A dual transcript-discovery approach to improve the delimitation of gene features from RNA-seq data in the chicken model

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**ABSTRACT**

The sequence of the chicken genome, like several other draft genome sequences, is presently not fully covered. Gaps, contigs assigned with low confidence and uncharacterized chromosomes result in gene fragmentation and imprecise gene annotation. Transcript abundance estimation from RNA sequencing (RNA-seq) data relies on read quality, library complexity and expression normalization. In addition, the quality of the genome sequence used to map sequencing reads, and the gene annotation that defines gene features, must also be taken into account. A partially covered genome sequence causes the loss of sequencing reads from the mapping step, while an inaccurate definition of gene features induces imprecise read counts from the assignment step. Both steps can significantly bias interpretation of RNA-seq data. Here, we describe a dual transcript-discovery approach combining a genome-guided gene prediction and a de novo transcriptome assembly. This dual approach enabled us to increase the assignment rate of RNA-seq data by nearly 20% as compared to when using only the chicken reference annotation, contributing therefore to a more accurate estimation of transcript abundance. More generally, this strategy could be applied to any organism with partial genome sequence and/or lacking a manually-curated reference annotation in order to improve the accuracy of gene expression studies.

**KEY WORDS:** Chicken genome annotation, *Gallus gallus*, Gene prediction, Genome-guided transcript discovery, RNA sequencing, Transcriptome reconstruction

**INTRODUCTION**

Since its first release in 2004 and despite significant improvements over the last past decade, the *Gallus gallus* genome is presently incomplete and highly fragmented (Hillier et al., 2004). The chicken karyotype is composed of 38 autosomal chromosomes (1-38) and two additional sex chromosomes (W, Z) (Bloom et al., 1993). Out of these autosomal chromosomes, 10 are macrochromosomes (1-10), with lengths similar to those in mammals, and 28 are microchromosomes (11-38), with lengths ranging from 2 to 25 Mb (Hillier et al., 2004). Chicken microchromosomes display a high recombination rate, contain an elevated number of repetitive elements and are GC-rich, which induces significant bias and sequencing errors when using high-throughput technologies (Chen et al., 2013; Dohm et al., 2008). In addition, microchromosomes are gene dense and enriched in CpG islands, which is the result of short intronic sequences (McQueen et al., 1998; Smith et al., 2000). The fourth version of the *Gallus gallus* genome (galGal4), released in November 2011, has not fully overcome these issues. Out of the 40 chromosomes, 31 are (1-28, 32, W, Z) and contain more than 9000 gaps, while nine chromosomes remain missing (29-31, 33-38). The genome is also composed of ~16,000 additional contigs that are not assigned to any chromosome or assigned with low confidence. In total, the galGal4 genome sequence has a size of 1.05 Gb.

RNA sequencing (RNA-seq) data processing and results are highly dependent on the quality of the genome sequence and the associated gene annotation model. Read mapping is one of the critical steps that will further influence sample normalization, gene expression quantification and the identification of relevant genes. Gene expression profiles rely on the alignment of RNA-seq reads along the available reference genome or transcriptome, followed by their assignment to gene features. An incomplete genome sequence coupled with an inaccurate definition of gene features induce a bias in the gene expression quantification and transcript abundance estimation (Jiang and Wong, 2009; Trapnell et al., 2010). Whole transcriptome sequencing offers valuable resources to detect novel genes and transcripts as well as to identify alternative splicing variants (Denoeud et al., 2008; Wang et al., 2008). Depending on the context, two main strategies are widely used to analyze RNA-seq data (Garber et al., 2011). One approach consists of the mapping of reads along the reference genome followed by gene prediction (Guttman et al., 2010; Trapnell et al., 2010; Yassour et al., 2009). This method can be combined with an existing reference annotation in order to detect new transcripts with respect to the provided gene annotation model (Roberts et al., 2011). The second approach aims at reconstructing the whole transcriptome independently of the reference genome (Birol et al., 2009; Grabherr et al., 2011; Robertson et al., 2010). This method is particularly suitable to study models with partial or missing genome sequence. The choice between these approaches greatly depends on the biological question and whether a reference genome is available (Conesa et al., 2016).

