

# Fly away Peter, come back Paul: the gains and losses of avian Non-coding RNAs

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## Abstract

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Here we present the results of a large-scale bioinformatic annotation of non-coding RNAs in 48 avian genomes. Our approach uses probabilistic models of hand-curated families from the Rfam database to infer conserved RNA families within each avian genome. We supplement these annotations with predictions from the tRNA annotation tool, tRNAscan-SE and microRNAs from miRBase. Although we show extensive conservation of classical ncRNAs (e.g., tRNAs and rRNAs) and more recently discovered ncRNAs (e.g., snoRNAs and miRNAs) in birds, we also demonstrate apparent “losses” in several RNA families. These include the divergence of some classical ncRNAs and the loss of several snoRNAs and microRNAs. In contrast, we show a significant number of lncRNAs are surprisingly well conserved between birds and mammals including several intriguing cases where the reported mammalian lncRNA function is not conserved in birds. These combined results illustrate the utility of applying homology based methods for annotating vertebrate genomes and illustrate many complex evolutionary patterns within the avian ncRNA cohort.

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## Introduction

Non-coding RNAs (ncRNAs) are an important class of genes. However, they pose serious research challenges, particularly for the field of genomics. They lack the strong statistical signals associated with protein coding genes, e.g. open reading frames, G+C content and codon-usage biases [1].

Non-coding RNAs are classified into hierarchical groupings by the Rfam database. The basic units are “families” which are groups of homologous, alignable sequences; “clans” which are groups of un-alignable (or functionally distinct), homologous families; and “classes” which are groups of clans and families with related biological functions e.g. spliceosomal RNAs, miRNAs and snoRNAs [2–6]. The major RNA families include the classical, highly conserved RNAs, sometimes called “molecular fossils”, such as the transfer RNAs, ribosomal RNAs, RNA components of RNase P and the signal recognition particle [7]. Other classes appear to have evolved more recently, e.g. the small nucleolar RNAs (snoRNAs), microRNAs (miRNAs) and the long non-coding RNAs (lncRNAs) [8]. The publication of avian genomes provides exciting opportunities to explore ncRNA conservation in unprecedented detail.

Despite promising techniques such as RNA-seq [9], homology based methods namely covariance models (CMs) are state of the art for ncRNA analyses [10–12]. The CM based approach for annotating ncRNAs in genomes requires reliable alignments and consensus secondary structures of representative sequences of RNA families. These are used to train probabilistic models for each family. These models can be used to generate sequences with similar properties, score the likelihood that a sequence is was generated by the same evolutionary processes as the training sequences and to build alignments based upon sequence and structural information [10–12]. The tRNAscan-SE software package uses CMs to accurately predict transfer RNAs [13, 14]. The Rfam database contains thousands of curated alignments and consensus structures for diverse classes of ncRNAs [2–6]. Independent benchmarks of bioinformatic annotation tools have shown that the CM approaches dramatically out-perform alternative methods [15].

The CM based approach works well for almost all classes of ncRNA, but the long non-coding RNAs (lncRNAs) are a particular challenge [16]. Recent technological advances have led to dramatic speed and memory-usage enhancements for CM analyses [12, 17–19]. However, CMs cannot model the exon-intron structures of spliced lncRNAs, nor can they deal simply with the repeats that many lncRNAs host.

Consequently in the latest release of Rfam the lncRNA families that were added were composed of local conserved and structured elements within lncRNAs, analogous to the “domains” housed within protein sequences [6]. The functions determined to date for lncRNAs range from regulating chromatin status to chromosomal inactivation [20, 21]. Yet functional characterisation of these genes is a lengthy and expensive

process [16].

## Results

In the following we explore the conservation patterns of the major classes of ncRNA in further detail. The collection of ncRNA sequences is generally biased towards model organisms [8, 22]. We focus our analysis on the unusual results within the avian lineages. These are either unexpectedly well-conserved RNAs or unexpectedly poorly-conserved RNAs. The former are RNAs we would not have expected to be conserved between the birds and the organisms these genes were initially identified in; Usually, this hypothesis is based upon the function of the RNA which is not conserved in avian species. The latter are apparent losses of RNA genes that were expected to be conserved; Usually, this hypothesis is based upon the conservation of these RNAs in other vertebrate species. We have three models of “loss” that we use to explain the data: Firstly, these could be genuine cases of an ancestral gene-loss along the avian lineage. Secondly, this could be a case of “divergence” where a RNA gene has undergone significant sequence and structural alterations, so much so, that our homology search tools can no longer detect a relationship between vertebrate exemplars and the avian varieties. Thirdly, we consider the possibility that the available genome assemblies have independently failed to capture these genes.

