RNA-Seq analysis reveals expression regulatory divergence of W-linked genes between two contrasting chicken breeds

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Abstract

The regulation of gene expression is a complex process involving organism function and phenotypic diversity, and is caused by cis- and trans- regulation. While prior studies identified the regulatory pattern of the autosome rewiring in hybrids, the role of gene regulation in W sex chromosomes is not clear due to their degradation and sex-limit expression. Here, we developed reciprocal crosses of two chicken breeds, White Leghorn and Cornish Game, which exhibited broad differences of gender-related traits, and assessed the expression of the genes on W chromosome to disentangle the contribution of cis- and trans-factors to expression divergence. We found that there was not appear to be an association between female fecundity and W chromosome gene expression, that 44% of expressed genes had divergent expression between breeds in both tissues, with only 17% of them showing greater expression in White Leghorn. We observed that the proportion of trans-acting genes in W chromosome was higher than cis-regulatory divergence. There were most parental divergence expression genes in muscle, also more heterosis compared with other two tissues. A strong dominant impact of Cornish alleles in brain, while obvious crosses-specific regulatory patterns appeared in liver. Taken together, this work describes the regulatory divergence of W-linked genes between two contrasting breeds and indicates sex chromosomes have a unique regulation and expression mechanism.

1. Introduction

Changes in genetic architecture can affect gene expression and their protein products, and further shape phenotypic variation [1–3]. As a form of genetic changes, the variability of gene expression, referred to as transcriptional regulatory factors, were categorized as cis-regulatory elements and trans-regulatory factors [4–6]. Cis-regulatory elements are present in the vicinity of genes on the same molecule of DNA, whereas trans-regulatory elements can regulate or modify genes distant from the gene from which they were transcribed by combining with their target sequences [7, 8]. The persistence of cis- and trans-acting, and the mechanism by which it occurs is particularly important because they play a large role in gene-expression novelty and phenotypic mutation [9, 10]. The current approach that distinguishes regulatory changes in the animal genome is based on gene expression divergence with fixed paraments, which estimates comparing the extent of the expression difference between two parents to the relative allelic expression in their hybrids [7]. To be specific, for autosomal in diploid individuals, the effect of cis-regulatory elements has allele-specific and quantified as additive inheritance of genes [11]. By comparison, trans-regulatory factors regulate both alleles derived from parental breeds, and thus hybrids alleles cannot inherit expression level origin from parents respectively, so trans-regulatory divergence is enriched for dominant effect [12].

Birds have a special sex chromosome system, in which females have heterogametic sex chromosomes (ZW in females and ZZ in males) [13–15]. Bird W chromosomes lack recombination except for the pseudo-autosomal regions (PARs) [16], thus recombination suppression between the Z/W chromosome determines the female W-linked genes (except for PARs genes) existing independently and without alleles [17]. The analyzed methods that identify gene regulatory divergence and/or inheritance pattern in diploid, can provide us with analogy insights for studying the regulatory changes of sex-limited W chromosome [18–22]. Theoretically, since there is no concept of allelic expression, there may be discrepancies that the criterion that categorizes regulatory divergence in the W chromosome. When hybrids expression level inherits its mother, we think cis-regulatory element acting on W-linked genes. While genes that are not explained by cis-regulatory divergence are attributed to trans-regulatory divergence. In other words, trans-regulatory divergence in the W chromosome always causes inconsistencies in expression levels between female progeny and her maternal parent. For regulatory changes, the inheritance pattern and the mechanism of regulatory divergence are often related. In W chromosome, the effect of cis-regulatory variants is maternal dominant, whereas trans-regulatory differences are more likely to be all patterns except maternal dominant.

Modern chickens have been subjected to artificial directional selection [23–25], and thus they were exposed to the different sex-specific effects and finally resulting in many phenotypic differences among breeds, such as egg number, body size, and female fecundity [26, 27]. This divergence in reproductive capacity is also related to female fitness, and therefore affects W-linked genes expression and their inheritance. The artificial multi-events that have known the explicit direction led to rapid change under domestication of chicken, also offered us an ideal model for revealing the relative contribution of the cis- and trans-regulatory variation in W-linked genes. Here, we used two chicken breeds, White Leghorn (WL) and Cornish (Cor), which have undergone varying sex-specific selection, to assess the parental expression difference and the role of two regulatory variations of W-linked genes in brain, liver, and muscle at 1-day-old.

2. Results

2.1. Divergence in gene expression among parental breeds

We chose two chicken breeds, Cor and WL, which exhibit varying female fecundity, to represent the effects of elevated and reduced female-specific selection respectively. Previous studies had proved that female-specific selection is an important force in shaping the evolution of gene expression on the W chromosome, we used the different sex-specific selection regimens in hopes of observing a wider range of W-linked genes regulatory divergence.

We characterized differential gene expression between the two parental breeds, and finally obtained 162 expressed W-linked genes in all three tissues, and there were 71 differential expressed genes (DEGs) (fold change > 1.25, FDR<0.05). Most of DEGs (85%) had a less than two-fold difference in expression, indicating that the expression divergence was subtle (Fig. 3). Also contrary to our conjecture, only 12 DEGs showed greater expression in WL (elevated female-specific selection breeds). There was a stark contrast in expression of the W-linked genes between three tissues, convergent patterns of gene expression were detected in brain which only 28% showed significant divergence, while genes in muscle showed more discrete expression which 55% were classified as DEGs.