When analyzing RNA-seq data obtained from chick embryonic limb cell cultures (so-called micromass cultures) by using the galGal4 reference genome and annotation, we observed that only 62.2% of sequencing read pairs were assigned to gene features, while 86.7% of the read pairs were mapped against the genome sequence. By comparison with the human genome, which has been nearly completely sequenced and accurately annotated, a similar analysis of RNA-seq data obtained from human blood samples

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RESULTS

We performed RNA-seq of two independent biological replicates of chick micromass cultures infected for 5 days with empty RCAS-BP (A) replication-competent retroviral particles. We generated 61.3 and 70.3 million strand-specific read pairs and mapped them against the galGal4 version of the chicken genome by using TopHat2 (Kim et al., 2013) (Table 1). Read assignment was performed by using featureCounts (Liao et al., 2014) and a gene annotation model composed of 17,318 genes resulting from the combination of both UCSC and Ensembl reference annotations that were available at the time of analysis. Surprisingly, while 86.7% of read pairs were mapped against the chicken genome, only 62.2% of read pairs were assigned to gene features (Table 1). Therefore, 28.3% of mapped read pairs were not counted, including 93.7% of these read pairs that were not overlapping with any gene feature (Table 1). Close investigation of these unassigned read pairs highlighted genes that seemed to be absent or partially covered by the UCSC and Ensembl reference annotations (Fig. 1A,B), as well as transcripts with missing or partial exon features (Fig. 1C).

In order to improve the read assignment rate, we first performed a genome-guided transcript discovery by using Cufflinks (Trapnell et al., 2010). This approach was intended to determine more accurately exon-intron junctions, to correct or to complete existing annotated genes, and to identify unannotated gene candidates from the UCSC/Ensembl gene annotation model (Fig. 1D,E). Following this approach, 77.9% of the sequencing read pairs were assigned to gene features, corresponding to 89.8% of the read pairs that were mapped against the genome (Table 1). Therefore, the genome-guided transcript discovery enabled us to raise the read assignment rate by 15.7% as compared to when using both UCSC and Ensembl reference annotations (Table 1). In contrast to genome-guided transcript prediction, de novo transcriptome reconstruction relies on overlaps between the sequencing reads to build consensus transcripts, independently of the genome sequence. We therefore applied a genome-independent strategy by using Trinity (Grabherr et al., 2011), in combination with the genome-guided approach, in order to detect transcripts or regions that were not recovered from the genome sequence, such as those located within gaps or uncharacterized chromosomes (Fig. 1D,E). Reconstructed transcripts thus generated were then compared to the gene candidates obtained with the genome-guided approach in order to remove redundant sequences. Full-length transcripts or transcript regions of at least 400 bp that were not assigned to any gene candidate were extracted and grouped as an artificial chromosome. We found that 4.0% of read pairs were mapped against this additional chromosome and 90.2% of these mapped read pairs were assigned to gene features (Table 1). By considering both transcript-discovery approaches, 90.7% of the total read pairs were mapped against the galGal4 chicken genome (86.7%) and reconstructed chromosome (4.0%) (Table 1), and 77.9% and 3.6% of the read pairs were assigned to gene features from the genome-guided and de novo transcript-discovery approaches, respectively (Fig. 2A, Table 1). Therefore, 81.5% of the read pairs were assigned to gene features by using this newly established gene annotation model. Given that 62.2% of the sequencing read pairs were assigned to gene features by using both UCSC and Ensembl reference annotations, our transcript reconstruction model enabled us to assign 19.3% more read pairs to gene features (Fig. 2A, Table 1).

The genome-independent transcript assembly also enabled us to correct for gene fragmentation by gathering gene regions located on multiple chromosomes and contigs together (Fig. 1D,E). In contrast to genome-guided transcript discovery, de novo reconstruction of transcripts was not limited by the quality of the reference genome sequence. By comparing transcripts generated from both reconstruction approaches, we were able to group dispersed gene features belonging to the same gene candidate together. Although 19,376 (90.8%) gene candidates were found exclusively on a single chromosome or unplaced contig, 1971 (9.2%) gene candidates were identified as being fragmented (Fig. 2B). These fragmented gene candidates included 478 (2.2%) gene candidates that were located on multiple ordered chromosomes, 462 (2.2%) gene candidates split among multiple unplaced contigs, and 1031 (4.8%) gene candidates with regions located on an ordered chromosome and additional unplaced contigs (Fig. 2B).