### Unusually, well conserved RNAs

The bulk of the “unusually well conserved RNAs” belong to the long non-coding RNA (lncRNA) group. The lncRNAs are a diverse group of RNAs that have been implicated in a multitude of functional processes [16, 20, 21]. These RNAs have largely been characterised in mammalian species, particularly human and mouse. Consequently, generally do not expect these to be conserved outside of mammalia. Notable examples include Xist [23] and H19 [24].

In general Rfam cannot include the entire length of any large, spliced RNAs. This is a limitation of the covariance-models used for the homology-searches Rfam runs [12]. Consequently, only short, well-conserved regions with evolutionarily conserved secondary structures are included in Rfam. By analogy to protein-domains, we refer to these as RNA-domains [6].

When analysing the RNA-domain annotations it is striking that many of the lncRNAs with multiple RNA-domains are consistently preserved in the birds. The annotations of these domains lie in the same genomic region, in the same order as in the mammalian homologs. Thus supporting a high degree of evolutionary conservation for the entire lncRNA. In particular the HOXA11-AS1, PCA3, RMST, Six3os1,

SOX2OT and ST7-OT3 lncRNAs have multiple, well conserved RNA-domains (See Figure 1).

The HOX cluster lncRNAs, HOTAIRM1, HOTTIP and HOXA11-AS1, are remarkably well conserved. In the human genome the corresponding RNA-domains are located at chr7:27135743-27245922 (hg19) and are modelled by 15 RNA-domains in total, the lncRNAs are ordered HOTAIRM1 (5 RNA-domains) → HOXA11-AS1 (6 RNA-domains) → HOTTIP (4 RNA-domains). In the alligator and turtle genomes five and six of the HOX RNA-domains are conserved, each supporting an ancestral gene order of HOTAIRM1 → HOXA11-AS1 → HOTTIP. In the birds, where two or more of the HOX cluster lncRNA RNA-domains were predicted on the same scaffold, this gene order was preserved.

Many of the lncRNAs have been associated with cancer, sparking a minor review industry [25,26]. Three examples of these that are also conserved in the birds are described below.

The RMST (Rhabdomyosarcoma 2 associated transcript) RNA-domains 6, 7, 8, and 9 are conserved across the birds. In each bird the gene order was also consistent with the human ordering. In the alligator and turtle an additional RNA-domain was predicted in each, these were RNA-domains 2 and 4 respectively, again the ordering of the domains was consistent with human. This suggests that the RMST lncRNA is highly conserved. However, little is known about the function of this RNA. It was originally identified in a screen for differentially expressed genes in two Rhabdomyosarcoma tumor types [27].

In addition, the lncRNA DLEU2, is well conserved across the vertebrates, it is a host gene for two miRNA genes, miR-15 and miR-16, both of which are also well conserved across the vertebrates (See Supplemental Figure 2). DLEU2 is thought to be a tumor-suppressor gene as it is frequently deleted in malignant tumours [28,29].

The NBR2 lncRNA and BRCA1 gene share a bidirectional promotor [30]. Both are expressed in a broad range of tissues. Extensive research on BRCA1 has shown that it is involved in DNA repair [31]. The function of NBR2 remains unknown, yet its conservation across the vertebrates certainly implies a function (See Figure 1).

#### **more detail on the prostate lncRNAs?**

Of the other classes of RNAs, none showed an unexpected degree of conservation or expansion within the avian lineage. The only exception being the snoRNA SNORD93 which we show has 92 copies in the tinamou genome, whereas it only has 1-2 copies in all the other vertebrate genomes.

### RNA Losses, divergence or missing data?

Much of the number of apparent losses and reduction in genomic sequence has been extensively discussed elsewhere [32]. Unsurprisingly, this reduction is reflected in the copy-number of RNA genes. Some of the most dramatic examples are the transfer RNAs and pseudogenes which average  $\approx 900$  and  $\approx 580$  copies in the human, turtle and alligator genomes, the average copies numbers of these drop to  $\approx 280$  and  $\approx 100$  copies in the avian genomes.

The absence of several well-conserved ncRNA families from many or even most bird genomes is unlikely to represent true gene losses. This concerns in particular the telomerase RNA, the RNA components of RNase P and MRP, the minor spliceosomal snRNAs U4atac and U11, the selenocystein tRNA (tRNA-Sec) as well as the vault RNAs. 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 selenocystein 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 [33]. The poor return thus does not come as a surprise. Since **blastn** searches remained unsuccessful we constructed a sauropsid-specific CM for the vault RNA. 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 also a well-know difficult problem for homology search [34].

Our ability to find additional homologs for several RNA families that fill gaps in the abundance matrices (Supplemental Figure 5) strongly suggests that 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.