2.2. The profile of the parental and hybrid W-linked genes expression in different tissues

For each hybrid cross, we collected RNA-Seq data from the brain, liver, and muscle tissue of 23 F1 progenies 1-day post-hatching. On average, we recovered 29.17 million mappable reads per sample. We only kept female individuals for studying the W chromosome. According to the criteria in the previous step, the unexpressed genes had been removed. We observed significant differences in gene expression between different tissues, between maternal-origin. Tissue was the most significant factor acting on W-linked gene expression (Fig. 4). For the three tissues, the maternal origin seemed the most powerful because samples were clustered based on it. Under these circumstances, the effect of maternal origin of Cor /CL was more obvious than WL/LC judging by the degree of dispersion of the two clusters. The taxa could basically be separated according to breeds, which was basically in line with our expectations.

2.3. Contribution of cis- and trans-acting effects base on the classification of regulatory divergence

Both the parental and hybrid data sets were analyzed for evidence of differential expression using the binomial exact test and fold change parament. According to the differential expressed situation, expressed genes were classified into different categories of regulatory divergence (Fig. 5, Table 1). In our experimental conditions, trans-acting genes were

more extensive compared with cis-regulatory divergence, with averages of 66.7% vs. 33.3% in all three tissues. Most genes exhibited dominant expression we speculate that it might be due to the strong effect of the allele from the single parent. In order to verify our conjecture, we further examined the W-linked genes for evidence of skewed expression of one allele. All 21 expressed dominant genes showed Cor-skewed dominant in brain. Such biased inheritance patterns are not extreme in muscle (around 62%). Interestingly, this strong heredity power of the Cor allele seems to lose its efficacy in the liver that around 52% of dominant genes showed Cor-skewed. Heterosis widely exists in hybridization events, corresponding to the improvement of production performance of hybrids. Although hybridization disadvantages occasionally appear, they are avoided in the breeding process as much as possible. As expected, we observed heterosis was ~3 times more common than the hybrid disadvantage. Specially, up-regulation of W-linked genes in hybrids was far more common in muscle, 14 expressed genes showed over-dominant. Only 1 and 4 loci were expressed at higher levels compared with both parents in brain and liver. Within tissues, few of these w-linked genes showed consistent regulatory divergence category between two groups, the proportion of these genes were accounting for around 18%, 10%, and 25% of the total in the brain, liver, and muscle.

Table 1
Statistical results of regulatory divergence categories of W-linked genes in three tissues

Tissues	Groups	Number of genes					
		Cis	Trans				Conserved
		Cis (dominant)	Trans (dominant)	Trans (additive)	Trans (overdominant)	Trans (underdominant)	
Brain	1	11	0	4	1	0	1
	2	0	10	3	1	1	2
Liver	1	8	6	6	0	1	0
	2	7	6	1	1	3	3
Muscle	1	11	6	2	5	8	0
	2	7	10	7	0	6	2

3. Discussion

Previous studies found that the direction and magnitude of sexual selection can partially shape the evolution of gene expression on the W chromosome [27]. Two breeds were selected as the samples representing distinct sexual selection modes. Given its differences in reproductive performance and other female fitness traits, we expected an apparent diversity of W-linked transcriptome abundance was also exist between WL and Cor. Surprisingly, our result was the opposite of the assumption in all three tissues. Specially, only less than half of the expressed genes (44%) showed significantly different expressed in the three tissues (FDR<0.05, fold change >1.25), and 17% of these genes are WL-skewed. We inferred that there are three main reasons. First, biased expression is not necessarily a fixed property of genes, expression level can vary greatly among tissues [28–31]. Generally, somatic tissues show much less dimorphism than gonads [32–34]. We chose three somatic tissues instead of gonads as the experimental samples because the gonads of 1-day-old chicks are extremely small and may easily be mixed in by other tissues. Second, gene expression is also highly variable over the course of development. Previous evidences showed there are expression changes with minor divergence in embryonic stages and high-level divergence in sexually mature adults [35, 36]. Nonetheless, we still selected 1-day-old chicks because female-specific selection in birds is strongest during

this developmental time point [35]. Third, known W-linked genes do play an important role in sex determination, but there is no evidence that their functions are patently associated with sexual fitness [37], the shaping effect of sexual selection on the W chromosome may also be insignificant.

Although the assumption of parental expression divergence deviated from our original intention, it did not affect the procedure and results of our exploration of regulatory changes. Before identifying regulatory variations, we observed expression clusters between tissues, and between breeds. The different sample clusters of each tissue indicated that tissues played the most significant role in gene expression for all individuals. Within tissues, the breeds-specific patterns could both be observed clearly, of which the most typical in muscle. We speculated that this obvious pattern in muscle might be related to the disparity in growth and development performance between breeds [38, 39]. Our results suggested that maternal origin was an important contributor to the cluster in gene expression in our dataset. This clustering feature was in line with our prediction due to the maternally inherited mode of W chromosome. We also hastened to point out that this maternal origin mode is more obvious in Cor♀/CL♂, but its shaping effect in WL♀/LC♂ did not seem to be significant. This evidence reflected from the side that Cor W-linked genes had stronger hereditary capabilities. The PCA overview results proved the genetic differences between the reciprocal crosses, and further demonstrated the necessity of identifying the regulatory divergence according to it.