Table 1. RNA-seq read pair assignment

| Sample | RCAS-BP(A) (Trinity) | chapel reference genome (galGal4) | De novo assembly (Trinity) |
|--------|---------------------|---------------------------------|---------------------------|
|        | Read pairs          | Mapped pairs                    | Assigned pairs [UCSC/Ensembl] | Assigned pairs [Cufflinks] | Gain of assigned pairs | Mapped pairs | Assigned pairs | Total gain of read assignment |
| Rep1   | 61.3 M              | 1.7 M                           | 53.1 M | 38.0 M | 47.6 M | +9.6 M | 2.4 M | 2.2 M | +11.8 M |
|        |                     |                                 | Mapped pairs with no gene feature | 14.2 M | 4.6 M |                     |                 |       |       |               |
| Rep2   | 70.3 M              | 2.1 M                           | 61.0 M | 43.9 M | 55.0 M | +11.1 M | 2.9 M | 2.6 M | +13.7 M |
|        |                     |                                 | Mapped pairs with no gene feature | 16.0 M | 5.0 M |                     |                 |       |       |               |
| Average (Rep1/2) | 2.9%               | 86.7%                           | 62.2% | 77.9% | +15.7% | 4.0% | 3.6% | +19.3% | total pairs total mapped pairs |
|         |                     | Assigned mapped pairs           | 71.7% | 89.8% | 90.2% | 9.8% | | | |
|         |                     | Unassigned mapped pairs         | 28.3% | 10.2% | | | | | |
|         |                     | Mapped pairs with no gene feature | 26.5% | 8.4% | | | | | |

M, million read pairs.
Transcript prediction and reconstruction approaches did not provide any information on gene name and function. Therefore, gene candidates identified by the dual transcript-discovery approach were then annotated by database comparison and protein domain prediction (Fig. 1E). Gene candidates were first compared to bird gene sequences, taking advantage of the recent increase of available genomic data within avian species and their high DNA sequence conservation (Dalloul et al., 2010; Huang et al., 2013; Jarvis et al., 2014; Schmid et al., 2015; Shapiro et al., 2013; Warren et al., 2010; Zhan et al., 2013; Zhang et al., 2014). Undefined gene candidates were then compared at the protein level to mouse and human databases. Finally, prediction of open reading frames (ORFs) and protein domains was performed on remaining unannotated gene candidates by using homology search against SwissProt and Pfam databases, and sequence analysis tools to identify transmembrane domains and signal peptides. Overall, the computed gene annotation model was mostly constituted of protein-coding gene candidates (16,716, 78.3%) (Fig. 2C). However, 672 (3.1%) gene candidates were only partly annotated (putative proteins having at least one protein domain detected), while 1410 (6.6%) gene candidates remained unannotated (uncharacterized proteins with no protein domain identified but an ORF of at least 100 amino acids). Remaining gene candidates corresponded to miscellaneous genes (213, 1.0%; such as spliceosome complex members, ribosomal RNAs and pseudogenes) and non-coding RNAs (ncRNAs; 4418, 20.7%) for which no sufficient ORF could be predicted (Fig. 2C).

