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RNA-seq screening of cuticle protein genes in *Culex pipiens pallens* among cypermethrin-resistant populations

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Abstract

Background

A long-lasting overdependence on insecticides has led to the rapid spread of pyrethroid resistance in mosquito vectors, which poses a great risk to the general public. Although there are many studies on metabolic resistance and target resistance, few have investigated cuticle resistance and behaviour resistance. The cuticle of mosquitoes has been hypothesized to play a role in insecticide resistance by reducing penetration or sequestering insecticides.

Methods

We used RNA sequencing (RNA-seq) to analyse the transcriptome of cypermethrin-resistant and cypermethrin-susceptible strains of *Culex pipiens pallens*. We sequenced 6 samples using an Illumina HiSeq platform and generated approximately 6.66 Gb bases from each sample on average. Mapping the sequenced reads to a reference genome and reconstructing the transcripts via gene expression analysis, we detected differentially expressed genes (DEGs) among the samples. Followed Gene Ontology (GO) classification and functional enrichment. Finally, we screened the genes of cuticle proteins associated with drug resistance throughout the genome, selected the significant DEGs with a $\log_2$ fold change > 3.0 and Padj < 0.05, and applied real-time fluorescence quantitative polymerase chain reaction (PCR) to verify the DEGs.

Results

We obtained 13,517 novel transcripts, of which 8,653 were previously unknown splicing events for known genes, 665 were novel coding transcripts without any known features, and 4,199 were long non-coding RNA (lncRNA). A total of 1035, 944, and 657 genes were upregulated in comparisons between the samples, and 2680, 1215, and 975 genes were downregulated in comparisons between the samples. Finally, among all samples, 167 genes were upregulated and 145 genes were downregulated. The GO classification and functional enrichment of DEGs were as follows: molecular function, 224 genes; cellular component, 149 genes; and biological process, 272 genes.

The expression of *XM_001863852* and *XM_001845881* in resistant strains of *Culex pipiens pallens* was lower than that in the laboratory sensitive strain, with 0.177 and 0.548-fold change in expression, respectively; the expression of the *XM_001845883.1* in the resistant strain was higher than that in the susceptible strain, with a 2.281-fold change in expression.

Conclusions

Our results provide a reference for resistance mechanisms via the mosquito cuticle as well as a new perspective for disease vector control.

Background

Culex pipiens pallens is the most common mosquito in northern urban areas and townships in China. In addition to stings and bites, it is also the main vector of several arboviruses, such as West Nile virus (WNV), St. Louis encephalitis (SLE), Sindbis virus (SINV), Rift Valley fever (RVFV), and Japanese encephalitis (JEV), and is the main vector for lymphatic filariasis [1, 2]. Historically, chemical control is the most commonly used measure to control vector mosquitoes [3, 4]. Insecticides, particularly pyrethroids, due to their low mammalian toxicity, high insecticidal activity, fast action, and ease of decomposition in the environment, remain a mainstay for the control of mosquito vectors [5, 6].

However, with the long-term use of pyrethroids, the resistance of mosquitoes to pyrethroids is increasing [7]. Many studies have found that pyrethroid resistance in Culex pipiens larvae is a global problem, with resistance ratios of up to 7000, 710, 370, and 18 for permethrin, deltamethrin, cypermethrin, and λ-cyhalothrin, respectively [8]. Culex pipiens pallens/Cx. quinquefasciatus in southern China show different levels of resistance to pyrethroid insecticides. In Hainan and other provinces, the resistance level has reached several-thousand-fold [9]. The JPal-per strain of Cx. quinquefasciatus showed a marked resistance to permethrin (2500-fold compared to that of an insecticide susceptible strain)[10] and to other pyrethroids, such as phenothrin (2460-fold) and etofenprox (4160-fold) [11] during the larval stage. A recent study found that a cypermethrin-resistant strain of Culex pipiens pallens, Coq, showed a 283.06- and 80.68-fold resistance to cypermethrin and permethrin compared to that of susceptible strains [12]. Shi et al. found that the resistance of Culex pipiens pallens to insecticide increased from generation to generation with consistent exposure to insecticides. They selected a mild selection strain and administered deltamethrin at a constant concentration of 0.05 ppm for 24 generations, and found that the level of resistance grew exponentially, with an increase in the resistance ratio of over 8-fold [13].

