# Comparative transcriptome analysis of differentially expressed genes in the ovary and testis of Athetis dissimilis

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# Abstract

Insects have developed a variety of sex-determining regulatory mechanisms throughout their evolution. In this study, we analyzed differentially expressed genes (DEG) from the ovary and testis transcriptomes of *Athetis dissimilis*. A total of 11065 differentially expressed genes (DEGs) between the males and females were identified, of which 6685 genes were up-regulated and 4380 genes were down-regulated in females. A *Adistra-2* (525 bp) gene was obtained from the transcriptome of *A. dissimilis*, and sequence alignment with other related species revealed a highly conserved RRM domain. Phylogenetic analysis showed that the Adistra-2 protein is a close relative of the lepidopterous tra-2 protein. The qRT-PCR of the *Adistra-2* temporal and spatial expression pattern showed that it is more abundant during embryonic development than other stages, and its expression was higher in ovaries than in testes. This work provides a theoretical reference for the sex differentiation and genetic manipulation of this insect.


# Introduction

The regulation and evolution of sex determination have been an important issue in the study of developmental and evolutionary biology for a long time. Sex determination is the result of evolutionary selection in species and the basic characteristics of biological activities. The mechanisms of insect sex determination are diverse and complex, even among similar species and even different populations of the same species have evolved different mechanisms of sex determination (Marın & Baker, 1998; Matson & Zarkower, 2012; Peng et al., 2020). Commonly, sex determination in insects involves an embryonic sex determination primary signal that activates the sex-determination cascade of gene-specific splicing, where the cascade gene sex-specific product regulates the differentiation of individuals into males or females. The absence of a primordial signal leads to a default gene splicing pattern that regulates opposite sexual development (Schütt & Nöthiger, 2000; Salz & Erickson, 2010; Blackmon & Demuth, 2015). In the evolutionary process of the insect sex determination mechanism, the underlying genes of the sex determination cascade are highly conserved, while the original signals of sex determination are variable (Sánchez, 2008; Bopp et al., 2014). The primary signals of sex determination mainly include X chromosome-linked signal elements (XSE), Male determiner factor (M-factor), homozygous or heterozygous genes, and piwi-interacting RNA (piRNA). Gender-specific expression of the *transformer* (*tra*) gene, a key gene regulating the original signal of sex determination, its *tra* product, and the *transformer-2* (*tra-2*) gene, can achieve the specific expression of the underlying bisexual gene *doublesex* (*dsx*) through a sex-specific selective splicing mechanism. This, in turn, regulates the expression of genes related to male and female development in both sexes to achieve gender differentiation.

Previously, *tra* homologous genes have been found in Diptera (Belote et al., 1989; Pane et al., 2002), Hymenoptera (Li et al., 2013), and Coleoptera (Shukla & Palli, 2012), but not in Lepidoptera. The *tra-2* homologous gene has been identified in *Drosophila melanogaster*, *Bombyx mori*, *Sciara coprophila*, *Tribolium castaneum*, and *Apis mellifera* (Amrein et al., 1990; Niu et al., 2005; Martín et al., 2011; Nissen et al., 2012; Shukla & Palli, 2013). The amino acid sequence length of the tra-2 protein varies, but the RRM domain is relatively conserved, consisting of two ribonucleoprotein domains (RNP) or RNA-binding

domains (RBD) (Dauwalder et al., 1996). Different insects often exhibit variable splicing isoform numbers of the tra-2 gene, for example, *D. melanogaster* has four variable shear bodies of the tra-2 gene, where three of the variable shears are in male and female individuals, and one variable splicing isoform is male-specific (Amrein et al., 1990). The tra-2 gene of *B. mori* and *A. mellifera* has six splicing variants, the tra-2 gene of *Nasonia vitripennis* has four splicing variants and the tra-2 gene of *T. castaneum* has three splicing variants (Niu et al., 2005; Nissen et al., 2012; Suzuki et al., 2012; Geuverink et al., 2017). However, the variable tra-2 splices of the above insects were not expressed specifically in males or females. Alternatively, there is only one variable splice of the tra-2 gene in *Musca domestica*, *Lucilia cuprina*, *Bactrocera dorsalis*, *Ceratitis capilata*, and *Anastrepha obliqua* (Concha & Scott, 2009; Liu et al., 2015; Peng et al., 2015; Burghardt et al., 2005; Salvemini et al., 2009; Sarno et al., 2010).

*Athetis dissimilis* (Lepidoptera: Noctuidae) has gradually become a major pest in wheat and maize fields in recent years. Similar in appearance to *Athetis lepigone*, a voracious moth, it poses a serious threat to wheat and corn production (Wang et al., 2012). Additionally, the frequent use of chemical pesticides has led to higher pest resistance, and developing new pest control measures is imminent. In the present study, we carried out transcriptomic sequencing of the ovary and testis of *A. dissimilis* to identify differentially expressed genes to better understand the regulatory mechanisms associated with the reproductive processes in this species.

# Materials and methods

Insect rearing and sample preparation

The *A. dissimilis* samples were collected from corn fields in Luoyang, Henan in 2014. Colonies were maintained in an artificial climate chamber at 25 ± 1°C on a 16 h: 8 h light: dark cycle with 80 ± 5% relative humidity.

Eggs laid by mated females at 1, 2 and 3 days after sampling was collected. Larvae at the 1, 2, 3, 4, 5, and 6th instars were selected. Male and female pupae were collected 3 days after pupation. Male and female adults were collected 3 days after emergence and the head, midgut, malpighian tubule, testis, and ovaries of male and female adults, and carcass (residual body) were dissected for RNA extraction. All samples were stored in liquid nitrogen at -80°C.

Differential expression analysis

The transcriptome data of *A. dissimilis* ovaries and testis were obtained from previous work (SRA: PRJNA970546). Pearson's Correlation Coefficient r was used as an evaluation index of correlation among samples (Anders & Huber, 2010). The closer $r^2$ is to 1, the stronger the correlation between the two samples.

DESeq2 software was used for differential expression analysis between sample groups (Leng et al., 2013). The Benjamini-Hochberg (Benjamini & Hochberg, 1995) method was adopted to correct the *P*-

value obtained from the original hypothesis test, where the corrected *P*-value was adopted as the key index of differentially expressed genes (DEGs) screening. During the screening process, the screening criteria were set to: a False Discovery Rate (FDR) of less than 0.01 and a Fold change (FC) greater than or equal to 4. Here the FC represented the expression ratio between two samples. .

# Functional annotation and enrichment analysis of differentially expressed genes

Multiple public databases were used for homology annotation, including non-redundant protein databases (NR), Protein family (Pfam), eukaryotic Ortholog Groups (KOG), SwissProt, eggnog, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO).

Gene Ontology terms were assigned to each sequence annotated by BLASTX against the Nr database using the Blast2GO v2.5 program with an E-value threshold of 1E − 6 for further functional categorization. WEGO software (Ye et al., 2006) was used to plot the distribution of the GO functional classification of unigenes. The unigene sequences were also aligned to the COG database to predict and classify possible functions. The unigenes were assigned to KEGG pathway annotations to analyze inner-cell metabolic pathways and related gene functions using BLASTX.

# Identification and bioinformatics analysis of Adistra-2

The *Adistra*-2 of *A. dissimilis* was derived from the previous transcriptome library annotation. The NCBI blast showed that the *Adistra*-2 was more than 95% consistent with other insects. We also verified the accuracy of *Adistra*-2 sequences by PCR.