The resulting gene annotation model was composed of 21,347 unique gene candidates, encompassing 5989 additional gene candidates as compared to the UCSC and Ensembl reference annotations associated with the galGal4 genome version. We then compared our results with the most recent version of the chicken genome (galGal5), released in December 2015, which includes 200 additional Mb, three previously missing chromosomes (30, 31, 33) and 23,400 unplaced contigs (Warren et al., 2017). Firstly, strand-specific read pairs were mapped against the galGal5 genome version by using TopHat2 (Kim et al., 2013), and assigned to gene features.
by using featureCounts (Liao et al., 2014) according to a gene annotation model combining both UCSC and Ensembl annotations. This gene annotation model contained 6280 additional genes as compared to the galGal4 UCSC/Ensembl annotations. Surprisingly, we did not observe any significant improvement of read pair mapping (+1.5%) and assignment (−0.9%) rates despite the increased genome size (Table 2). This indicated that when using galGal5, similar issues will be encountered as with galGal4. Indeed, a comparable number of reads pairs (25.5%) was not associated with any gene feature when mapped against galGal5 (Table 2). Secondly, we compared the predicted gene candidates from our annotation model to the RefSeq annotated galGal5 transcripts. We found that only 52.7% of gene candidates were covered by at least 50% of their total length by galGal5 reference genes (Table 3). In addition, 3958 (18.5%) gene candidates were not detected at all in galGal5 reference genes (Table 3), and 3151 (79.6%) of these corresponded to gene candidates absent from galGal4 UCSC/Ensembl annotations. Lastly, we compared the gene names assigned to gene candidates with galGal5 reference genes that matched at least 50% of their length. Among the 15,358 gene candidates that were identified in the galGal4 UCSC/Ensembl annotations, 74.1% had a concordant gene name, while 17.9% did not significantly match any galGal5 reference gene (Table 4). Regarding the 5989 additional gene candidates, most of these were not significantly detected among galGal5 reference genes (76.8%) or matched an undefined gene (12.7%) (Table 4). However, 223 (1.0%) gene candidates remaining partly annotated with the dual transcript-discovery approach could be successfully assigned (Table 4).

| Table 2. RNA-seq read pair assignment against galGal5 |
|------------------------|------------------------|------------------------|
| Sample | Read pairs | Reference genome (galGal5) | Reference annotations (UCSC/Ensembl) |
| | Mapped pairs | As compared to galGal4 | Assigned pairs | As compared to galGal4 |
| Rep1 | 61.3 M | 53.9 M | +0.8 M | 37.3 M | −0.6 M |
| Rep2 | 70.3 M | 62.2 M | +1.2 M | 15.6 M | −0.4 M |
| Average (Rep1/2) | 88.2% | 61.3% | −0.9% | total pairs |

M, million read pairs.
Altogether, this dual transcript-discovery approach enabled us to define an annotation model of 21,347 gene candidates that includes additional genes as compared to the reference annotation of the chicken genome. Most importantly, it enabled us to retrieve 19.3% more information from the RNA-seq data.

**DISCUSSION**

The work presented here describes a dual transcript-discovery approach combining genome-guided gene prediction and de novo transcriptome reconstruction, which was applied to improve the assignment rate of RNA-seq data obtained from chicken samples. For the first approach, sequencing read pairs are mapped along the genome followed by a genome-dependent transcript discovery, which computes read coverage and exon-intron junctions from gapped alignments, and distance between both reads of each pair. By contrast, the second approach is carried out independently of the reference genome. Sequencing reads are de novo assembled by relying on their overlaps to reconstruct full-length transcripts. Genome-guided transcript discovery is more sensitive than de novo transcript reconstruction, but requires a reference genome along which RNA-seq reads are mapped for gene prediction (Garber et al., 2011; Roberts et al., 2011). Therefore, the choice of the latter method is obvious when no or incomplete genome sequence is available. In the case of the chicken model with its partial and fragmented genome sequence, the choice of a complementary transcript-discovery approach, combining both genome-guided and -independent methods, appears suitable to improve RNA-seq data quantification and analysis. While the genome-guided approach contributes to correct existing annotated genes and to identify novel gene candidates, the de novo transcript reconstruction compensates for gene fragmentation by associating gene parts located on multiple chromosomes or contigs together; and it identifies gene regions or complete gene candidates that do not belong to the genome sequence due to the presence of gaps or uncharacterized fragments. The new annotation model is composed of 21,347 gene candidates, accounting for 5989 additional gene candidates as compared to the UCSC and Ensembl reference annotations associated with the galGal4 genome version. Of these gene candidates, 1971 (9.2%) have parts spread on multiple locations, while 3340 (15.6%) are identified among the 16,000 unplaced contigs that are not assigned to any ordered chromosome. In addition, the resulting gene annotation model increased the assignment rate of RNA-seq read pairs by 19.3% as compared to when using both galGal4 reference annotations (UCSC and Ensembl), thus contributing to a more accurate estimation of transcript abundance.