## Y RNA clusters

Vertebrate Y RNAs typically form a cluster comprising four well-defined paralog groups Y1, Y3, Y4, and Y5. In line with *Mosig:07a* we find that the Y5 paralog family is absent from all bird genomes, while it is still present in both alligator and turtle, see Supplemental Figure 4. Within bird, we find an the conserved Y4-Y3-Y1 cluster. Apparently, broken-up clusters are in most cases consistent with breaks in the available sequence assemblies. In several genomes we observe one or a few additional Y RNA homologs unlinked to the canonical Y RNA cluster. These sequences can be identified unambiguously as derived members of one of the three ancestral paralog groups, they almost always fit less well to the consensus (as measured by the CM bit score of paralog group specific covariance models) than the paralog linked to cluster, and there is no indication that any of these additional copies is evolutionarily conserved over longer time scales. We therefore suggest that most or all of these interspersed copies are in fact pseudogenes.

## MicroRNA Clusters

Nearly all microRNAs that are broadly conserved in fish, amphibians and mammals are also conserved in the birds. Nevertheless, there are obvious instances of microRNAs lost in all birds. For example, mammalian and ambhibian genomes contain three loci of clustered microRNAs from the mir-17 and mir-92 families [35]. One of these clusters (cluster II, with families mir-106b, mir-93 and mir-25) was not found in turtles, crocodiles and birds, see Supplemental Figure 6.

The microRNA family let-7 is the most abundant microRNA family with 14 paralogs in human. These genes also localize in 7 genomic clusters, together with mir-100 and mir-125 miRNA families (see previous study on the evolution of the let-7 miRNA cluster in [36]). In Sauropsids we observed that cluster A - that is strongly conserved in vertebrates has been completely lost in the avian lineage. Another obvious lost in birds is cluster F, containing two let-7 microRNA paralogs. Cluster H, on the other hand has been retained in all oviparous animals and completely lost later, after the split of Theria. See Supplemental Figure 7 for details.

## Conclusions Methods

Bird genomes were searched using the cmsearch program from INFERNAL 1.1 and the covariance models from the Rfam database v11.0 [5,6]. All matches above the curated GA threshold were included. Subsequently, all hits with an E-value greater than 0.0005 were discarded, so only matches which passed

the model-specific GA threshold and had an E-value smaller than 0.0005 were retained.

In order to obtain good annotations of tRNA genes we also ran the specialist tRNA-scan version 1.3.1 annotation tool. This method also uses covariance models to identify tRNAs. However it also uses some heuristics to increase the search-speed, annotates the Isoacceptor Type of each prediction and uses sequence analysis to infer if predictions are likely to be functional or tRNA-derived pseudogenes [13,14]. Rfam matches and the tRNA-scan results for families belonging to the same clan were then “competed” so that only the best match was retained for any genomic region [5]. To further increase the specificity of our annotations we filtered out families that were identified in four or fewer of the 51 vertebrate species we have analysed in this work. These filtered families largely corresponded to bacterial contamination within the genomic sequences.

999 microRNA sequence families, previously annotated in at least one vertebrate, were retrieved from miRBase (v19). Individual sequences or multiple sequence alignments were used to build covariance models with INFERNAL (v1.1rc3), and these models were searched against the 48 bird genomes, and the genomes of the american alligator and the green turtle as outgroups. Hits with e-value  $\leq 10$  realigned with the query sequences and the resultant multiple sequence alignments manually inspected and edited using RALEE.

An additional snoRNA homology search was performed with snoStrip [37]. As initial queries we used deuterostomian snoRNA families from human [38], platypus [39], and chicken [40].

SnoRNA annotations overlapping miRNAs annotations were manually inspected [and?....](#)

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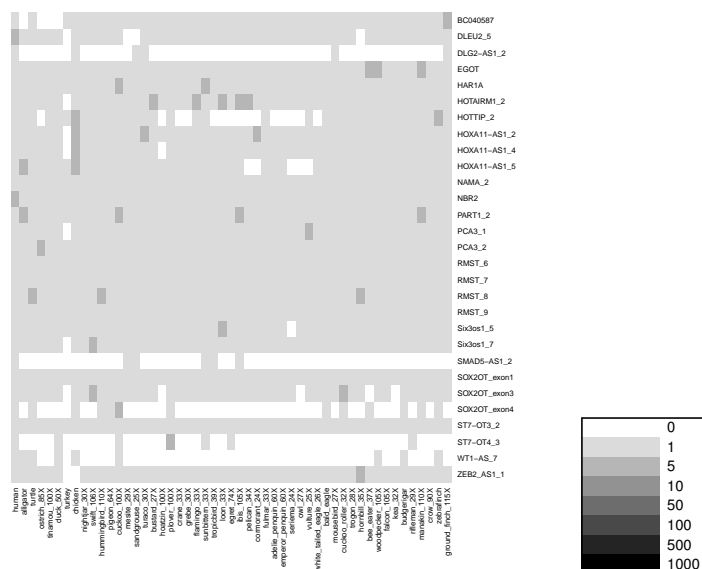


Figure 1: Heatmaps showing the prescence/abscece and approximate copy number of **lncRNA** families.