Regulatory changes and inheritance patterns are both based on gene expression dynamic changes in the hybridization process [12, 40]. Mechanisms of regulatory divergence may influence the inheritance of gene expression, recent studies showed that inheritance diversity may depend on the effects of trans-regulatory factors of one genome on the other genome [41–43], and hence the regulatory divergence between parental species. This assumption is based on the effect that dominance is caused by trans-regulatory factors in diploid hybrid [10]. Under the special circumstances in W chromosome, genes locate in the non-recombination region can be regarded as ‘single allele’, so cis-/trans- regulatory elements can also lead to dominant pattern. The difference is that cis-regulatory divergence will lead to a maternal-dominant, while trans-regulatory divergence contributes independently to paternal-dominant. Nevertheless, a large proportion of expressed genes showed dominant (both including caused by cis-/trans-regulatory elements), and previously plant-based studies showed that dominant pattern is prevalent and widespread among different natural populations, and maybe also closely related to the phenotypic novelty of hybrids [44–46]. The above evidences proved two conclusions. First, the special sex-limit characteristics of the W chromosome will cause a different regulatory changes mechanism compared with autosomes and Y chromosomes. Second, the result that the novel or the superiority phenotype has a certain link to dominant patterns in offspring is consistent with the classic concept in hybrid breeding.

For all classified genes, major of them were controlled by trans-regulatory factors rather than cis-regulatory elements, the ratios of "Cis" and "Trans" are 0.55, 0.63, and 0.41 in brain, liver, and muscle respectively. These results were consistent with the previous observation in autosomal genome, in which the ratios were 0.71, 0.53, and 0.25 in brain, liver, and muscle respectively [43]. These observations confirmed that the gene expression evolution of most W-linked genes might be controlled by loci on autosomes or Y chromosomes. The similar effect of regulatory changes on autosomal genes and sex-linked genes showed that the total efficacy of regulatory divergence along the entire genome was basically stable. To identify the relative contribution of “W single allele” originating from WL and Cor, we carefully observed whether there was a breed-skewed expression mode in dominant W-linked genes. Interestingly, all dominant genes in brain tissue exhibited Cor-skewed, regardless of the female paternal expression level. This extreme imbalance was not observed in liver and muscle tissues, the proportion of Cor-skewed expression genes only accounted for 52% and 62% respectively, only showed a slight advantage over WL-skewed expression. We previously thought that hybrids might inherit the excellent muscle growth characteristics of the Cor parent and thus be more Cor-skewed compared with the other two tissues. For this result that was contrary to our conjecture, there were at least

three, non-mutually exclusive, possible explanations. First, compared with liver and muscle, brain has the most conservative expression pattern [47], the expression regulation was not easily affected by reciprocal crosses and has a high consistency. Second, heterosis in hybrids may be mainly reflected in over-dominant rather than dominant [47, 48]. The evidence for this inference in this study was that muscles have the most “over-dominant” genes. Finally, unlike autosomal genes, the function of W-linked genes may be less related to muscle and body development.

Taken together, this study provided a significant advance in understanding regulatory evolution on a sex-limit genomic scale. We drew on the traditional methods that distinguish between cis- and trans-acting sources on autosomes, and used a new method to evaluate the regulatory changes of W chromosome for the first time. Our results also provided a systematic look at the evolution of cis- and trans-acting, and incorporated the inheritance pattern into the same research framework with regulatory divergence. In principle, this joint analysis approach had advantages because of the causal relationship between these two concepts. Cis-regulatory elements and trans-acting factors control nearby and distant gene expression. Meanwhile, the hereditary architecture of gene expression levels determines the inheritance pattern. Using the RNA-Seq data of hybridization model, we globally identified the features at the transcriptome level of W-linked genes and visualized them through the classification of regulatory divergence. More instances of trans-regulatory divergence than instances of cis-regulatory divergence were observed in W chromosome, this might be because the relatively short divergence history of Cor and WL [23, 49]. This low genetic diversity was also a potential cause that DEGs among parents only account for a small part of expressed genes.

What is certain is that although the regulatory pattern identifies based on the transcriptome level is reasonable, it does not mean that the result is completely accurate. A small number of organized, single-point, fixed-parent studies are not enough to allow us to understand the mechanism and laws of regulatory divergence from a broader perspective, also does not allow us to locate the sequence sites of these regulatory factors. What can be encountered is that when these limitations are broken, higher throughput and more accurate sequencing methods are applied, the problem will be solved, and the research on regulatory divergence will not just stop at the stage of description and statistical analysis.

In conclusion, our research used innovative methods to identify the genetic pattern and regulatory divergence of the W chromosome, which was not limited to a single tissue, and a single set of conditions. The results revealed an autosomal-like regulatory model, which implied a robust mechanism of regulatory divergence across whole sequences. Insights on W-linked gene expression regulation and evolution would expand such research at the species (or breed) and genome levels.