It is noteworthy to take into consideration that de novo assembly of short reads is prone to cause artefacts and to generate false chimeric transcripts (Yang and Smith, 2013). Such errors can be corrected for instance by comparing reconstructed transcripts with transcripts/ proteins of the same organism, closely related organisms, or more accurately annotated organisms. In addition, transcriptome assemblers tend to create multiple transcript sequences per gene, which would cause reads to map at multiple locations and be subsequently ignored during read counting. Several programs have been developed in order to cluster transcript sequences into genes and to remove redundancy. TIGCL (Pertea et al., 2003) and CD-HIT-EST (Fu et al., 2012), which were originally designed for clustering of expressed sequence tags (EST), can be used to create consensus gene sequences. However, since both programs perform their clustering based on all transcript sequences, paralogous genes may be erroneously merged. In contrast, Corset (Davidson and Oshlack, 2014) identifies sequence similarity between transcripts by identifying multi-mapped reads resulting from re-mapping of reads against the reconstructed transcriptome. Although this program accurately clusters transcripts into genes, it falls short of building consensus genes from transcript sequences. To overcome these limitations, we applied a strategy that consists in a pairwise comparison of transcript sequences belonging to the same gene candidates followed by incremental concatenation of identical and unique transcript sequences to build full-length gene candidates. Very recently, a similar approach has been reported under the name of superTranscripts (Davidson et al., 2017). We observed that 99.95% of consensus gene sequences generated by superTranscripts were identical to our results. However, we note that superTranscripts tends to remove sequences specific to a unique transcript that do not overlap with any other transcript sequences although being indicated as belonging to the same gene candidates.

**Table 3. Length coverage of gene candidates as compared to galGal5 reference genes**

| Length coverage | Number of gene candidates | Cumulative number | Cumulative percentage |
|-----------------|---------------------------|-------------------|----------------------|
| 100%            | 3620                      | 3620              | 17.0%                |
| ≥75% and <100%  | 4822                      | 8442              | 39.5%                |
| ≥60% and <75%   | 2801                      | 11,243            | 52.7%                |
| ≥25% and 50%    | 3282                      | 14,525            | 68.0%                |
| >0% and <25%    | 2864                      | 17,389            | 81.5%                |
| 0%              | 3958                      | 21,347            | 100.0%               |

**Table 4. Comparison of galGal4 gene candidates to galGal5 reference genes**

| Gene candidates | Number | Percentage |
|-----------------|--------|------------|
| galGal4 reference genes | 15,358 | 74.1% |
| -concordant assignment | 11,384 | 6.3% |
| -concordant and undefined assignments | 368 | 2.4% |
| -partly annotated with assignment | 41 | 0.3% |
| -assigned with different gene symbol | 126 | 0.8% |
| -undefined assignment | 441 | 2.9% |
| -discordant assignment | 244 | 1.6% |
| -without assignment | 2754 | 17.9% |
| galGal4 additional genes | 5989 | 36.9% |
| -concordant assignment | 376 | 6.3% |
| -concordant and undefined assignment | 29 | 0.5% |
| -partly annotated with assignment | 182 | 3.0% |
| -assigned with different gene symbol | 28 | 0.5% |
| -undefined assignment | 760 | 12.7% |
| -discordant assignment | 16 | 0.3% |
| -without assignment | 4598 | 76.8% |

*a* Gene candidates matching a correct gene and one or several undefined genes (LOC, ORF).

*b* Gene candidates resulting from ORF and protein domain prediction.

*A* Gene candidates matching one or several undefined genes (LOC, ORF).

*b* Includes highly repeated genes such as those encoding histone proteins and myosin heavy chains.
extensive insights into evolutionary and adaptive traits within birds. DNA conservation of protein-coding genes among avian species considerably facilitated the annotation of the 21,347 gene candidates identified by the dual transcript-discovery approach. By combining DNA sequence comparison against avian genes with protein sequence comparison against mammal species and protein domain prediction, 14,847 (69.6%) gene candidates could be assigned and 672 (3.1%) putative protein-coding gene candidates could be identified. The 5828 (27.3%) remaining gene candidates were divided between uncharacterized proteins and ncRNAs depending on the length of the predicted ORF. However, gene candidates encoding uncharacterized proteins could be also potentially non-coding since none of the protein domains investigated was detected within their putative ORF. On the other hand, ncRNAs remain challenging to annotate according to a recent study comparing an extensive repertoire of long multi-exonic ncRNAs across 11 tetrapods separated by up to 370 million years (Necsulea et al., 2014). Besides their overall weak conservation as compared to protein-coding sequences, long ncRNAs display high tissue specificity and rapidly diverge through evolution, which renders their annotation difficult by comparing with other species.