The sequence similarity between Adistra-2 and homologous species was analyzed using the NCBI-Blast network server (http://blast.ncbi.nlm.nih.gov/). The TMHMM Server v2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0/) predicted the transmembrane structure of the Adistra-2 protein. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) predicted the presence and the location of signal peptides in the prediction protein. The DNAMAN software was used to compare the multi-sequence alignment of the Adistra-2 protein with the tra-2 proteins from other insects [*Aedes albopictus* (AWW13909.1), *Cotesia congregata* (CAG5074056.1), *Bradysia coprophila* (CBX45938.1), *Drosophila melanogaster* (NP_476764.1), *Bombyx mori* (NP_001119705.1), *A. mellifera* (NP_001252514.1), *Tribolium castaneum* (NP_001280513.1), *Bemisia tabaci* (QAB02877.1), *Helicoverpa armigera* (XP_021199210.1), *Spodoptera litura* (XP_022838015.1), *Trichoplusia ni* (XP_026724995.1), *Ostrinia furnacalis* (XP_028159452.1), *Ostrinia furnacalis* (XP_028159452.1), *Ostrinia furnacalis* (XP_028159452.1), *Leguminivora glycinivorella* (XP_047991853.1)]. A phylogenetic tree was constructed using the Neighbor-joining (NJ) method in Mega 6.0, and the confidence values among species were calculated with 1,000 Bootstrap repeats (Zhang & Sun, 2008).

Temporal and spatial expression analysis of Adtra-2

Eggs at the first, second, third, fourth, fifth, and sixth days and larvae at the first, second, third, fourth, fifth, and sixth instar were selected to detect the temporal expression characteristics of *Adistra-2*. Total RNA was extracted from the head, midgut, malpighian tubule, testis, and ovary of male and female adults, and carcass (residual body) to detect the spatial expression characteristics of *Adtra-2*. Total RNA was extracted and transcribed to first-strand cDNA using RNAiso Plus kits and PrimeScript RT reagent with gDNA Eraser (TaKaRa, Dalian, China). Real-time quantitative PCR (RT-qPCR) was performed with SYBR® Premix Ex Taq II (TaKaRa). A GAPDH gene was used as the internal reference (Song et al., 2021). Adistra-F: GATGCTAAGACCGGTCGTTCTC and Adistra-R: CGGTAGTAGTCGTCTCTATC were used as primers. The total RT-qPCR reactions contained: 10 μL of SYBR Premix Ex Taq II, 2 μL of cDNA template, 0.8 μL of each primer, and 6.4 μL $ddH_2O$. The RT-qPCR cycling conditions were as follows: pre-denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 58°C for 30 s. Melt curve conditions were 95°C for 10 s and 65°C for 30 s. No-template controls (NTC) were included to detect possible contamination. The target gene relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method with three biological replicates. Significance was calculated with a *T*-test, where $p < 0.05$ was considered statistically significant.

# Results

Differential expression gene analysis

The $r^2$ values of the three replicates of the ovary and testis were 0.958, 0.946, 0.967, and 0.942, 0.949, 0.95, which were close to 1, indicating that the biological replicate data was reliable. The $r^2$ values between different samples of ovary and testis were 0.004, 0.009, 0.011, and 0.004, 0.009, 0.011, which were less than 0.05, indicating that there was a significant difference between the ovary and testis (Fig. 1). There were 11065 DEGs between the ovary and testis of *A. dissimilis*, including 6685 up-regulated genes and 4380 down-regulated genes in the ovary (Fig. 2).

Annotation and function analyses of DEGs

At the same time, 6656 DEGs were annotated in NR (6616), GO (1484), COG (1696), KEGG (2483), Swiss-Prot (3077), Pfam (3967), and eggnog (5808). Seven large databases were used to describe and classify their common functions (Table 1). In the COG databases, 1696 DEGs were annotated and classified into 25 subcategories. Among these annotated functional classes, the largest subcategory was carbohydrate transport and metabolism (229 DEGs); this was followed by the General function prediction only (200 DEGs) (Fig. 3).

Table 1
Statistics of annotation results

| Database | GO | KEGG | eggNOG | COG | NR | Pfam | Swiss-Prot |
|---|---|---|---|---|---|---|---|
| Unigenes | 1484 | 2483 | 5808 | 1696 | 6616 | 3967 | 3077 |
| Ratio (%) | 22.30 | 37.30 | 87.26 | 25.48 | 99.40 | 59.60 | 46.23 |

To identify the biological pathways of these DEGs, we also classified them according to the KEGG databases. In the KEGG pathway analysis, 2483 DEGs were annotated to 50 categories, of which 158 DEGs were annotated to purine metabolism and 137 DEGs were annotated to RNA transport (Fig. 4).

The GO enrichment analysis showed 15 GO terms were significantly enriched in the ovary and four in the testis (Table 2). For example, the GO term related to the structural constituent of chorion was significantly enriched (FDR = 6.41E-08) in the ovary-biased genes. Chorion-containing eggshell formation (FDR = 5.10E-06) was also found in ovary-biased genes. Overrepresentation of this term suggests that gene transcription is more active in the ovary in comparison with the testis. The GO enrichment of the testis-biased genes displayed significant odorant binding (FDR = 5.96E-31) and structural constituent of cuticle (FDR = 3.61E-11) categories.

Table 2
GO classification in sex differential expressed genes of *Athetis dissimilis*

| Term ID | Term name | Genes | FDR | *P* value |
|---|---|---|---|---|
| Ovary-biased | | | | |
| GO:0003735 | structural constituent of ribosome | 97 | 6.85E-37 | 7.60E-17 |
| GO:0051082 | unfolded protein binding | 25 | 2.49E-11 | 1.20E-07 |
| GO:0004812 | aminoacyl-tRNA ligase activity | 26 | 4.81E-26 | 1.80E-07 |
| GO:1901363 | heterocyclic compound binding | 723 | 1.08E-09 | 1.20E-06 |
| GO:0005213 | structural constituent of chorion | 11 | 6.41E-08 | 1.60E-06 |
| GO:0097159 | organic cyclic compound binding | 724 | 1.99E-05 | 3.20E-06 |
| GO:0007304 | chorion-containing eggshell formation | 13 | 5.10E-06 | 5.20E-06 |
| GO:0003678 | DNA helicase activity | 20 | 6.06E-09 | 4.30E-06 |
| GO:0003677 | DNA binding | 122 | 5.81E-13 | 5.10E-06 |
| GO:0003743 | translation initiation factor activity | 30 | 8.79E-14 | 5.40E-06 |
| GO:0003746 | translation elongation factor activity | 20 | 2.18E-29 | 6.20E-05 |
| Testis-biased | | | | |
| GO:0004568 | chitinase activity | 13 | 1.59E-131 | 2.70E-07 |
| GO:0003735 | structural constituent of ribosome | 1 | 5.68E-119 | 6.00E-07 |
| GO:0005549 | odorant binding | 11 | 5.96E-31 | 3.10E-06 |
| GO:0042302 | structural constituent of cuticle | 11 | 3.61E-11 | 5.40E-05 |

Molecular identification of Adtra-2 and sequence analysis

The cDNA sequence of *Adistra-2* (GenBank no. OQ799648) was obtained from our previous transcriptome database and validated by sequenced specific primers PCR. The open reading frame (ORF) of Adistra-2 was 525 bp and encoded 174 amino acids with a predicted molecular weight of 20753.98 Da and a theoretical isoelectric point of 9.75. The protein has no signal peptide or transmembrane structure.

Multiple alignment and phylogenetic analysis

The multi-sequence alignment of the Adistra-2 protein with the tra-2 proteins from other insects showed a high degree of sequence conservation within the RRM and Linker domain, which was located in the same position in all samples. Similar to other species, the RNP-1, and RNP2 were found in Adistra-2, which is very conserved in the RRM protein (Fig. 5).