4. Material And Methods

4.1. Sample preparation and sequencing

We used WL and Cor chickens from the National Engineering Laboratory for Animal Breeding of the China Agricultural University, as representative breeds of layers and broilers respectively, to obtain pure-bred and hybrid progeny. Three tissues, including brain, liver, and breast muscle tissues were collected from 23 1-day-old chickens, 3 males and 3 females were selected from the progeny, except for the Cor male × WL female cross that only has two female offspring (Fig. 1). The chicks are euthanized with high-concentration carbon dioxide, which makes the animals lose consciousness quickly and minimize pain. Because one-day-old chickens are too small for intravenous injection, we did not use pentobarbital sodium injection for euthanasia

The tissues were deposited in RNAlater (Invitrogen, Carlsbad, CA, USA), an RNA stabilization solution, at 4 degrees Celsius for one night and then moved to -20 degrees Celsius refrigerator, and we extracted total RNA using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was sequenced on Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) with 100-bp paired-end reads and 300-bp insert size. Finally, we obtained a total of 246.3 Gb of RNA-Seq data, corresponding to an average of 3.6 million mappable reads per sample.

4.2. RNA-Seq Analysis of genome

RNA-seq raw data were filtered using Fastp [50] c High-quality reads were aligned to the chicken reference genome (GCA_016699485.1) using Hisat2 [51, 52]. After that, we used Stringtie [53] to estimate high-quality transcript abundance, with the normalization methods of Transcripts Per Kilobase Million (TPM) [54]. To further determine the credibility of W-linked genes in the reference genome, we checked the expression data of W-linked genes in all individuals to ensure that they are not expressed only in males (TPM=0 or basically tends to 0).

4.3. Principal Components Analysis of W-linked genes

In order to investigate the patterns of gene expression for expressed W-linked genes in females, we analyzed the expression clusters of the three tissues by principal component analysis (PCA), as implemented in RStudio and visualized by R packages, factoextra and FactMineR [55]. Before PCA, we removed genes with TPM < 0.5 in all samples to ensure the reliability of the results.

4.4. Classification of cis- and trans- regulatory categories

We used two inbred chicken breeds (Cor and WL) to generate reciprocal cross progeny. Therefore, we could classify by cross when identifying regulatory categories. Cor♀, WL♂, CL were divided into one group (Group 1), WL♀, Cor♂, LC were divided into another group (Group 2). Since there is no W chromosome existing in the male genome, the males in the above groups should be replaced by females of the same breeds, that was, Cor♀, WL♂, CL♀ as Group 1, and Cor♂, WL♀, LC♀ as Group 2. After determining the group, we removed all males for the further analysis of regulatory divergence.

Different from autosome and Z chromosomes, the sex-specific W chromosome diverge from their counterparts, thus W-linked genes don't have alleles. Although the standard method to category regulatory variations could not apply in this study, the strategy of this method that identified differential expression between the two purebred progenies and hybrids could be referenced. Statistical thresholds of fold change>1.25 and false discovery rate (FDR) < 5% were both set so that we could determine if there were significant differences in expression level [12, 56]. The expressed genes were classified into three main categories according to the following criteria:

(1) Cis: Significant difference between parents (WL♀ and Cor♂), no significant difference between F1 and their maternal parents (Cor♀ and CL♀; WL♀ and LC♀), significant difference between F1 and their paternal parents (WL♂ and CL♂; Cor♂ and LC♂).

(2) Trans: Significant difference between parents, significant difference between F1 and their maternal parents.

(3) Conserved: Significant difference between parents, no significant difference between F1 and their maternal parents, no significant difference between F1 and their paternal parents.

As shown in Figure (Fig. 2), all 5 sub-categories (Subdivide the 'Cis' and 'Trans' main categories, 'Conserved' main category was not subdivided) are listed according to expression level.

Genes that were not significantly different between parents were not taken into consideration in this classification, because the effect of cis/trans would be masked due to their expression pattern.

Declarations

Data availability

All raw data during the current study are available in the NCBI BioProject (https://submit.ncbi.nlm.nih.gov/subs/bioproject/) with accession number PRJNA591354.

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Author contributions

HG analyzed and interpreted the sequencing data and drafted the manuscript. LW and XL participated in the reciprocal cross experiment. WY, YC, KL and JZ provided some suggestions for the improvement of the study and substantively revised the manuscript. YJ participated in the collection of samples. ZN participated in the design of the study. LQ conceived the study, and participated in its design and coordination. All the authors have read and approved the final manuscript.

Ethics approval

Our animal experiments were approved by the Animal Care and Use Committee of China Agricultural University (Approval ID: XXCB-20090209). All the animals were fed and handled according to the regulations and guidelines established by this committee, and all efforts were made to minimize suffering. All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

Declaration of Competing Interest

The authors declare that they have no competing interest.