Mosquito resistance mechanisms include metabolic resistance, target resistance, cuticle resistance, and behavioural resistance [14]. Metabolic resistance refers to the degradation, isolation, or transportation/excretion of insecticides from cells prior to binding to the target. Metabolic resistance results from increased detoxification caused by the overexpression of or conformational changes in the enzymes involved in chemical insecticide metabolism, sequestration, and excretion. P450-monooxygenases, glutathione S-transferases, and carboxy/cholinesterases are the main enzymes involved in this process [15–18]. Target-site resistance, or mutations in target binding sites for insecticides, is caused by a modification of the chemical insecticide site of action, which reduces or prevents insecticide binding at that site. Mutations in the voltage sensitive sodium channel (Vssc) gene are the most common causes of target-site resistance [19]. Behavioural resistance results from selection pressure of mosquitoes under long-term exposure to pesticides, such that mosquitoes show a series of behavioural changes to avoid pesticides. For example, the long-term application of indoor residual spraying (IRS) and insecticide-treated nets (ITNs) was found to cause mosquitoes to change from endophagy to exophagy and from endophily to exophily, resulting in the peak bloodsucking time throughout the day to change from late at night to dusk [20].

Cuticle thickening is implicated in insecticide resistance by reducing the uptake of the insecticide that reaches the target site in response to the modification of the chemical composition of the cuticle [21]. However, this mechanism remains poorly understood, and its importance in the Aedes species is yet to be confirmed [16, 22, 23]. A previous study found that this mechanism may play a major role in the development of resistance, where it normally occurs simultaneously with other mechanism(s) [24], causing resistance to single or multiple insecticides [25]. It has been reviewed elsewhere that cuticle thickening is associated with metabolic detoxification, whereby a thicker cuticle leads to a gradual insecticide absorption rate that will increase the effectiveness of metabolic detoxification in Anopheles funestus [26]. Moreover, it is crucial to note that insects with cuticular resistance will display a resistance level of not more than 3-fold in comparison to that of susceptible insects, but the co-occurrence of other resistance mechanisms will lead to a marked surge in the insecticide resistance level [27]. This is demonstrated by Anopheles gambiae in Benin [28], in which the overexpression of cuticular genes and P450 genes gave rise to a relatively high resistance level.

In order to screen the cuticle protein genes (CPGs) responsible for cypermethrin resistance in Culex pipiens pallens, we sequenced the transcriptome of cypermethrin-resistant strains and cypermethrin-susceptible strains. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed, as well as the functional analysis and real-time polymerase chain reaction (PCR) validation of three important differentially expressed genes (DEGs). Our aim was to provide a reference for the cuticle resistance mechanism of Culex pipiens pallens and provide a novel perspective on mosquito control and management.

Materials And Methods

Mosquito sample collection

We collected laboratory sensitive and resistant strains of Culex pipiens pallens in the following developmental stages: I, II, III, and IV instar larvae, pupa, and female Culex pipiens pallens 3 days after hatching that had not fed on blood. A total of 200 mg of Culex pipiens pallens was collected at each developmental stage and placed into a 1.5 ml Eppendorf (EP) tube, to which 150 μl of TRIzol lysis buffer was added to soak the mosquitoes. The samples were immediately stored in a −80 °C freezer, and the RNA was extracted and sent to the BGI group (Shenzhen, China) for transcriptome sequencing. The resistant strain was screened from sensitive strains in our laboratory in accordance with the larvae dipping method recommended by the World Health Organization (WHO).

RNA Sequencing

Three RNA-seq libraries per population (3 biological replicates) were prepared using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The extracted RNA was treated with RNase-free DNase (Qiagen GmbH, Hilden, Germany) and purified using an RNeasy MinElute Cleanup Kit (Qiagen GmbH) to remove DNA. The amount of total RNA was measured in a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The quality of the extracted RNA was verified by agarose gel electrophoresis. Subsequently, the reaction systems to synthesize the first and second strands of cDNA were constructed. After the second strand of cDNA was synthesized, the ends of the double-stranded cDNA (dscDNA) were blunt-ended using the EcoRI restriction sequence. After terminal phosphorylation and XhoI digestion, the dscDNA was recovered using a recovery kit. An Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were then used for quality tests. The Illumina HiSeq platform was used for RNA-seq after the quality of the dscDNA was confirmed.