For phylogenetic analysis, the Adistra-2 protein was aligned with homologous sequences from other species. The phylogenetic tree was divided into different branches according to insect orders. The orthologs between different orders were distant (Fig. 6).

Spatial and temporal expression patterns of the Adistra-2 gene

The expression patterns of the temporal and spatial *Adistra-2* gene were examined with qRT-PCR analyses. The results showed that the *Adistra-2* gene had the highest relative transcription levels throughout embryonic development, compared to other stages (Fig. 7). The spatial expression pattern of *tra2* in *A. dissimilis* showed that *Adistra-2* was expressed in most tissues of females and males. Interestingly, *Adistra-2* showed especially enriched expression in the ovaries of *A. dissimilis* (Fig. 8).

# Discussion

There is very little genomic information available about insect reproduction. In the present study, we provide an important genomic resource for understanding the functional roles of major candidate genes involved in controlling the reproduction of *A. dissimilis.*

A total of 11065 differentially expressed genes (DEGs) between the males and females were identified, of which 6685 genes were up-regulated and 4380 genes were down-regulated in females. A total of 6656 (60.15%) DEGs were annotated in seven large databases. Similar studies have been done in invertebrates and insects (Gao et al., 2014; Li et al., 2016; Du et al., 2017; Wang et al., 2017; Yan et al., 2018; Guo et al., 2019; Cao et al., 2022; Guo et al., 2022; Pan et al., 2022; Cao et al., 2023). The GO enrichment analysis showed 15 GO terms were significantly enriched in the ovaries and four in the testes. Chorion-containing eggshell formation and structural constituent of chorion were significantly enriched in the ovaries, while odorant binding and chitinase activity were significantly enriched in the testes, suggesting that different regulation mechanisms are present in female egg development and male spermatogenesis in *A. dissimilis*. There have been many reports about the phenomenon of high expression of odor-binding protein in the testis (Brown er al., 2023; Han et al., 2023; Song et al., 2021), but the specific physiological function in the testis is still unclear.

Through an online BLAST search, an *Adistra-2* gene was identified in *A. dissimilis*. The open reading frame of *Adistra-2* was 525 bp which encoded 174 amino acids. Adistra-2 has a very conserved RRM domain (containing the RNP-1 and RNP2) and linker region, which are common features of tra-2 proteins. The RNP-1 and RRM domains are generally considered to be involved in the recognition of single-stranded RNA and can affect pre-mRNA selective splicing. Amino acid sequence alignment found that the homology and conservation of the RRM domain and linker domain in bivalves are similar, suggesting the functional similarity of tra-2 in these species (Wang et al., 2021). According to the evolutionary tree analysis, the evolution of tra-2 in different orders of insects or other invertebrates differs, whereas the homology relation of tra-2 is more closely related to the same order of insects. This is consistent with results from *Aedes albopictus* and *Hyriopsis cumingii* (Li et al., 2019; Wang et al., 2021).

The qRT-PCR results showed that the *Adistra-2* gene had the highest relative transcript levels throughout embryonic development compared to other stages. The results of a previous study found that knockdown of tra-2 in embryos of *B. mori* and *A. mellifera* can cause abnormal testis formation in some larvae, even leading to embryonic lethality, implying that tra-2 is vital during the early embryonic stages (Nissen et al., 2012; Suzuki et al., 2012). The spatial expression pattern of *tra-2* in *A. dissimilis* showed that *Adistra-2* was expressed in most male and female tissues, however, the *Adistra-2* expression level in the ovary was significantly higher than that in the testis. This expression pattern is consistent with that of *Aedes albopictus* (Li et al., 2019). This suggests that *Adistra-2* may play a potential regulatory role in the gonadal development of *A. dissimilis*. In the future, the specific function of the tra-2 gene needs to be confirmed by CRISPR/Cas9 gene knockout technology.

# Declarations

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**Conflict of interest** There is no conflict of interest between the authors.

# References

1. Amrein, H., Maniatis, T., & Nöthiger, R. (1990). Alternatively spliced transcripts of the sex-determining gene tra–2 of Drosophila encode functional proteins of different size. *EMBO Journal*, *9*, 3619–3629.
2. Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology*,*11*, R106.
3. Belote, J. M., McKeown, M., Boggs, R. T., Ohkawa, R., & Sosnowski, B. A. (1989). Molecular genetics of transformer, a genetic switch controlling sexual differentiation in *Drosophila*. *Developmental Genetics*, *10*, 143–154.
4. Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B-statistical*

*Methodology*, *57*, 289–300.

5. Blackmon, H., & Demuth, J. P. (2015). Genomic origins of insect sex chromosomes. *Current Opinion in Insect Science*, *7*, 45–50.
6. Bopp, D., Saccone, G., & Beye, M. (2014). Sex determination in insects: variations on a common theme. *Sexual Development*, *8*, 20–28.
7. Brown, N. C., Gordon, B., McDonough-Golgstein, C. E., Misra, S., Findlay, G. D., & Clark, A. G. (2023). The seminal odorant binding protein Obp56g is required for mating plug formation and male fertility in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, *31*, 543-551.
8. Burghardt, G., Hediger, M., Siegenthaler, C., Moser, M., Dübendorfer, A., & Bopp, D. (2005). The transformer2 gene in Musca domestica is required for selecting and maintaining the female pathway of development. *Development Genes and Evolution*, *215*, 165–176.
9. Cao, H. R., Gao, H. H., Li, Z. P., Peng, G. F., Chen, Y. M., Jin, T. Q., Zhu C., Ji H., & Dong W. (2022). Comparative transcriptome provides insights into differentially expressed genes between testis and ovary of *Onychostoma macrolepis* in reproduction period. *General and Comparative Endocrinology*, *326*, 114066.
10. Cao, H. R., Li, L., Li, Z. P., Gao, H. H., Peng, G. F., Zhu, C., Chen Y., Yang F., & Dong W. (2023). Denovo RNA-Seq analysis of ovary and testis reveals potential differentially expressed transcripts associated with gonadal unsynchronization development in *Onychostoma macrolepis*. *Gene Expression Patterns*, *47*, 119303.
11. Concha, C., & Scott, M. (2009). Sexual development in *Lucilia cuprina* (Diptera, Calliphoridae) is controlled by the transformer gene. *Genetics*, *182*, 785–798.
12. Dauwalder, B., Amaya-Manzanares, F., & Mattox, W. (1996). A human homologue of the *Drosophila* sex determination factor *transformer–2* has conserved splicing regulatory functions. *PNAS*, *93*, 9004–9009.
13. Du, X. X., Wang, B., Liu, X. M., Liu, X. B., He, Y., Zhang, Q. Q., & Wang X. B. (2017). Comparative transcriptome analysis of ovary and testis reveals potential sexrelated genes and pathways in spotted knifejaw *Oplegnathus punctatus*. *Gene*, *637*, 203–210.
14. Gao, J., Wang, X. W., Zou, Z. H., Jia, X. W., Wang, Y. L., & Zhang, Z. P. (2014). Transcriptome analysis of the differences in gene expression between testis and ovary in green mud crab (*Scylla paramamosain*). *BMC Genomics*, *15*, 585.
15. Geuverink, E., Rensink, A. H., Rondeel, I., & Beukeboom, L. W. (2017). van de Zande, L., Verhulst, E.C. Maternal provision of *transformer–2* is required for female development and embryo viability in the wasp *Nasonia vitripennis*. *Insect Biochemistry and Molecular Biology*, *90*, 23–33.
16. Gou, P. P., Wang, Z. C., Yang, J., Wang, X. L., & Qiu, X. M. (2022). Comparative transcriptome analysis of differentially expressed genes in the testis and ovary of sea urchin (*Strongylocentrotus intermedius*). *Fishes*, *7*, 152.
17. Guo, W., Lü, J., Guo, M. J., Chen, S. M., Qiu, B. L., Sang, W., Yang, C. X., Zhang, Y. J, & Pan, H. P. (2019). De Novo Transcriptome Analysis Reveals Abundant Gonad-specific Genes in the Ovary and Testis of

*Henosepilachna vigintioctopunctata*. *International Journal of Molecular Sciences*, *20*, 4084.