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Tables

Table 1. Statistical results of regulatory divergence categories of W-linked genes in three tissues

Tissues	Groups	Number of genes					
		Cis	Trans				Conserved
		Cis (dominant)	Trans (dominant)	Trans (additive)	Trans (overdominant)	Trans (underdominant)	
Brain	1	11	0	4	1	0	1
	2	0	10	3	1	1	2
Liver	1	8	6	6	0	1	0
	2	7	6	1	1	3	3
Muscle	1	11	6	2	5	8	0
	2	7	10	7	0	6	2

Figures

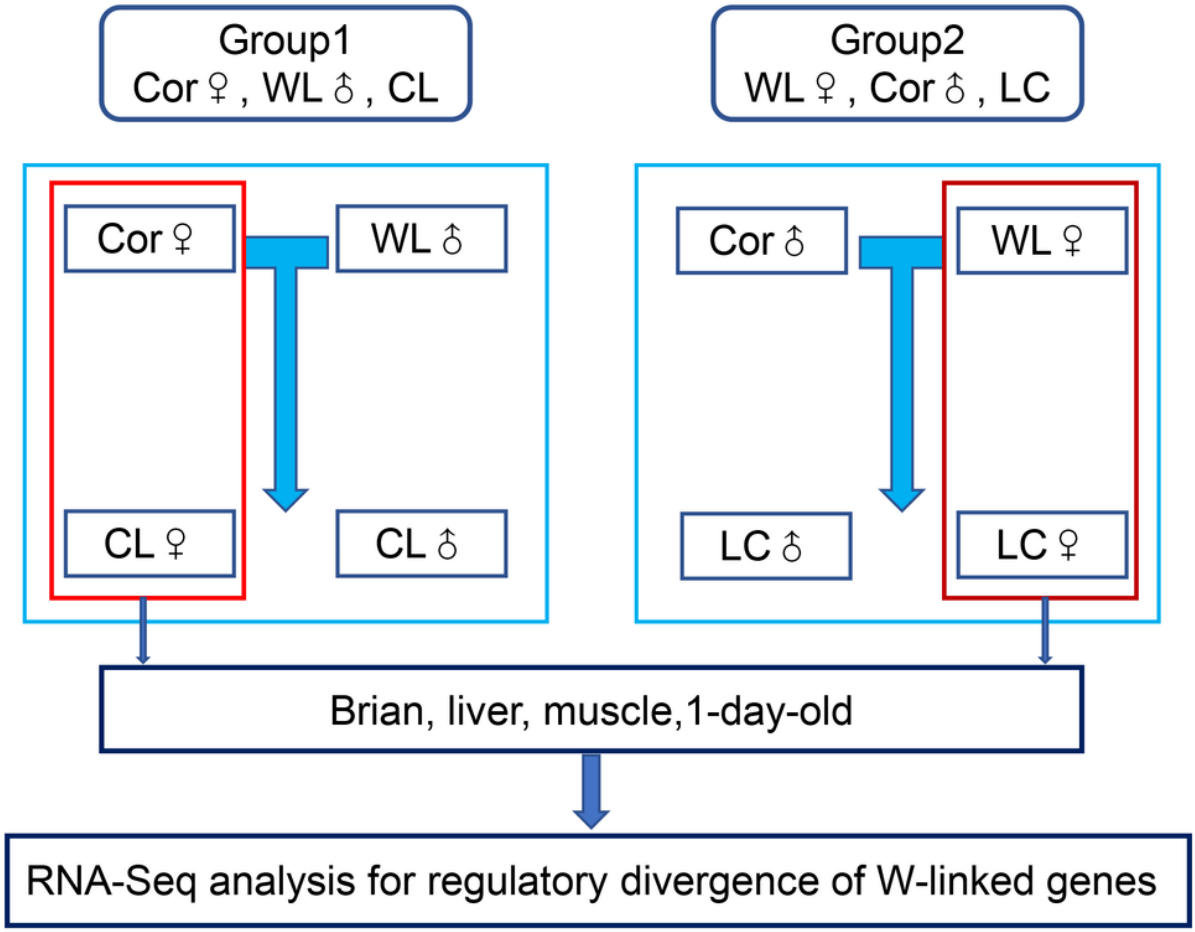

Figure 1

Samples and experiment design. Group 1 and Group 2 represent reciprocal crosses, respectively. All analyses use female individuals (Group 1: Cor♀, WL♀, CL♀, Group 2: Cor♀, WL♀, LC♀). Samples of three tissues (brain, liver, and muscle) were collected from all chicks one day after hatching for transcriptome sequencing