Sequenced reads were assigned to each sample (unplexing), and adaptors were removed. The read quality was assessed for each sample using FastQC. Reads were then filtered based on their length, pairing, and quality using Trimmomatic [29] with the following parameters: Leading, 25; Trailing, 25; Minlen, 60; Slidingwindow, 4–25. Only paired reads were kept. The reads were then mapped to the Culex pipiens quinquefasciatus genome using Tophat2 [30] with the following parameters: do not report discordant pair alignments; final read mismatches = 3; intron length = 45-300000; use coverage search. Only read pairs mapping at a unique location (mapQ > 50) were retained. The quantification of the transcription levels was performed using the Cuffdiff2 module of Cufflinks implemented in Galaxy pipeline (http://galaxyproject.org) based on fragment per kilobase exon model (FPKM) values obtained for each gene across all samples.

The transcription ratios between each resistant and each susceptible strain were computed across all biological replicates using Cuffdiff. Genes showing an FC ≥ 3 (in either direction) and a q-value ≤ 0.001 between a given resistant population and all 3 susceptible strains were considered DEGs.

The DESeq2 and PossionDis algorithms were used to perform DEG detection. DESeq2 is a differential analysis software based on the principle of negative binomial distribution, and the analysis was conducted according to the method reported by Michael et al. [31]. The PossionDis difference analysis algorithm is based on the Poisson distribution model, and the analysis was conducted according to the method described in Audic et al. [32].

Screening and verification of DEGs between sensitive and resistant strains

Based on the results of the transcriptome sequencing analysis, we analysed the genes related to cuticle proteins among the susceptible and resistant strain DEGs and identified 3 mRNAs with a fold change greater than 2 ($-\log_2$Ratio ≥ 1) and FDR ≤ 0.001 for subsequent real-time PCR verification.

Referring to the Culex pipiens pallens genome data in the gene library, real-time quantitative PCR primers were designed using Primer Premier 5 software and the nucleotide sequences of the selected mRNAs. β-actin was used as the quantitative internal mRNA reference. The primers were synthesized by Shenzhen BGI. The base sequences of the specific primers are provided in Table 1. The RNA was extracted using TRIzol reagent, and the cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) using oligo(dT)18. Quantitative reverse transcription PCR (qRT-PCR) was performed using a CFX96 Touch (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Twenty-five nanograms of cDNA and 500 nM of each forward and reverse primer were used in each reaction. The relative expression of each gene in the resistant and susceptible mosquitoes was calculated using the ΔΔCt method [33] with actin (ADIR001186-RA) as a control. The real-time PCR data were analysed using reliability simulation tool (REST) software and hypothesis testing; other data were expressed as the mean ± standard deviation (XJ ± S) and analysed with STATA7.0 software. Student's t-test was used to perform comparisons between groups. $p < 0.05$ was used as a basis for determining statistical significance.

Table 1
Nucleotide sequence of each gene primer and related information

Gene ID	Primer sequence (5'-3')	Primer length	Product size
XM_001845883.1-F	TGCCATCCGTTTCTTCCA	18	103
XM_001845883.1-R	GGGCTCAACCAGGGAGTAAG	20	
XM_001863852-F	ATGCCATCGTGAAGGGTGT	19	93
XM_001863852 -R	GACTCTTGATGTCTCCGGTGTG	22	
actin-F	AGGACTCGTACGTCGGTGAC	20	-
actin-R	TGGTGCCAGATCTTCTCTCCAT	22	
XM_001845881-F	CACATTCGGATTACAAAATG	20	196
XM_001845881-R	GTGGTAGCTGTAGCTGTACTG	21	

Results

Overview of RNA-seq data

We selected 3 samples from different physiological stages of the sensitive and resistant strains, for a total of 6 samples. Each sample produced an average of 6.66 Gb of data. The sequenced clean reads were compared with the reference genome of Culex pipiens pallens, and the transcripts were reintegrated. A total of 13,517 new transcripts were detected, of which 8,653 were new alternative splicing isoforms of existing known protein-coding genes, 665 were transcripts of unknown protein-coding genes, and 4,199 were long non-coding RNAs (lncRNAs) (see Tables 2, 3).