Since the first draft released in 2004, considerable efforts have been made to improve the Gallus gallus reference genome and its annotation (Hillier et al., 2004; Kuo et al., 2017; Schmid et al., 2015; Thomas et al., 2014; Warren et al., 2017). In December 2015, the fifth version of the chicken genome (galGal5) was released (Warren et al., 2017). As compared to the fourth version, this release is 200 Mb longer and includes three additional chromosomes (30, 31, 33) but remains highly fragmented. Indeed, this fifth version is still composed of 15,400 unassigned contigs and 8000 contigs assigned with low confidence, accounting for ~17% of the total genome size. While we found that some gene candidates still remain missing or partly annotated in this new release, our gene prediction is consistent with other comparisons identifying novel genes absent from galGal4 reference annotation but present in galGal5 reference annotation or other birds (Borneliv et al., 2017; Hron et al., 2015; Lovell et al., 2014; Warren et al., 2017). Improvement of the chicken genome is an ongoing project and a new version should be released within the next few years. It is reasonable to believe that continuing efforts will contribute to elucidate the full sequence of the chicken genome in a near future. Until then, applying the dual transcript-discovery approach described here prior to the analysis of RNA-seq data per se enhances the sensitivity of gene discovery.

A second transcript-discovery approach was led independently of the genome culture. Low-quality RNA-seq reads from each replicate of micromass cultures were first filtered out by using the FASTX-Toolkit v0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). Reads with a median quality value lower than 28 were discarded. Filtered read pairs were then trimmed by using Trimomatic v0.32 (Bolger et al., 2014) (parameters: ILLUMINACLIP TruSeq3 paired-end for HiSeq, seedMismatches 2, palindromeClipThreshold 30, simpleClipThreshold 10; LEADING 5; TRAILING 5; MINLEN 36). Complete read pairs were then assembled by using Trinity r20140717 (Grabherr et al., 2011) (default parameters except for the strand-specific library orientation set at RF).

Gene fragmentation correction

Contigs resulting from the de novo assembly were compared to the gene candidates sequences obtained by the first approach by using BLASTN from BLAST+ v2.2.31+ (Camacho et al., 2009) (parameters: -strand plus; -dust no; -soft_masking no). Contigs were assigned to a given gene candidate if they matched at least 40 bp that were not covered by a previous hit with a percentage of identities higher than 90%. Assigned contigs that were not fully covered by a given gene candidate were further processed to extract continuous uncovered regions of at least 400 bp. Remaining contigs were mapped against the galGal4 genome by using BLASTN (parameters:
-perc_identity 90; -dust no; -soft_masking no). Contigs were assigned to a
given gene candidate if they were located between two gene features,
potentially corresponding to an exon missed by Cufflinks, or in the vicinity
of a first or last exon, potentially corresponding to a missing 5' or 3'-
untranslated region (UTR), respectively. Remaining unmapped contigs were
retrieved as they could correspond to non-defined genomic regions.
Unmapped, unassigned and non-covered contigs or regions of at least
400 bp were further processed to remove redundant sequences between
multiple isoforms. This step was necessary to prevent read pairs to be
mapped on multiple gene features and to be consequently discarded during
fragment counting. Isoforms belonging to the same gene candidates defined
by Trinity were compared to the longest isoforms by using BLASTN
(parameters: -perc_identity 90; -strand plus; -dust no; -soft_masking no;
-ungapped). Sequence alignments were then examined to build consensus
gene sequences by merging identical sequences between two isoforms and
by adding sequences unique to each isoform. Pairwise sequence comparison
was performed until all isoforms of the same gene candidates were
processed and concatenated. Resulting contig sequences were gathered
assembled as an artificial chromosome and separated to each other by 250 bp
of nucleotides N, corresponding to the total length of read pairs (50 bp for
each read and 150 bp as insert size).