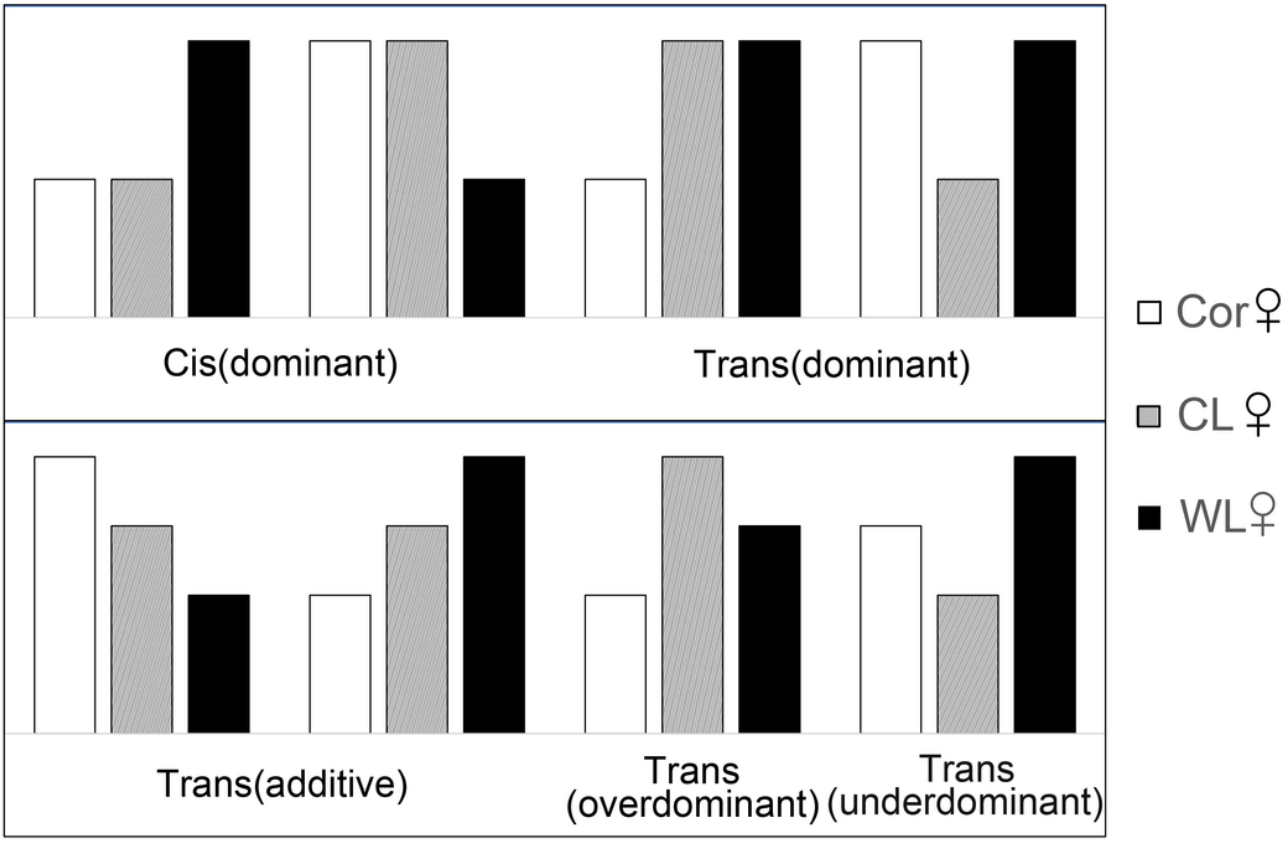

Figure 2

Hypothetical classification of regulatory divergence. Classification according to the expression level in Cor, WL, and F1 hybrids. Only the categories in Group 1 (Cor, WL, CL) are shown here, as an example.