Table 2
Summary of differentially expressed genes

VS	Upregulated	Downregulated
Cx_S_strain-VS-Cx_R_strain.DEseq2	167	145
Cx_S_strain-VS-Cx_R_strain.DEseq3	1035	2680
Cx_S_strain-VS-Cx_R_strain.DEseq4	944	1215
Cx_S_strain-VS-Cx_R_strain.DEseq5	657	975

Table 3
Summary of whole genome expression

Sample name	Total gene number	Number of known genes	Number of novel genes	Total transcript number	Known transcript number	Novel transcript number
Cx_R_strain_1	14597	14036	561	19094	11413	7681
Cx_R_strain_2	14507	13921	586	19460	11415	8045
Cx_R_strain_3	14592	14016	576	19733	11533	8200
Cx_S_strain_1	14603	14040	563	19381	11440	7941
Cx_S_strain_2	14551	13980	571	19671	11562	8109
Cx_S_strain_3	14568	13999	569	19679	11501	8178

Prediction Of New Transcripts

After comparing the clean reads to the Culex pipiens pallens genome, we used the StringTie [34] software to perform transcript reintegration for each sample, and then used Cuffmerge and Cuffcompare software (both are packages in Cufflinks [35]) to compare the reintegrated transcripts with the annotation information for the Culex pipiens genome. We selected transcripts with a class code type of u, i, o, and j as candidates for novel transcripts. A total of 13,517 new transcripts were detected. Detailed statistical information is provided in Table 4.

Table 4
Statistical results for new transcript types

Total Novel Transcript	Coding Transcript	Noncoding Transcript	Novel Isoform	Novel Gene
13,517	9,318	4,199	8,653	665
Novel Isoform: A novel isoform means that the transcript is a new isoform of a known protein-coding gene.				

Detection of single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs)

After comparing the clean reads to the Culex pipiens pallens genome, we used Genome Analysis Toolkit (GATK) [36] software to call each chromosome, identify SNPs and INDEL sites for each sample, and store the final results in variant call format (VCF). The SNP statistical information for all samples is provided in Table 5. We then analysed the site information for each SNP and INDEL, as shown in Fig. 1 and Fig. 2.

Table 5
Single nucleotide polymorphisms (SNPs)

Sample	A-G	C-T	Transition	A-C	A-T	C-G	G-T	Transversion	Total
Cx_R_Strain_1	54,869	55,073	109,942	13,839	17,339	12,324	13,482	56,984	166,926
Cx_R_Strain_2	60,813	60,772	121,585	14,894	17,862	13,807	14,513	61,076	182,661
Cx_R_Strain_3	65,303	64,814	130,117	15,921	19,131	14,640	15,508	65,200	195,317
Cx_S_Strain_1	57,605	57,458	115,063	14,116	17,777	12,968	14,035	58,896	173,959
Cx_S_Strain_2	65,253	65,174	130,427	15,922	19,003	14,525	15,541	64,991	195,418
Cx_S_Strain_3	64,017	63,988	128,005	15,481	18,436	14,451	15,141	63,509	191,514

Transition: Substitution of a purine with a purine or substitution of a pyrimidine with a pyrimidine; Transversion: Substitution of a purine with a pyrimidine.

Numbers, functional categorization, and pathway analysis of DEGs

DEGs were obtained by comparing the expression levels of differential genes between the sample groups. The results are shown in Fig. 3.

Validation of the 3 target cuticle protein genes

To ensure the amplification of the target genes and the housekeeping gene, we performed primer verification. The results showed that the amplification curve for the primers was good and that the melting curve was monomodal, while the electrophoresis results revealed specific target fragments. The average Ct value for the actin gene in the sensitive group was 25.905; the average Ct value of the actin gene in the resistant group was 26.227; the average Ct value of the XM_001863852 gene in the sensitive group was 26.813; the average Ct value of the XM_001863852 gene in the resistant group was 29.633; the average Ct value of the XM_001845883.1 gene in the sensitive group was 36.797; the average Ct value of the XM_001845883.1 gene in the resistant group was 35.93; the average Ct value of the XM_001845881 gene in the sensitive group was 32.647; and the average Ct value of the XM_001845881 gene in the resistant group was 33.837 (see Tables 6 and 7 for details).