18. Han, W. K., Tang, F. X., Yu, N., Zhang, Y. X., & Liu Z. W. (2023) A non-sensory OBP plays an important role in the larval development and adult mating of *Spodoptera frugiperda*. *Insect Science.* https://doi.org/10.1111/1744-7917.13178.
19. Leng, N., Dawson, J. A., Thomson, J. A., Ruotti, V., Rissman, A. I., Smits, B. M. G., & Kendziorski, C. (2013). EBSeq: An empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics*, *29*, 1035–43.
20. Li, F., Vensko, S. P., Belikoff, E. J., & Scott, M. J. (2013). Conservation and sex–specific splicing of the *transformer* gene in the Calliphorids *Cochliomyia hominivorax*, *Cochliomyia macellaria* and *Lucilia sericata*. *PLoS ONE*, *8*, e56303.
21. Li, X. C., Jin, B. B., Dong, Y. Q., Chen, X. G., Tu, Z. J., & Gu, J. B. (2019). Two of the three transformer-2 genes are required for ovarian development in Aedes albopictus. *Insect Biochemistry and Molecular Biology, 109*, 92–105.
22. Li, Y. P., Zhang, L. L., Sun, Y., Ma, X. L., Wang, J., Li, R. J., Zhang, M. W., Wang, S., Hu, X. L., & Bao, Z. M. (2016). Transcriptome sequencing and comparative analysis of ovary and testis identifies potential key sex–related genes and pathways in scallop *Patinopecten yessoensis*. *Marine Biotechnology*, *18*, 453–465.
23. Liu, G., Wu, Q., Li, J., Zhang, G., & Wan, F. H. (2015). RNAi–mediated knock-down of transformer and transformer 2 to generate male–only progeny in the Oriental Fruit Fly, *Bactrocera dorsalis* (Hendel). *PLoS ONE*, *10*, e0128892.
24. Marın, I., & Baker, B. S. (1998). The evolutionary dynamics of sex determination. *Science*, *281*, 1990–1994.
25. Martín, I., Ruiz, M. F., & Sánchez, L. (2011). The gene *transformer–2* of Sciara (Diptera, Nematocera) and its effect on *Drosophila* sexual development. *BMC Developmental Biology, 11*, 1–13.
26. Matson, C., & Zarkower, D. (2012). Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. *Nature Reviews Genetics, 13*, 163–174.
27. Nissen, I., Muller, M., & Beye, M. (2012). The *Am–tra2* gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee. *Genetics*, *192*, 1015–1026.
28. Nissen, I., Muller, M., & Beye, M. (2012). The Am-tra2 gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee. *Genetics*, *192*, 1015–1026.
29. Niu, B. L., Meng, Z. Q., Tao, Y. Z., Lu, S. L., Weng, H. B., He, L. H., & Shen, W. F. (2005). Cloning and alternative splicing analysis of *Bombyx mori transformer–2* gene using silkworm EST database. *Acta Biochimica et Biophysica Sinica*, *37*, 728–736.
30. Pan, L. X., Hu, W. W., Cheng, F. P., Hu, X. F., & Wang, Z. L. (2022). Transcriptome analysis reveals differentially expressed genes between the ovary and testis of the honey bee *Apis mellifera*. *Apidologie*, *53*, 1–12.

31. Pane, A., Salvemini, M., & Bovi, P. D. (2002). The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development*, *129*, 3715–3725.
32. Peng, W., Yu, S. N., Alfred, M. H., Tu, Z. J., Giuseppe, S., Xi, Z.Y., & Zhang, H. Y. (2020). miRNA-1-3p is an early embryonic male sex determining factor in the Oriental fruit fly *Bactrocera dorsalis. Nature Communications*, *11*, 1–11.
33. Peng, W., Zheng, W., Handler, A. M., & Zhang, H. (2015). The role of the transformer gene in sex determination and reproduction in the tephritid fruit fly, *Bactrocera dorsalis* (Hendel). *Genetica*, *143*, 717–727.
34. Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L. C., & Saccone, G. (2009). Ceratitis capitata transformer–2 gene is required to establish and maintain the autoregulation of Cctra, the master gene for female sex determination. *International Journal of Developmental Biology*, *53*, 109–120.
35. Salz, H. & Erickson, J. W. (2010). Sex determination in *Drosophila*: the view from the top. *Fly*, *4*, 60–70.
36. Sánchez, L. (2008). Sex–determining mechanisms in insects. *International Journal of Developmental Biology*, *52*, 837–856.
37. Sarno, F., Ruiz, M.F., Eirín-López, J.M., Perondini, A.L., Selivon, D., & Sánchez, L. (2010). The gene transformer–2 of Anastrepha fruit flies (Diptera, Tephritidae) and its evolution in insects. *BMC Evolutionary Biology*, *10*, 140.
38. Schütt, C., & Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in *Dipteran* insects. *Development*, *127*, 667–677.
39. Shukla, J. N., & Palli, S. R. (2012). Sex determination in beetles: production of all male progeny by parental RNAi knockdown of *transformer*. *Scientific Reports*, *2*, 602–602.
40. Shukla, J. N., & Palli, S. R. (2013). *Tribolium castaneum Transformer-2* regulates sex determination and development in both males and females. *Insect Biochemistry and Molecular Biology*, *43*, 1125–1132.
41. Song, Y. Q., Song, Z. Y., Dong, J. F., Lv, Q. H., Chen, Q. X., & Sun, H. Z. (2021). Identifcation and comparative expression analysis of odorant-binding proteins in the reproductive system and antennae of *Athetis dissimilis*. *Scientific Reports*, *11*, 13941.
42. Suzuki, M. G., Suzuki, K., Aoki, F., & Ajimura, M. (2012). Effect of RNAi–mediated knockdown of the *Bombyx mori transformer-2* gene on the sex–specific splicing of *Bmdsx* pre-mRNA. *International Journal of Developmental Biology*, *56*, 693–699.
43. Wang, Y. Y., Wang, X. Q., Ge, J. Y., Wang, G. L., & Li, J. L. (2021). Identifcation and functional analysis of the sex–determiner transformer–2 homologue in the freshwater pearl mussel, *Hyriopsis cumingii*. *Frontiers in Physiology*, *12*, 704548.
44. Wang, Z. C., Qiu, X. M., Kong, D. R., Zhou, X. X., Guo, Z. B., Gao, C. F., Ma, S., Hao, W., Jiang, Z., & Liu, S. (2017). Comparative RNA–Seq analysis of differentially expressed genes in the testis and ovary of

*Takifugu rubripes*. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *22*, 50–57.

45. Wang, Z. Y., Shi, J., & Dong, J. G. (2012). Reason analysis on *Proxenus lepigone* outbreak of summer corn region in the Yellow River, Huai and Hai Rivers plain and the countermeasures suggested. *Maize Science*, *20*, 132–134.
46. Yan, H. W., Cui, X., Shen, X. F., Wang, L. S., Jiang, L. N., Liu, H. Y., Liu, Y., Liu, Q., & Jiang, C. (2018). De novo transcriptome analysis and differentially expressed genes in the ovary and testis of the Japanese mantis shrimp *Oratosquilla oratoria* by RNASeq. *Comparative biochemistry and physiology. Part D, Genomics & proteomics*, *26*, 69–78.
47. Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., Wang, J., Li, S., Li, R., & Bolund, L. (2006). WEGO: a web tool for plotting GO annotations. *Nucleic Acids Research*, *34*, 293–297.
48. Zhang, W., & Sun, Z. (2008). Random local neighbor joining: a new method for reconstructing phylogenetic trees. *Molecular Phylogenetics and Evolution*, *47*, 117–128.

