-depths for these 3 snoRNAs.

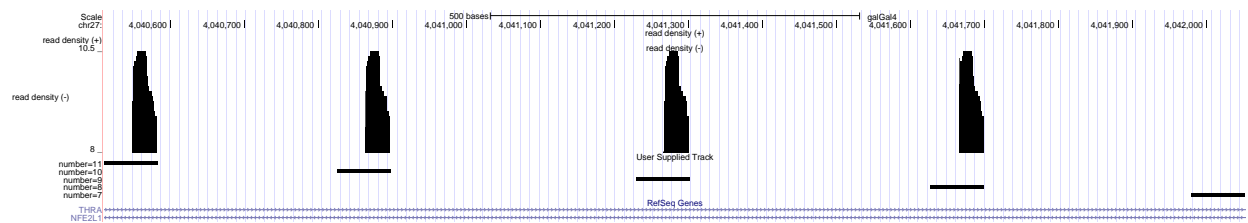


Figure 11: Cysteine tRNA cluster. The figure indicates the genomic location and the RNA-seq read-depths for these 5 transfer RNAs.

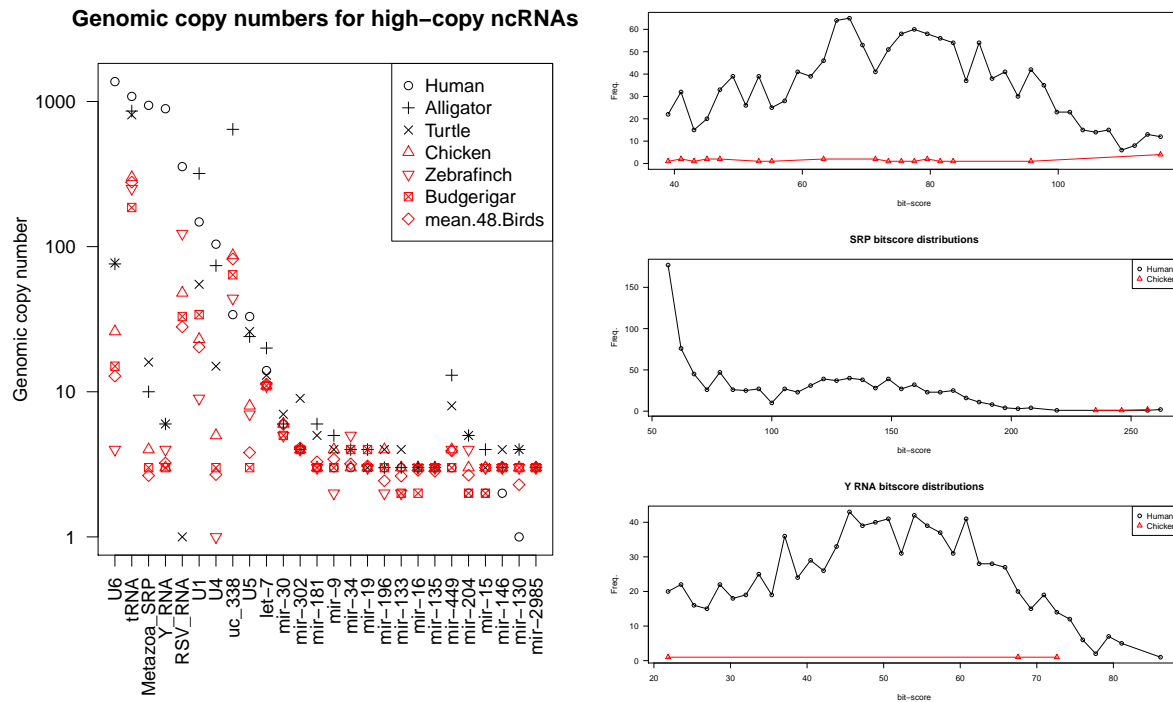


Figure 12: **Left:** A comparison of the genomic copy numbers for 26 of the most abundant RNAs found in human, reptile and the bird genomes. **Right:** Bit-score distributions for the Rfam U6, Metazoa SRP and the Y RNA covariance models for human and chicken paralogues/pseudogenes. Loci with high bit-scores are more likely to be functional than those with low bit-scores (due to excess variation that conflicts with the canonical RNA models).