Table 6

Quantitative PCR results

Target ID	Target Name	Sample ID	Sample Name	Ct Avg (SDM)	Rel. Qty (SDM)
T001	Actin	S001	S (sensitive) 1	25.475	1.00E + 00
T001	Actin	S002	S 2	26.205	1.00E + 00
T001	Actin	S003	S 3	26.035	1.00E + 00
T001	Actin	S004	R (resistance) 1	25.37	1.00E + 00
T001	Actin	S005	R 2	26.19	1.00E + 00
T001	Actin	S006	R 3	27.12	1.00E + 00
T002	XM_001863852	S001	S 1	31.79	1.00E + 00
T002	XM_001863852	S002	S 2	24.00	3.67E + 02
T002	XM_001863852	S003	S 3	24.65	2.08E + 02
T002	XM_001863852	S004	R 1	30.235	2.73E + 00
T002	XM_001863852	S005	R 2	30.61	3.72E + 00
T002	XM_001863852	S006	R 3	28.055	4.16E + 01
T003	XM_001845883.1	S001	S 1	36.19	1.00E + 00
T003	XM_001845883.1	S002	S 2	37.07	9.01E-01
T003	XM_001845883.1	S003	S 3	37.13	7.68E-01
T003	XM_001845883.1	S004	R 1	35.43	1.58E + 00
T003	XM_001845883.1	S005	R 2	35.94	1.95E + 00
T003	XM_001845883.1	S006	R 3	36.42	2.67E + 00
T004	XM_001845881	S001	S 1	36.48	1.00E + 00
T004	XM_001845881	S002	S 2	29.33	2.36E + 02
T004	XM_001845881	S003	S 3	32.13	3.01E + 01
T004	XM_001845881	S004	R 1	33.04	1.01E + 01
T004	XM_001845881	S005	R 2	35.15	4.13E + 00
T004	XM_001845881	S006	R3	33.32	2.80E + 01

Table 7
Calculation of the relative quantitative Ct values for target genes and the internal reference gene

	β-actin	XM_001863852	XM_001845883.1	XM_001845881
Average Ct value in the sensitive group	25.905	26.813	36.797	32.647
Average Ct value in the resistant group	26.227	29.633	35.93	33.837
△ Ct value in the sensitive group	-----	0.908	10.892	6.742
△ Ct value in the resistant group	-----	3.406	9.703	7.61
△△ Ct value	-----	2.498	-1.189	0.868
2^-△△ Ct value	-----	0.177	2.281	0.548

Subsequently, we used the $2^{-\triangle\triangle Ct}$ method to analyse the expression of target gene mRNA in the extracted RNA and normalized the result based on the housekeeping gene.

The specific calculation method used was as follows:

△△Ct = (Ct target gene – Ct housekeeping gene) experimental group – (Ct target gene – Ct housekeeping gene) control group

The relative expression level of the target gene = $2^{-\triangle\triangle Ct}$, which indicates the fold change in the expression of the target gene in the experimental group relative to the control group.

The calculation results are presented in Tables 6 and 7.

As shown in Table 7, the expression levels of the target genes XM_001863852 and XM_001845881 were similar between the sensitive and resistant strains of Culex pipiens pallens; the fold changes in expression were 0.177 and 0.548, respectively. The expression level of the target gene XM_001845883.1 in the resistant strain was higher than that in the sensitive strain, with a fold change in expression of 2.281.

Discussion

In 1963, a survey of houseflies (Fannia canicularis) identified the cause of insecticide resistance: the penetration of chemical pesticides in resistant lines was slower than in sensitive lines, suggesting that a slower penetration could be the cause for dichlorodiphenyltrichloroethane (DDT) and pyrethroid resistance [37]. The cuticular protein (CP) family was first discovered in 2007 by the tandem mass spectrometry analysis of epidermal exfoliation from Anopheles gambiae [38]. The majority of the gene family members have the prefix CPLC (cuticular protein of low complexity) and often play an important role in protein-protein interaction networks [39, 40]. Studies have shown that pyrethroid-resistant female Anopheles sinensis have thicker cuticles than pyrethroid-sensitive female Anopheles sinensis, and that female mosquitoes also have thicker cuticles their male counterparts [26]. In addition, Lily et al. confirmed that cuticle thickening is present in the pyrethroid-resistant strains of Cimex lectularius [41]. Cuticle analysis by electron microscopy and the characterization of lipid extracts showed that resistant mosquitoes had a thicker outer skin layer and a higher hydrocarbon content (approximately 29%) [42].

These findings suggest that insect cuticle proteins play an indispensable role in mosquito resistance. The expression of the target genes XM_001863852 and XM_001845881 in the Culex pipiens pallens resistant strain was lower than that in the sensitive strain, with fold changes in expression of 0.177 and 0.548, respectively, while the expression of

XM_001845883.1 in the resistant strain was higher than that in the sensitive strain, with a 2.281-fold change in expression. GO function analysis indicated that all 3 were genes responsible for cuticle structural components, and a non-redundant (NR) database comparison also showed that these genes code cuticle proteins in Culex pipiens.