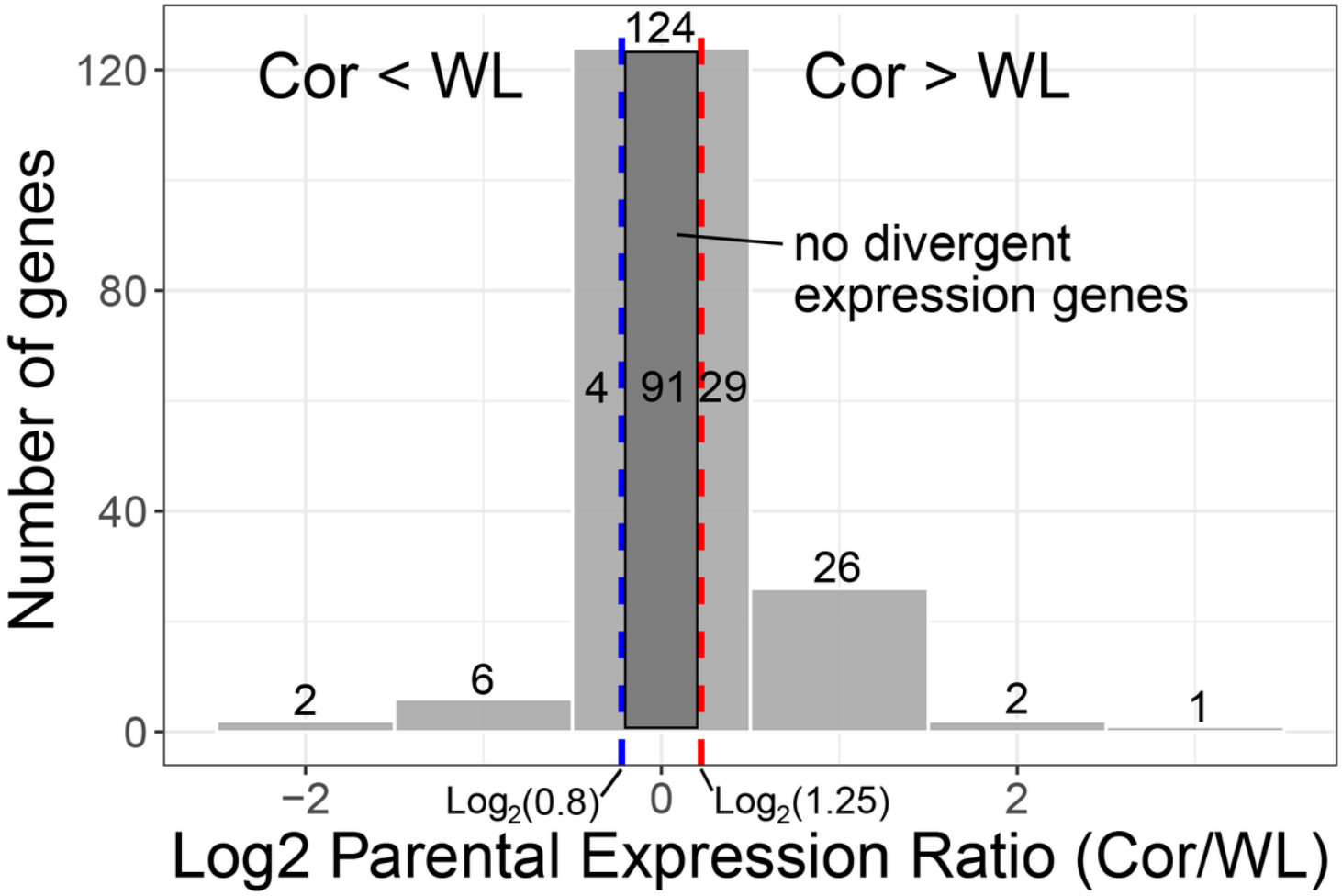

Figure 3

Differences in gene expression between Cor and WL. The histogram shows the direction and magnitude of changes in expression in genes exhibiting divergent parental expression. The two vertical dashed lines represent the expression thresholds of parental divergence. Negative values indicate up-regulated expression of WL, and positive values indicate up-regulated expression of Cor.