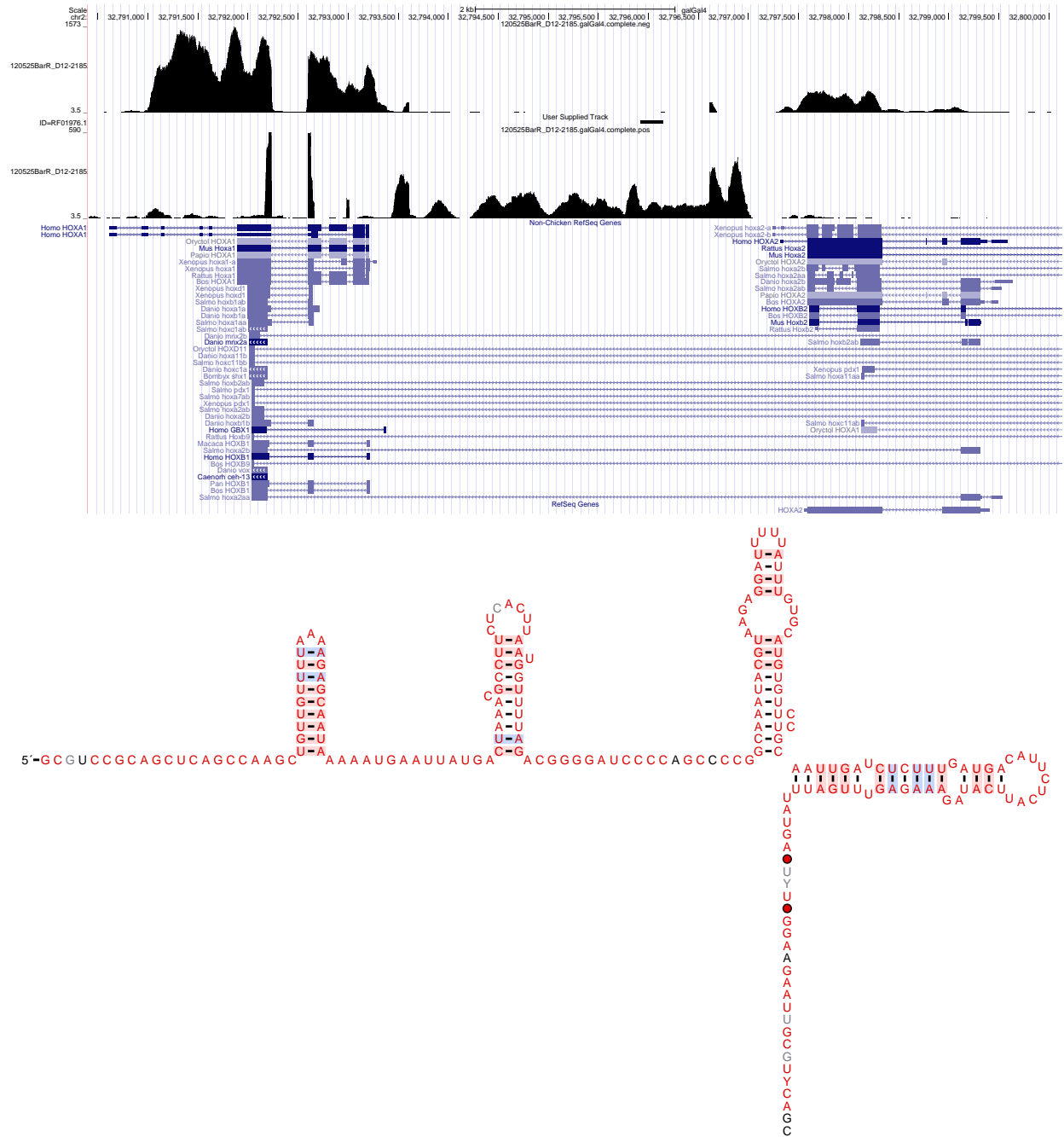


Figure 13: Above: Expression of the chicken HOTAIRM1 (RF01976) locus and the surrounding genes: HOXA1, HOXA2, and HOXA3. Below: The predicted secondary structure of the Avian HOTAIRM1 domain.