In fact, when we screened the genes, we initially identified 3 different genes in the XM_001845 series: XM_001845880, XM_001845881, and XM_001845883.1. However, when designing the primers, the ones designed for these 3 were not ideal, with varying degrees of non-specific amplification. We compared the designed primer fragments to the BLAST database and found that the primers were highly consistent with an unknown conserved hypothetical protein. We wondered whether the 3 different genes in the XM_001845 series were different splicing isoforms of the same gene, and if so, whether such frequent splicing could promote resistance in mosquitoes. After considering the experimental cost, experimental significance, and feasibility of the experiment, we selected 2 of the genes to conduct further validation, and the experimental results for XM_001845881 and XM_001845883.1 were opposite to that of each other. Therefore, we questioned whether the 2 genes had antagonistic effects in the genetic pathways leading to the formation of cuticle resistance in mosquitoes; for example, XM_001845883.1 is responsible for promoting the formation of cuticle resistance, while XM_001845881 is responsible for regulating the expression of other upstream or downstream cuticle protein genes, preventing the overexpression of other related cuticle proteins.

Since the 3 identified target genes in our study are novel, the original identification process was relatively complicated and innovative; however, the specific regulatory networks and the in vivo function of the respective genes will need to be further investigated.

Conclusions

These data provide transcriptomic information related to the resistance of Culex pipiens pallens and preliminarily verify the relationship between the identified cuticle protein genes and mosquito drug resistance, partially explaining the specific mechanism of mosquito cuticle resistance, providing a scientific basis for the study of new target insecticides and a novel perspective for the mosquitoes control and management.

Abbreviations

RNA-seq RNA sequencing

CPGs Cuticular protein genes

CP Cuticular protein

DEGs Differentially expressed genes

GO Gene Ontology

PCR Polymerase Chain Reaction

WNV West Nile virus

SLE St. Louis encephalitis

SINV Sindbis virus

RVFV Rift Valley fever

JEV Japanese encephalitis

Vssc Voltage sensitive sodium channel

IRS Indoor residual spraying

ITNs Insecticide-treated nets

ncRNAs Non-coding RNAs

sRNAs Small RNAs

EP Eppendorf

WHO World Health Organization

dscDNA Double strands cDNA

FPKM Fragment per kilobase exon model

REST Reliability simulation tool

lncRNAs Long non-coding RNAs

GATK Genome Analysis Toolkit

SNPs Single nucleotide polymorphisms

INDEL Insertion and deletion

VCF Variant call format

KEGG Kyoto Encyclopaedia of Genes and Genomes

DDT Dichlorodiphenyltrichloroethane

CPLC Cuticular protein of low complexity

NR Non-redundant

bp Base pair

qRT-PCR Quantitative reverse transcription Polymerase. ChainReaction

Declarations

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Availability of data and materials

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive (SRA) repository, the accession number is: PRJNA601003.

Authors' contributions

QQS, PC, CXZ, IJL, XS, XXG, HFW, YW, and HML conducted the sample collection and wrote the manuscript. HWW and MQG reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