# Figures

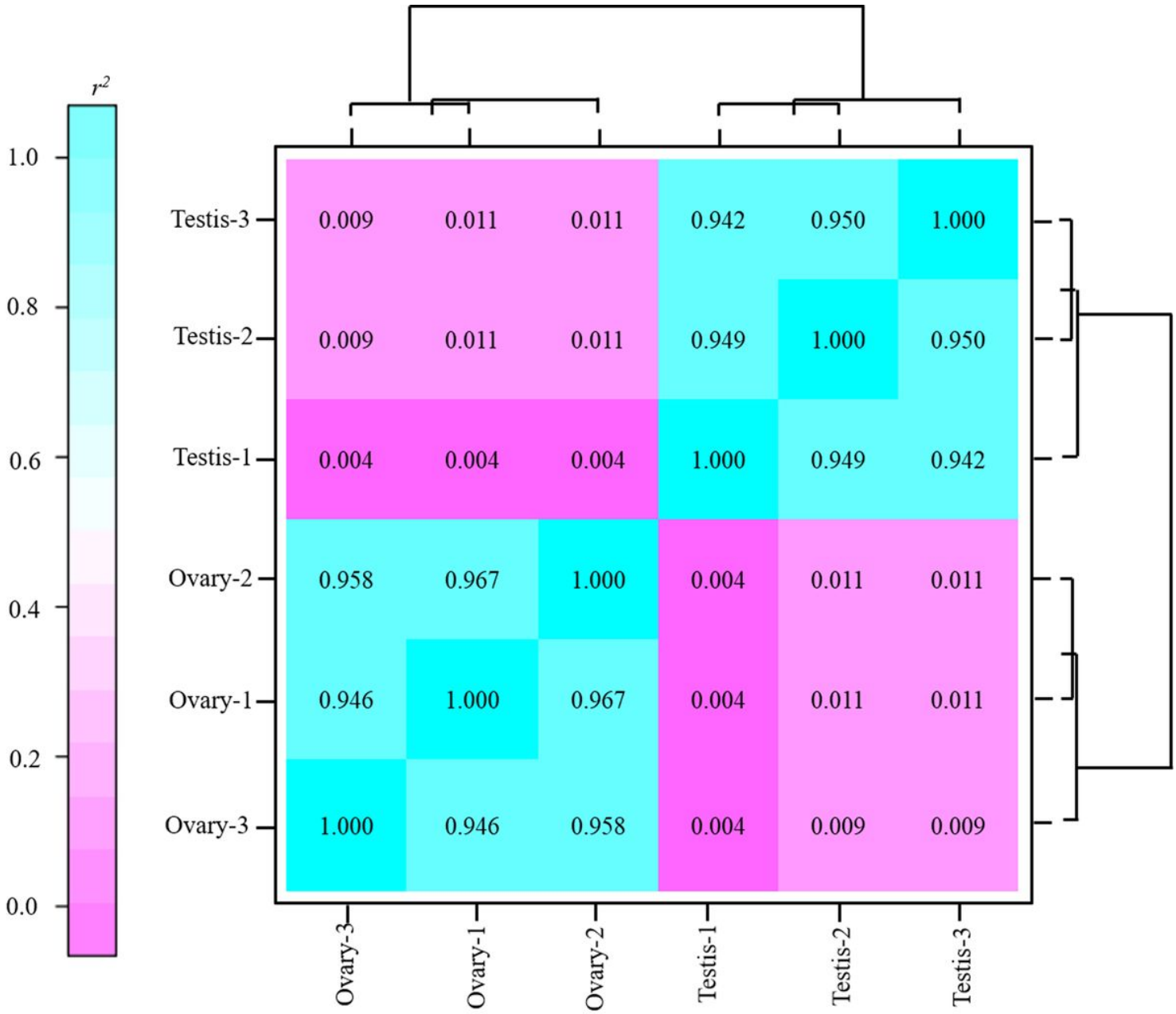



**Figure 1**

Correlation of gene expression levels in the transcriptome of the ovary and testis of *Athetis dissimilis*

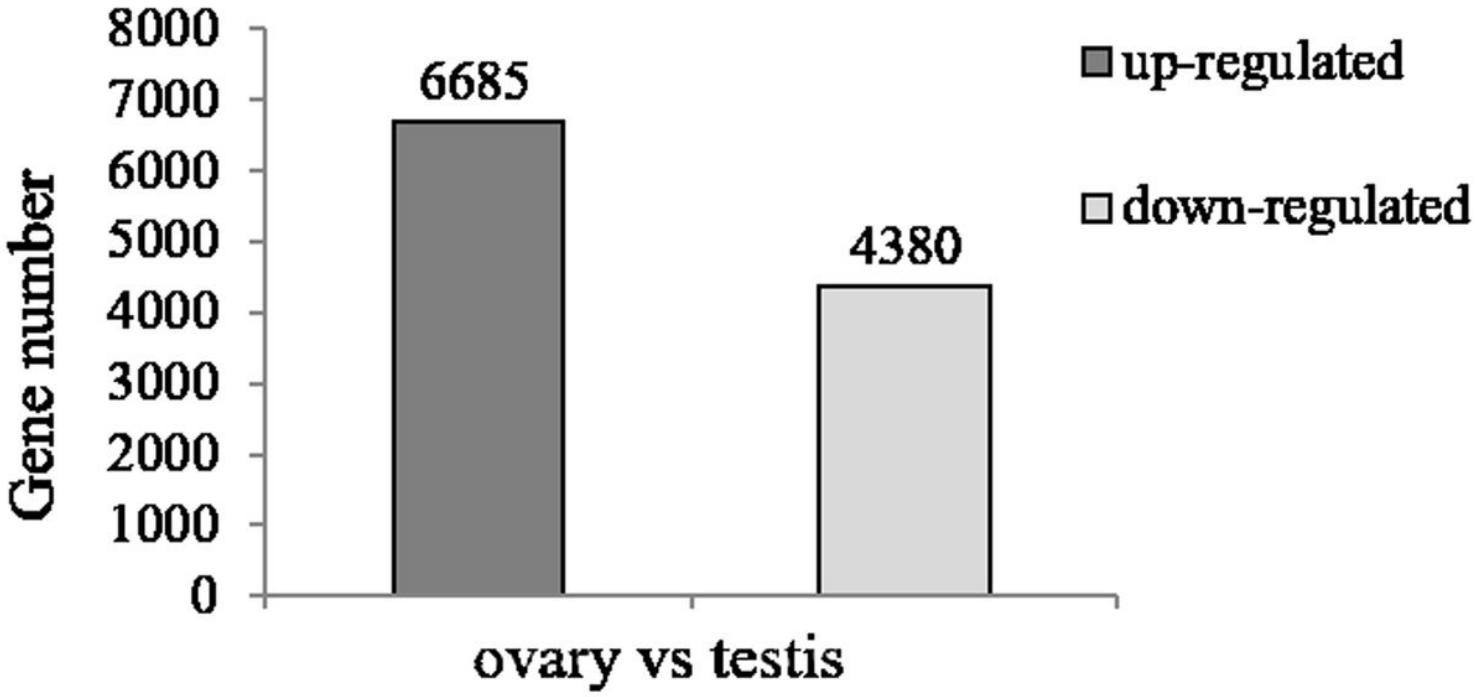



Figure 2

Up- and down-regulated DEGs in the ovary compared to the testis in *Athetis dissimilis*.