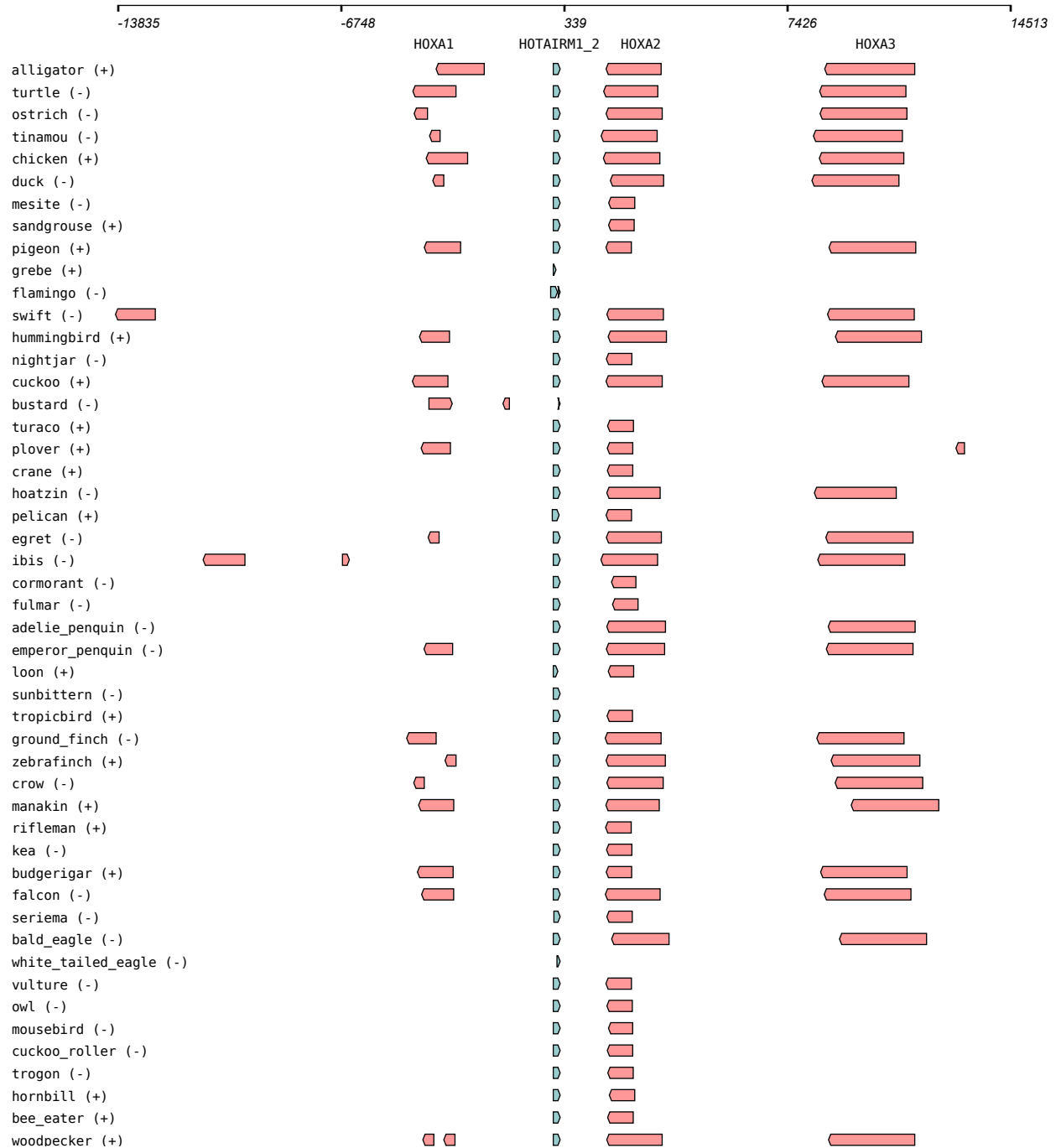


Figure 14: The preservation of gene order (synteny) surrounding the HOTAIRM1 (RF01976) locus across the Avian and other Vertebrate lineages.

RNA family (Rfam ID)	Chromosome	Coordinates	Strand
Macro-chromosomes:			
RNase MRP (RF00030)	chr4	39276202-39276488	-
Micro-chromosomes:			
U4atac snRNA (RF00618)	chr7	25702714-25702831	+
RNase P RNA (RF00009)	chr8	6609347-6609668	-
Telomerase RNA (RF00024)	chr9	19429028-19429084	+
Vault RNA (RF00006)	chr13	380747-380840	+
U11 snRNA (RF00548)	chr23	2216932-2217058	-

Table 1: The location of “missing” RNA families in the chicken genome (galGal4).