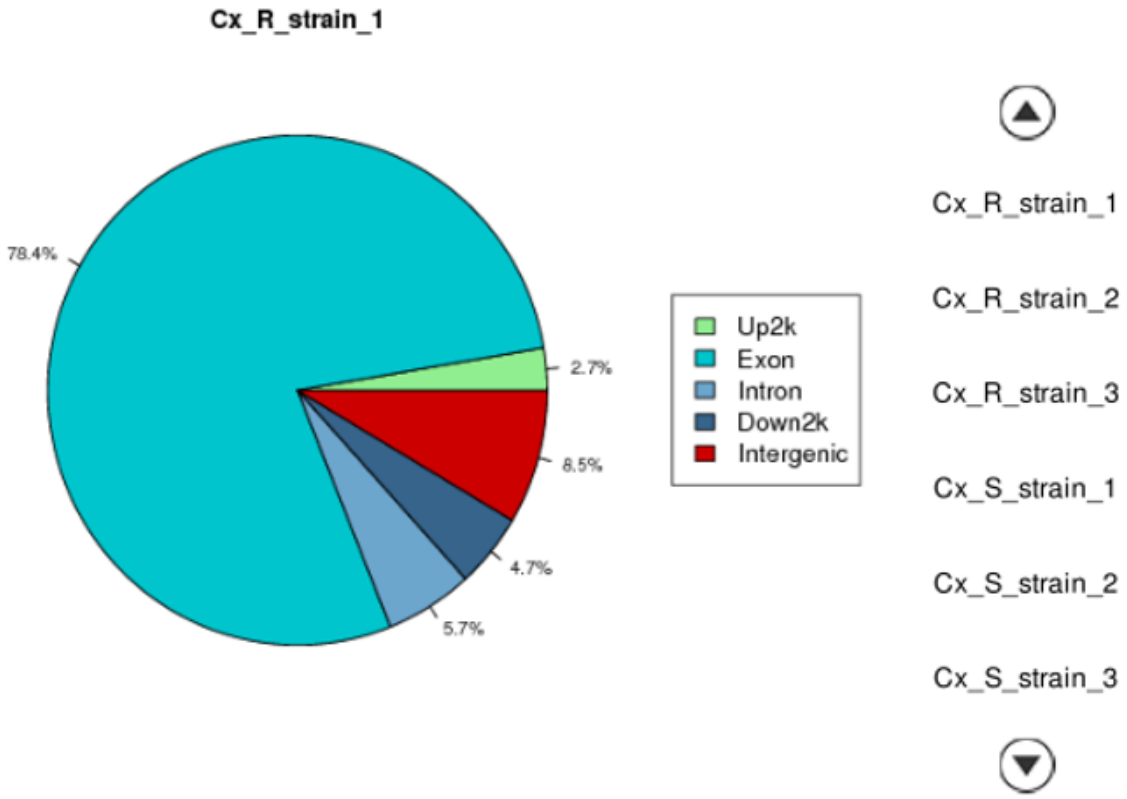

Figure 1

Distribution of SNP sites. Up2k refers to the area within 2000 bp upstream of a gene, and Down2k refers to the area within 2000 bp downstream of a gene.

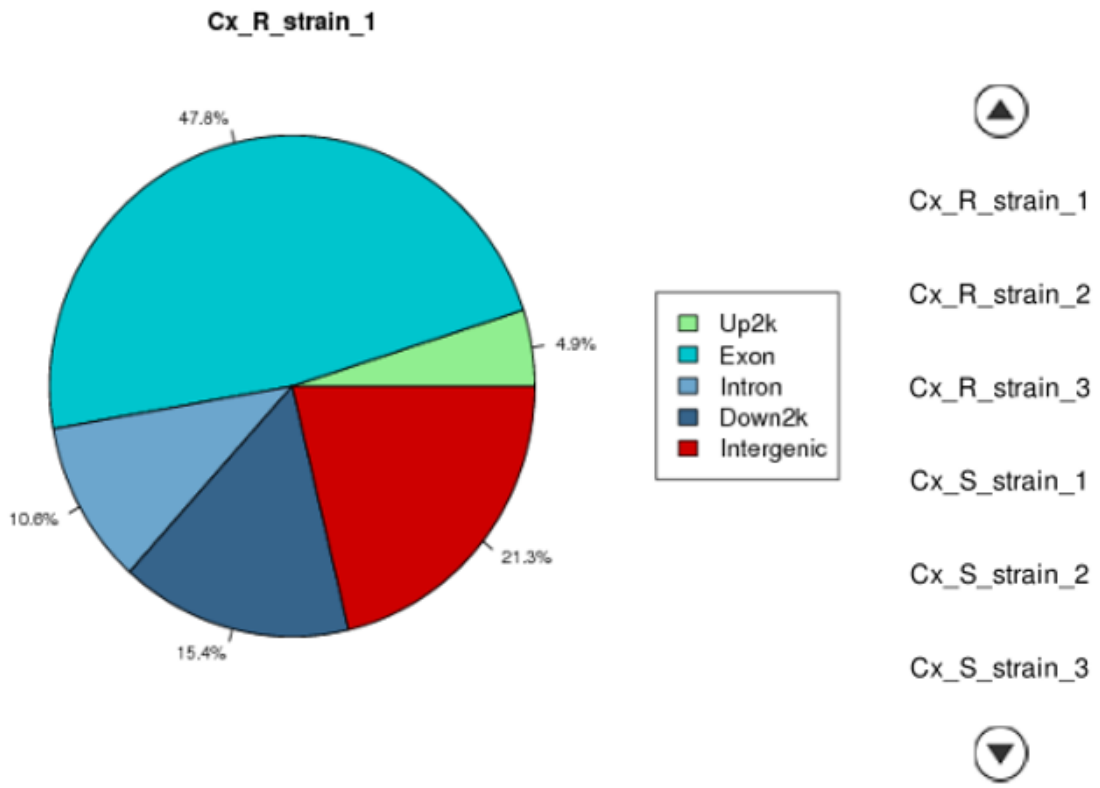

Figure 2

Figure 2 Distribution of INDEL sites. Up2k refers to the area within 2000 bp upstream of a gene, and Down2k refers to the area within 2000 bp downstream of a gene.