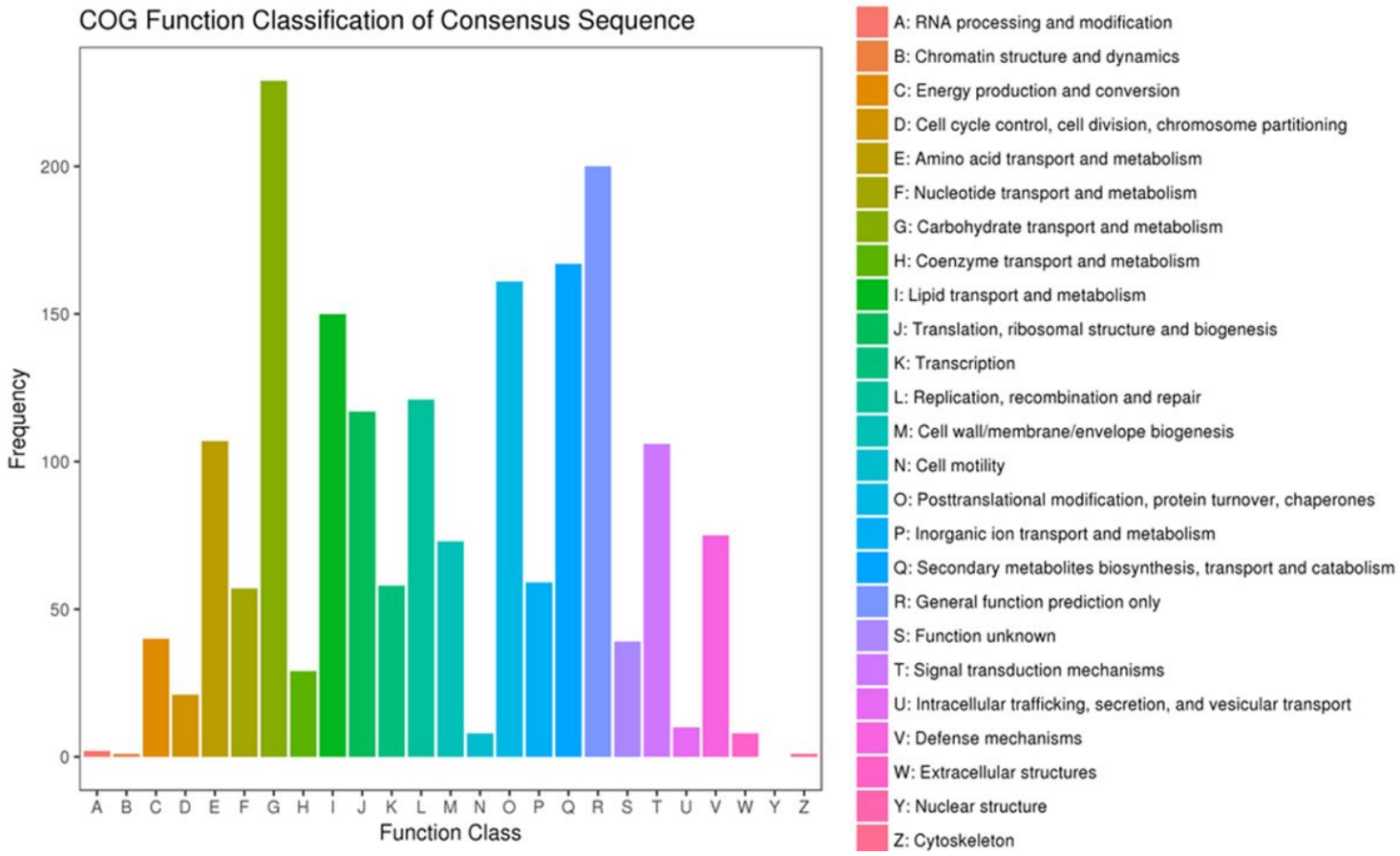
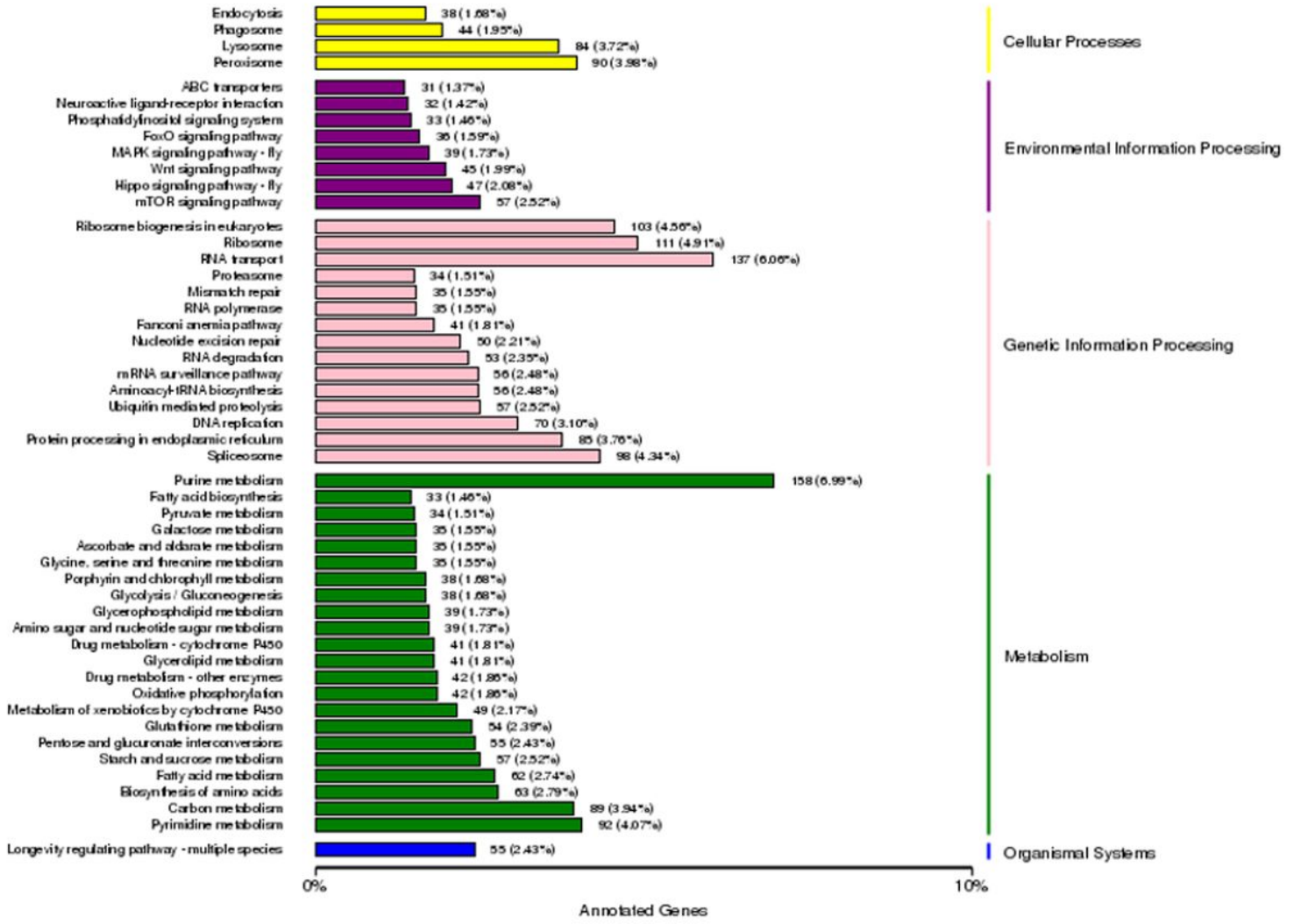