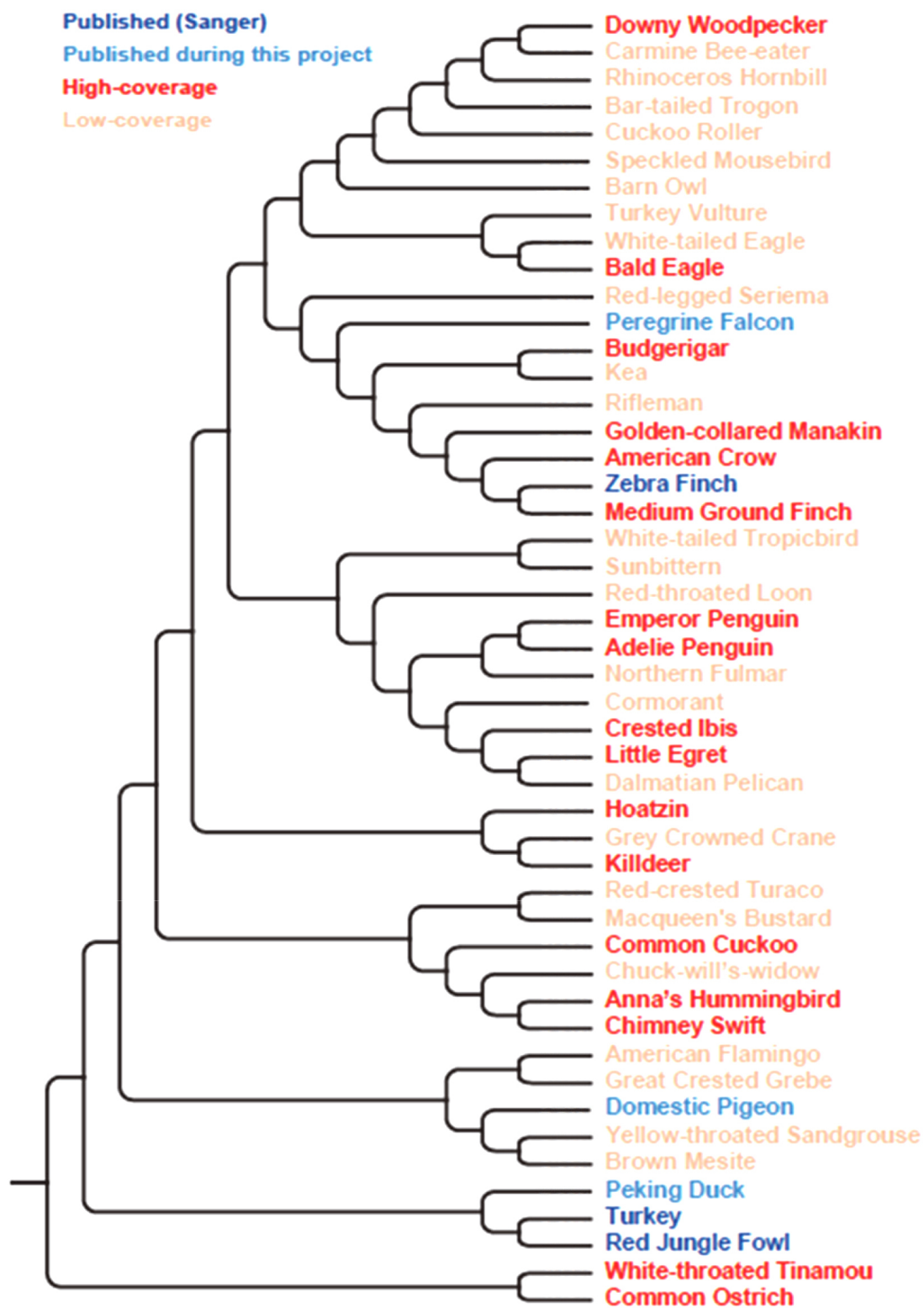


Figure 15: The phylogenetic relationships between the Avian species used in this study. This figure has been reproduced, with permission from [48].

Database accessions

The NCBI BioProject, SRA and Study IDs for the genomes used in this study are listed below.