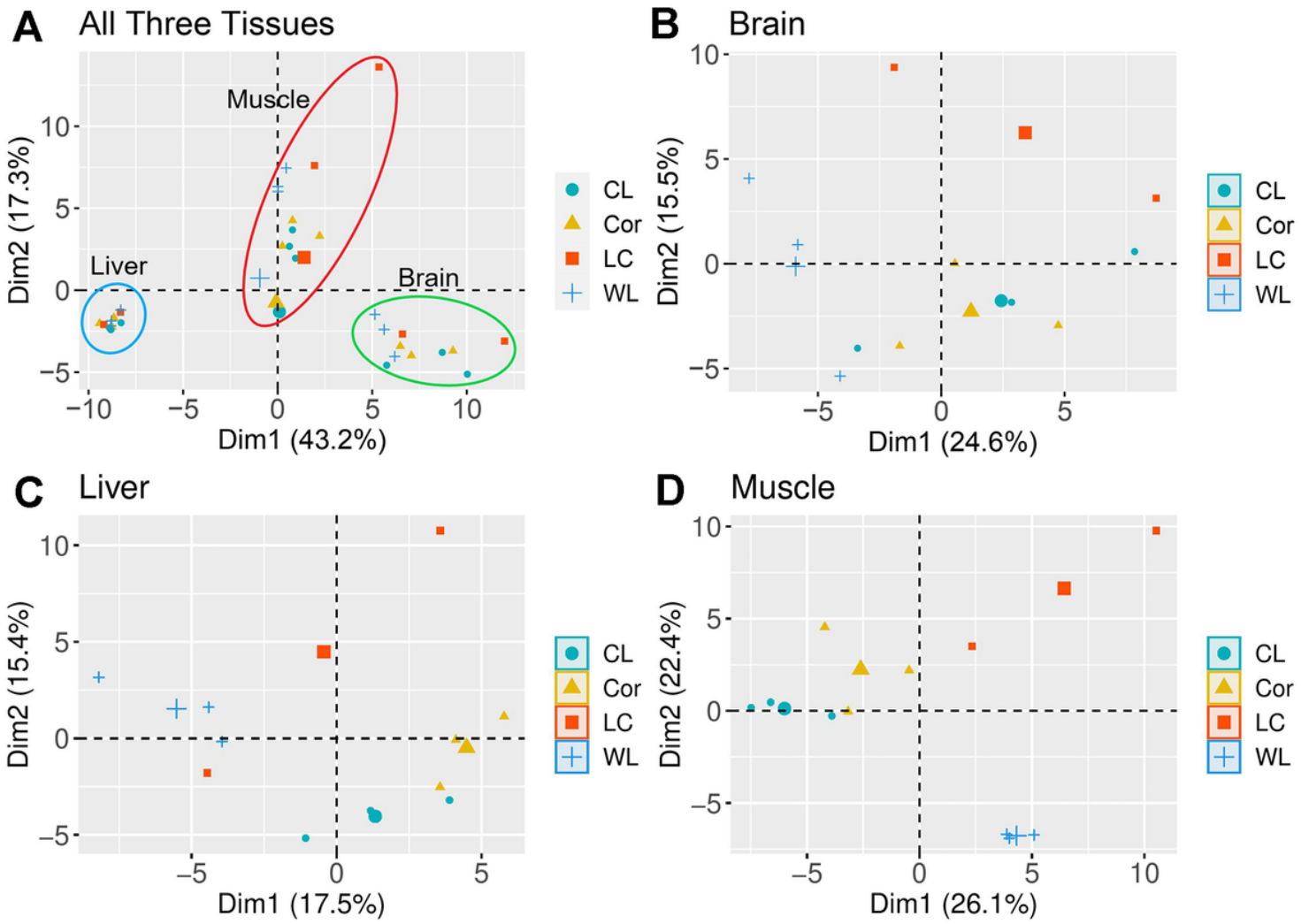

Figure 4

Principal Component Analysis using RNA-Seq data. A) PCA results of all three tissues. B) PCA results of brain. C) PCA results of liver. D) PCA results of muscle. Each dot represents an individual, and different varieties use dots with different shapes and colors to indicate.

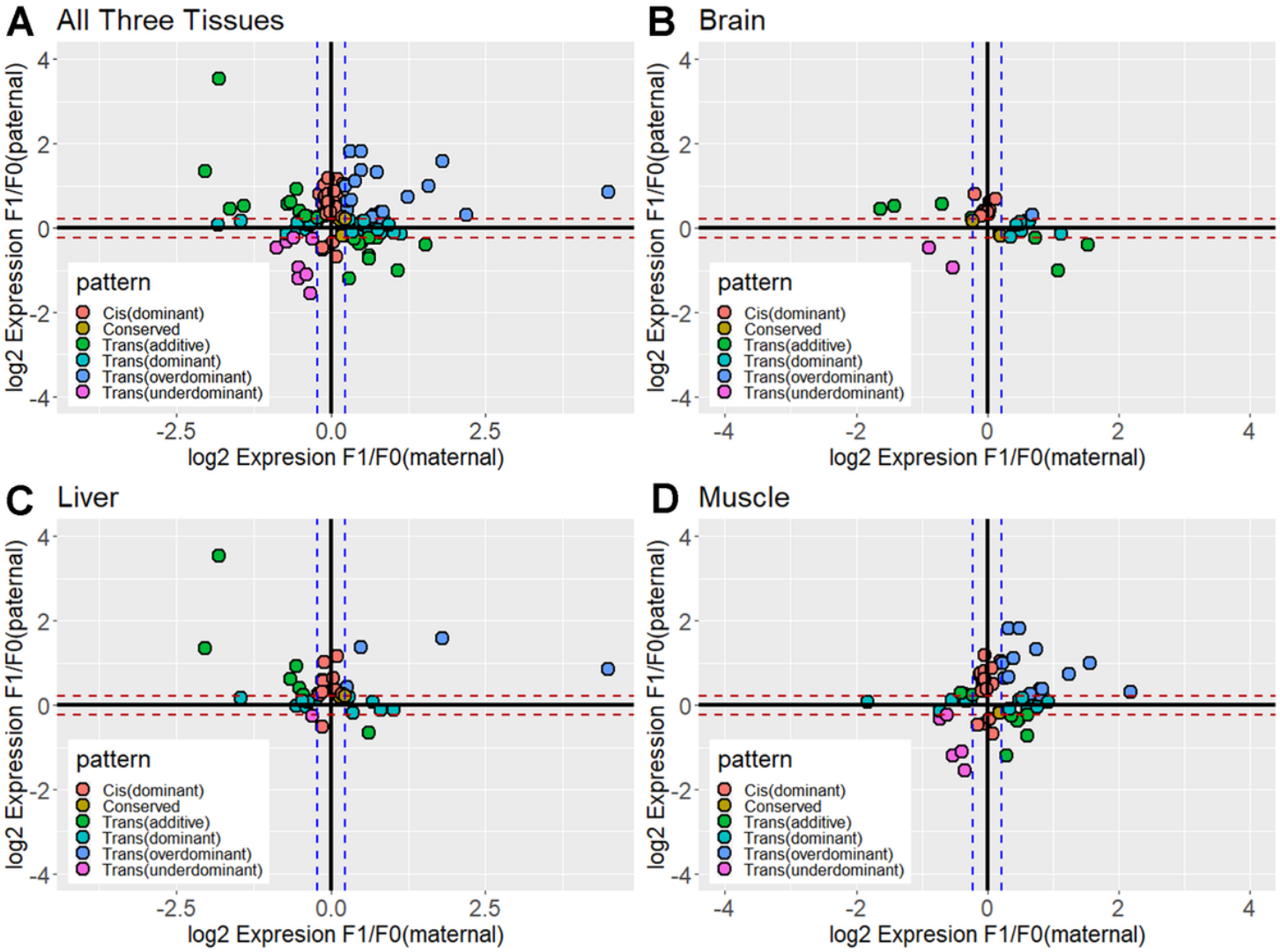

Figure 5

The scatterplot compares the W-linked expressional differences between hybrids and their parental breeds. The visualized results are respectively displayed in A) all three tissues, B) brain, C) liver, D) muscle (WL on the x-axis, Cor on the y-axis). The different colored dots represent the different regulatory divergence categories.