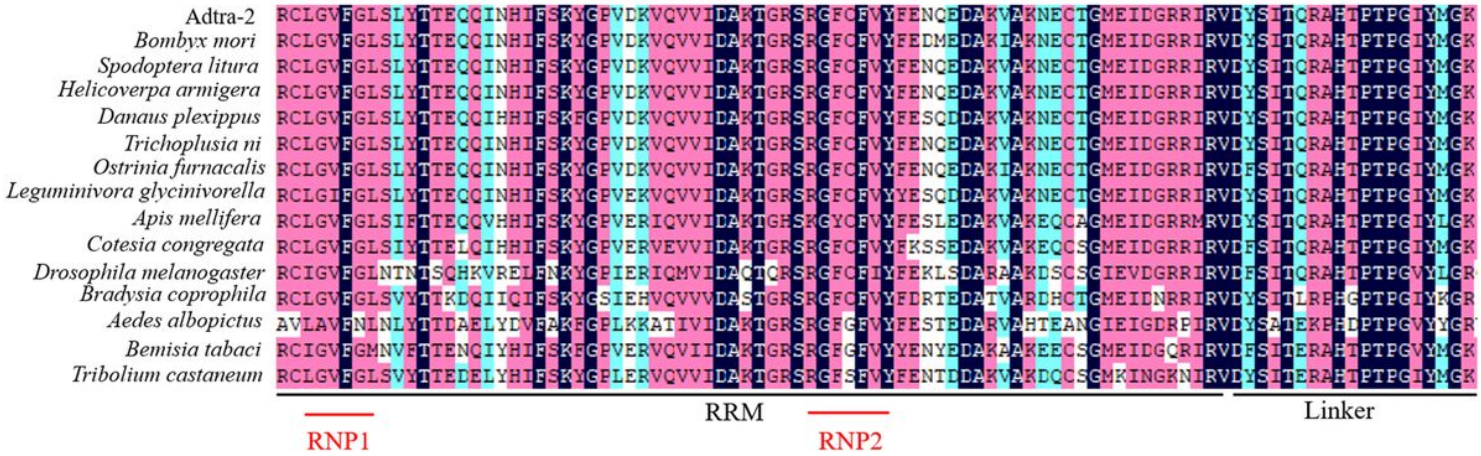

Figure 3

COG classification of all DEGs.




## Figure 4

Distribution of the mapped KEGG pathways from all DEGs.




## Figure 5

Multiple alignment of Adistra-2 amino acid sequence from *Athetis dissimilis*with other species. A consensus RRM structural core sequence is indicated with black line at the bottom of the alignment. Also indicated within the RRM are the positions of two ribonucleoprotein identifier sequences, RNP-1 and RNP-2.

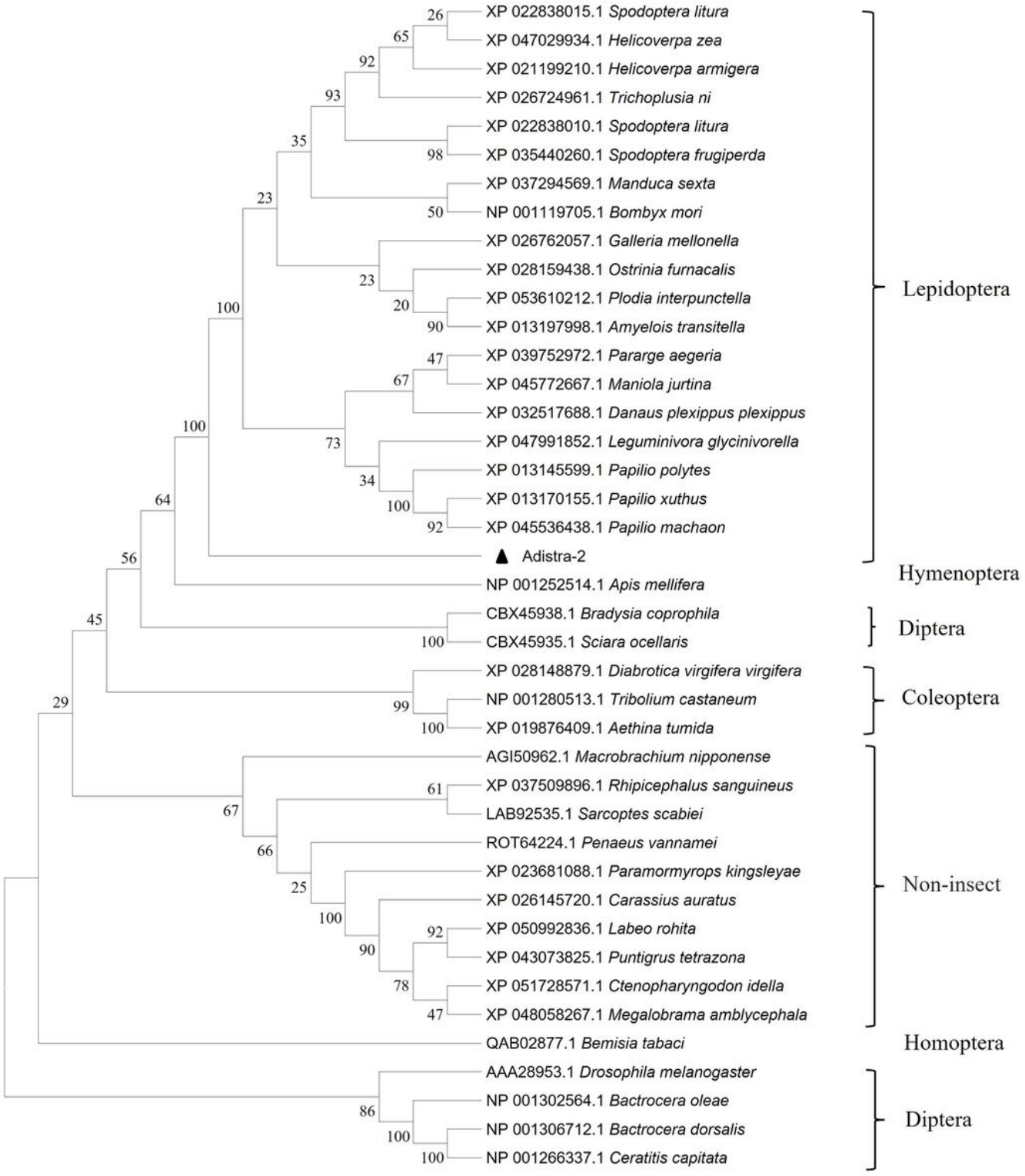



**Figure 6**

NJ phylogenetic tree of amino acid sequences of Adistra-2 gene in different species. NJ law contribution was made by MEGA6.0; the number on the node was bootstrap test confidence value repeated 1000 times.