Functional annotation
Gene candidate sequences retrieved from both transcript-discovery
approaches were then compared to existing databases for gene name
assignment. First, gene candidates were compared to the NCBI RefSeq
transcript database by using BLASTN (parameters: -strand plus; -dust no;
-soft_masking no). Comparison was limited to Aves (birds) sequences (taxid
8782). Gene candidates with a percentage of identities >90% for chicken
genes or 75% for bird genes, and bidirectionally covered on at least 50% of
their length were assigned to the corresponding hits. Gene candidates
matching several discordant gene names, such as chimeric and fused gene
candidates, were manually investigated and corrected. Non-annotated gene
candidate sequences were then compared to the NCBI human (taxid 9606)
and mouse (taxid 10090) non-redundant protein database by using BLASTX
from BLAST+ v2.2.31+ (Camacho et al., 2009) (parameters: -strand, plus;
-seq, no). Gene candidates with a percentage of homology of at least 30% and
covered by at least 50% of their length were filtered. Matching protein
accession numbers were converted into gene accession numbers by using the
Hyperlink Management System (Imanishi and Nakaoka, 2009). ORF
prediction was finally performed on remaining gene candidates by using
TransDecoder v2.1.0 (Haas et al., 2013) (strand specificity parameter: -S).
ORFs of at least 100 amino acids were annotated by using Trinotate v3.0.1
(https://trinotate.github.io/). Functional annotation was based on the following
protein predictions: (i) BLASTX and BLASTP homology search against the
SwissProt database (Bairoch et al., 2001); (ii) protein domain prediction
against the Pfam database (Punta et al., 2012) by using HMMER v3.1b2
(Finn et al., 2011); (iii) signal peptide prediction by using SignalP v4.1
(Petersen et al., 2011); and (iv) transmembrane domain prediction by using
tmHMM v2.0c (Krogh et al., 2001). Resulting functional annotation was
divided into three categories: (i) putative proteins, for which at least one
protein domain could be identified; (ii) uncharacterized proteins,
corresponding to ORFs for which no protein domain could be identified;
and (iii) ncRNAs, corresponding to genes with an ORF shorter than
100 amino acids.

Comparison with galGal5
UCSC (galGal5) and Ensembl (release 89) reference annotations associated
with the galGal5 genome version were downloaded from the UCSC browser
and merged by using the Cufflinks tool Cuffmerger v2.1.1 (Trapnell et al.,
2010). RNA-seq strand-specific read pairs were mapped against the chicken
genome galGal5 (Warren et al., 2017) by using TopHat2 v0.14 (Kim et al.
2013) (parameters: -perc_identity 90; -strand plus; -dust no; -soft_masking no). On one hand,
the total length coverage of predicted gene candidates was assessed by
identifying all regions matching with galGal5 gene sequences. On the other
hand, gene name assignment between predicted gene candidates and
annotated galGal5 genes was compared by retrieving only the hits that
matched at least 50% of their length.

Fragment counting
Strand-specific read pairs mapped against the chicken genome and the
artificial chromosome generated from the de novo transcript discovery were
first split by strand by using SAMtools v1.2 (Li et al., 2009) according to their
FLAG field (strand plus: -f 128 -F 16, -f 80; strand minus: -f 144, -f 64 -F 16). Fragments (both reads of a pair) mapped on gene features were counted by using
featureCounts v1.4.6-p3 (Liao et al., 2014) (parameters: -p; -s 2;
ignoreDup, -B; -R). Chimeric fragments aligned on different chromosomes
were taken into consideration to overcome the gene fragmentation due to the
location of gene parts on multiple chromosome contigs.

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Data availability
RNA-seq data have been deposited on the Gene Expression Omnibus (GEO)
database under the SuperSeries accession number GSE100517. Both samples that
have been used for this study are available under the SubSeries GSE100516 via the
accession numbers GSM2685833 and GSM2685834. Gene annotation model in
GTF format associated with the galGal4 version of the chicken genome and
sequence of the artificial chromosome in FASTA format are available at https://
sourceforge.net/projects/dualtranscriptdiscovery/

Supplementary information
Supplementary information available online at
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