Species	BioProject ID	SRA ID	Study ID
<i>Chaetura pelagica</i>	PRJNA210808	SRA092327	SRP026688
<i>Calypte anna</i>	PRJNA212866	SRA096094	SRP028275
<i>Charadrius vociferus</i>	PRJNA212867	SRA096158	SRP028286
<i>Corvus brachyrhynchos</i>	PRJNA212869	SRA096200	SRP028317
<i>Cuculus canorus</i>	PRJNA212870	SRA096365	SRP028349
<i>Manacus vitellinus</i>	PRJNA212872	SRA096507	SRP028393
<i>Ophisthocomus hoazin</i>	PRJNA212873	SRA096539	SRP028409
<i>Picoides pubescens</i>	PRJNA212874	SRA097131	SRP028625
<i>Struthio camelus</i>	PRJNA212875	SRA097407	SRP028745
<i>Timamus guttatus</i>	PRJNA212876	SRA097796	SRP028753
<i>Acanthisitta chloris</i>	PRJNA212877	SRA097960	SRP028832
<i>Apaloderma vittatum</i>	PRJNA212878	SRA097967	SRP028834
<i>Balearica regulorum</i>	PRJNA212879	SRA097970	SRP028839
<i>Buceros rhinoceros</i>	PRJNA212887	SRA097991	SRP028845
<i>Antrostomus carolinensis</i>	PRJNA212888	SRA098079	SRP028883
<i>Cariama cristata</i>	PRJNA212889	SRA098089	SRP028884
<i>Cathartes aura</i>	PRJNA212890	SRA098145	SRP028913
<i>Chlamydotis macqueenii</i>	PRJNA212891	SRA098203	SRP028950
<i>Colinus striatus</i>	PRJNA212892	SRA098342	SRP028965
<i>Eurypyga helias</i>	PRJNA212893	SRA098749	SRP029147
<i>Fulmarus glacialis</i>	PRJNA212894	SRA098806	SRP029180
<i>Gavia stellata</i>	PRJNA212895	SRA098829	SRP029187
<i>Haliaeetus albicilla</i>	PRJNA212896	SRA098868	SRP029203
<i>Haliaeetus leucocephalus</i>	PRJNA237821	SRX475899	SRP038924
	SRX475900		
	SRX475901		
	SRX475902		
<i>Leptosomus discolor</i>	PRJNA212897	SRA098894	SRP029206
<i>Merops nubicus</i>	PRJNA212898	SRA099305	SRP029278
<i>Mesitornis unicolor</i>	PRJNA212899	SRA099409	SRP029309
<i>Nestor notabilis</i>	PRJNA212900	SRA099410	SRP029311
<i>Pelecanus crispus</i>	PRJNA212901	SRA099411	SRP029331
<i>Phaethon lepturus</i>	PRJNA212902	SRA099412	SRP029342
<i>Phalacrocorax carbo</i>	PRJNA212903	SRA099413	SRP029344
<i>Phoenicopiterus ruber</i>	PRJNA212904	SRA099414	SRP029345
<i>Podiceps cristatus</i>	PRJNA212905	SRA099415	SRP029346
<i>Pterocles gutturalis</i>	PRJNA212906	SRA099416	SRP029347
<i>Tauraco erythrolophus</i>	PRJNA212908	SRA099418	SRP029348
<i>Tyto alba</i>	PRJNA212909	SRA099419	SRP029349
<i>Nipponia nippon</i>	PRJNA232572	SRA122361	SRP035852
<i>Egretta garzetta</i>	PRJNA232959	SRA123137	SRP035853

Table 2: The NCBI BioProject/SRA and Study IDs used in this study.

Species	BioProject ID	SRA ID	Study ID
Genomes released before			
<i>Aptenodytes forsteri</i>	PRJNA235982	SRA129317	SRP035855
<i>Pygoscelis adeliae</i>	PRJNA235983	SRA129318	SRP035856
<i>Gallus gallus</i>	PRJNA13342	SRA030184	SRP005856 (galGal4)
<i>Taeniopygia guttata</i>	PRJNA17289	SRA010067	SRP001389
<i>Meleagris gallopavo</i>	PRJNA42129	Unknown	Unknown
<i>Melopsittacus undulatus</i>	PRJEB1588	ERA200248	ERP002324
<i>Anas platyrhynchos</i>	PRJNA46621	SRA010308	SRP001571
<i>Columba livia</i>	PRJNA167554	SRA054954	SRP013894
<i>Falco peregrinus</i>	PRJNA159791	SRA055082	SRP013939
<i>Geospiza fortis</i>	PRJNA156703	SRA051234	SRP011940
Outgroups			
<i>Homo sapiens</i>	PRJNA104937		HG19/GRCh37
<i>Alligator mississippiensis</i>			allMis1
<i>Chelonia mydas</i>			
RNA-seq data			
<i>Gallus gallus</i>	PRJNA204941	NA	NA
<i>Gallus gallus</i>	NA	NA	SRP041863

Table 3: The NCBI BioProject/SRA and Study IDs for the previously published genomes used in this study.

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