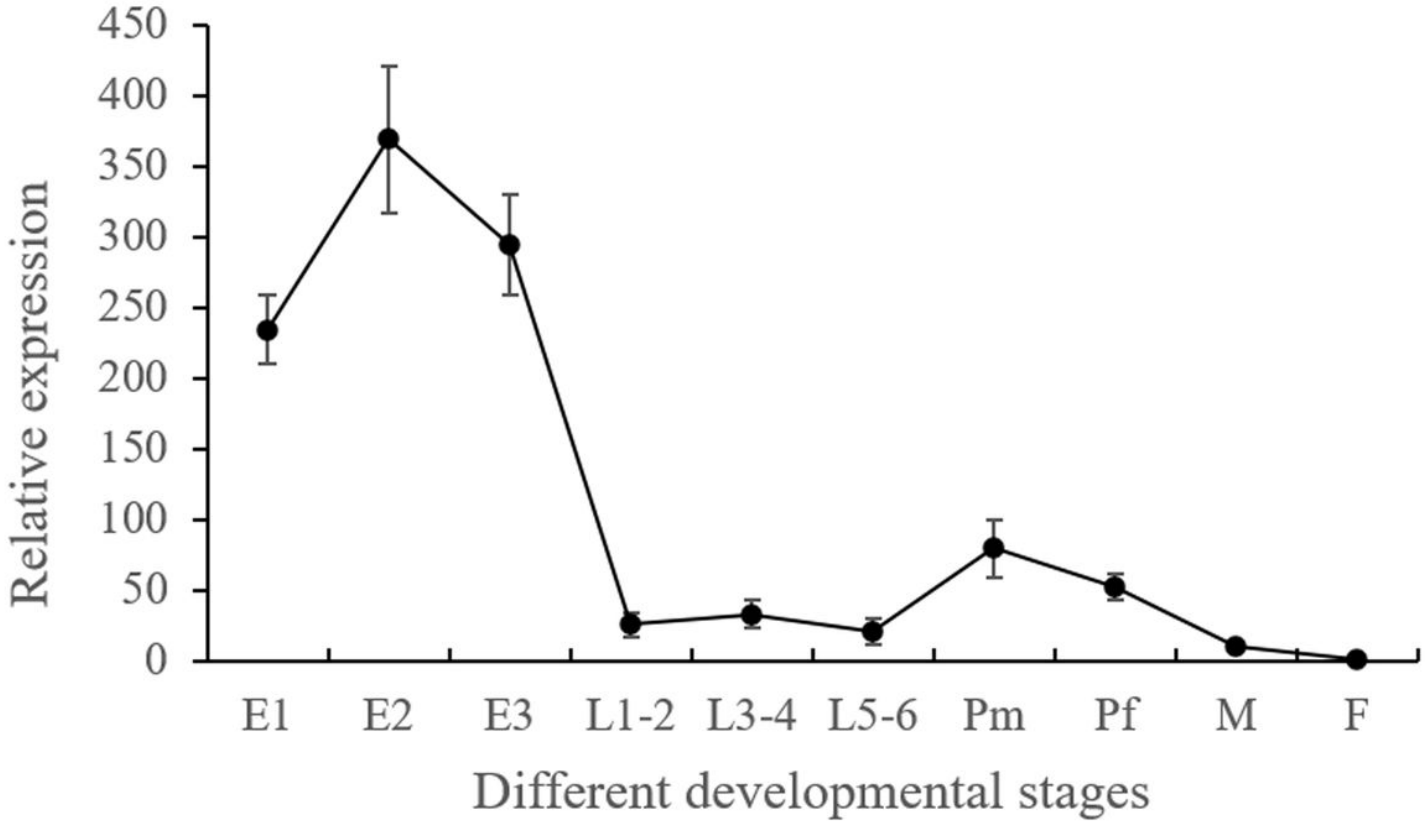



**Figure 7**

Relative expression of Adistra-2 gene in different developmental periods of *Athetis dissimilis*. E1 = 1 d eggs; E2 = 2 d eggs; E3 = 3 d eggs; L1-2 = $1^{st}$-$2^{nd}$ instar larvae; L3-4 = $3^{rd}$-$4^{th}$ instar larvae; L5-6 = $5^{th}$-$6^{th}$ instar larvae; Pm = the pupa of male; Pf = the pupa of female M = male; F = female.

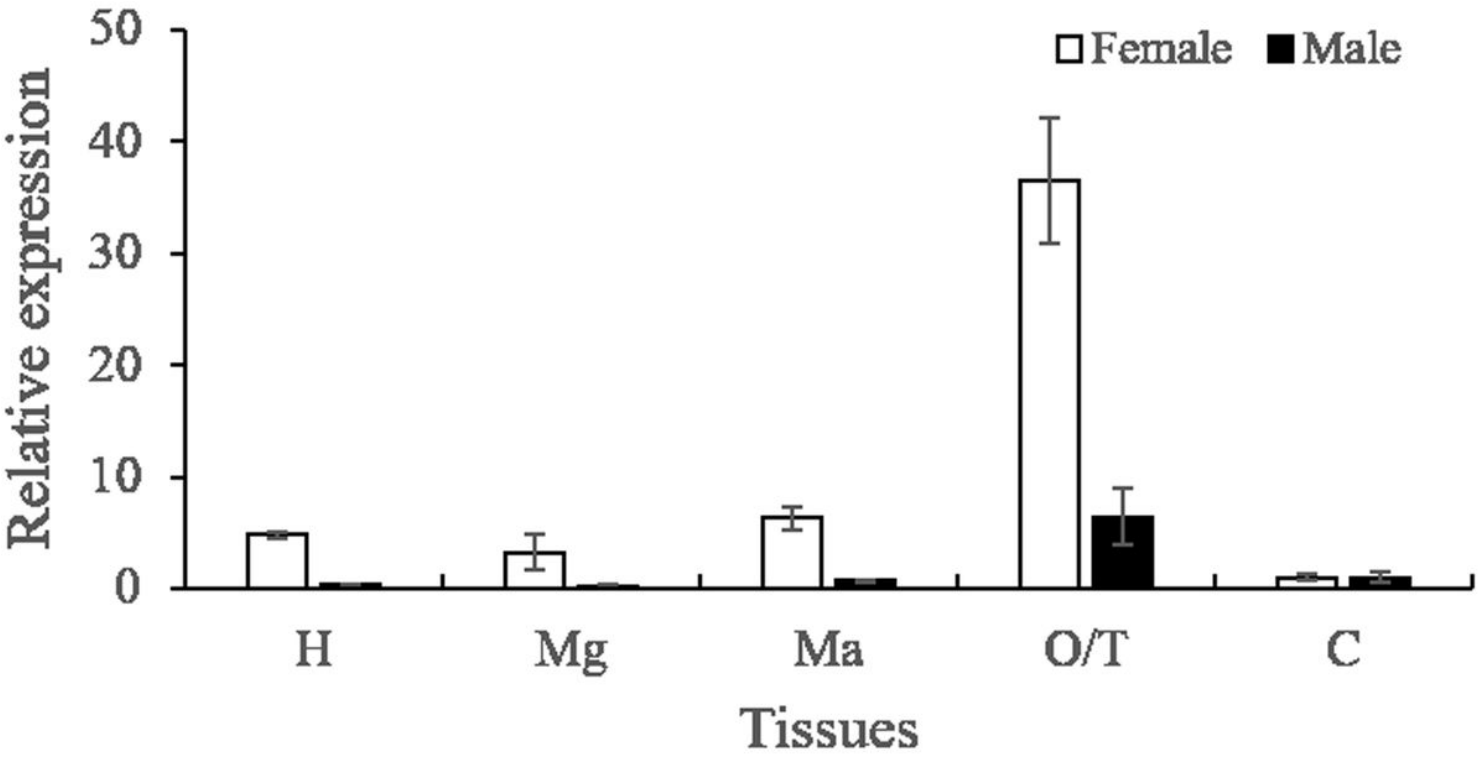



**Figure 8**

Relative expression of Adistra-2 gene in adult male and female of *Athetis dissimilis*. H = head; Mg = midgut; Ma = malpighian; O = ovary; T = testis; C = remaining carcass.

## Supplementary Files

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