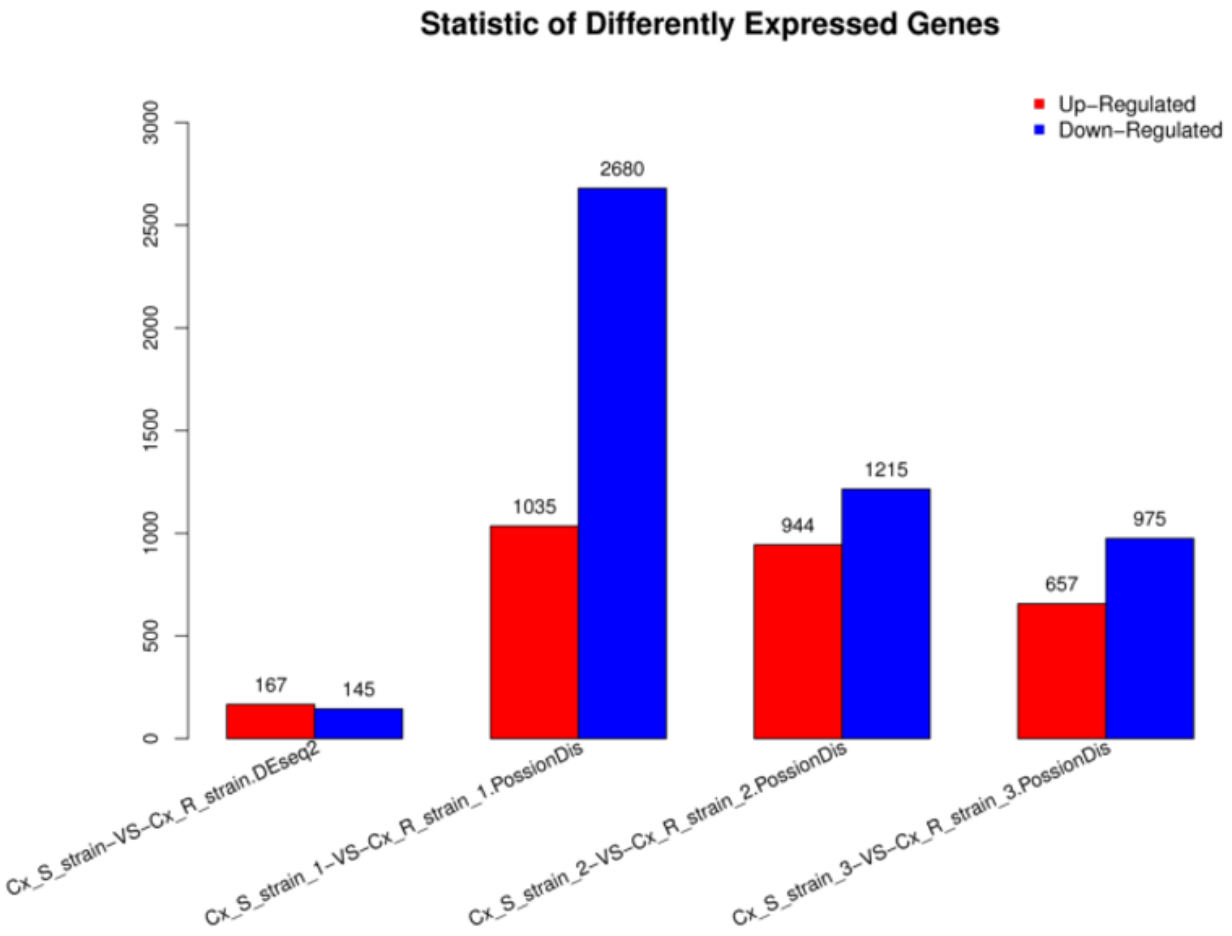

Figure 3

Comparison of DEGs between groups. The abscissa indicates the pairs of samples for differential comparison, and the ordinate indicates the corresponding number of DEGs.

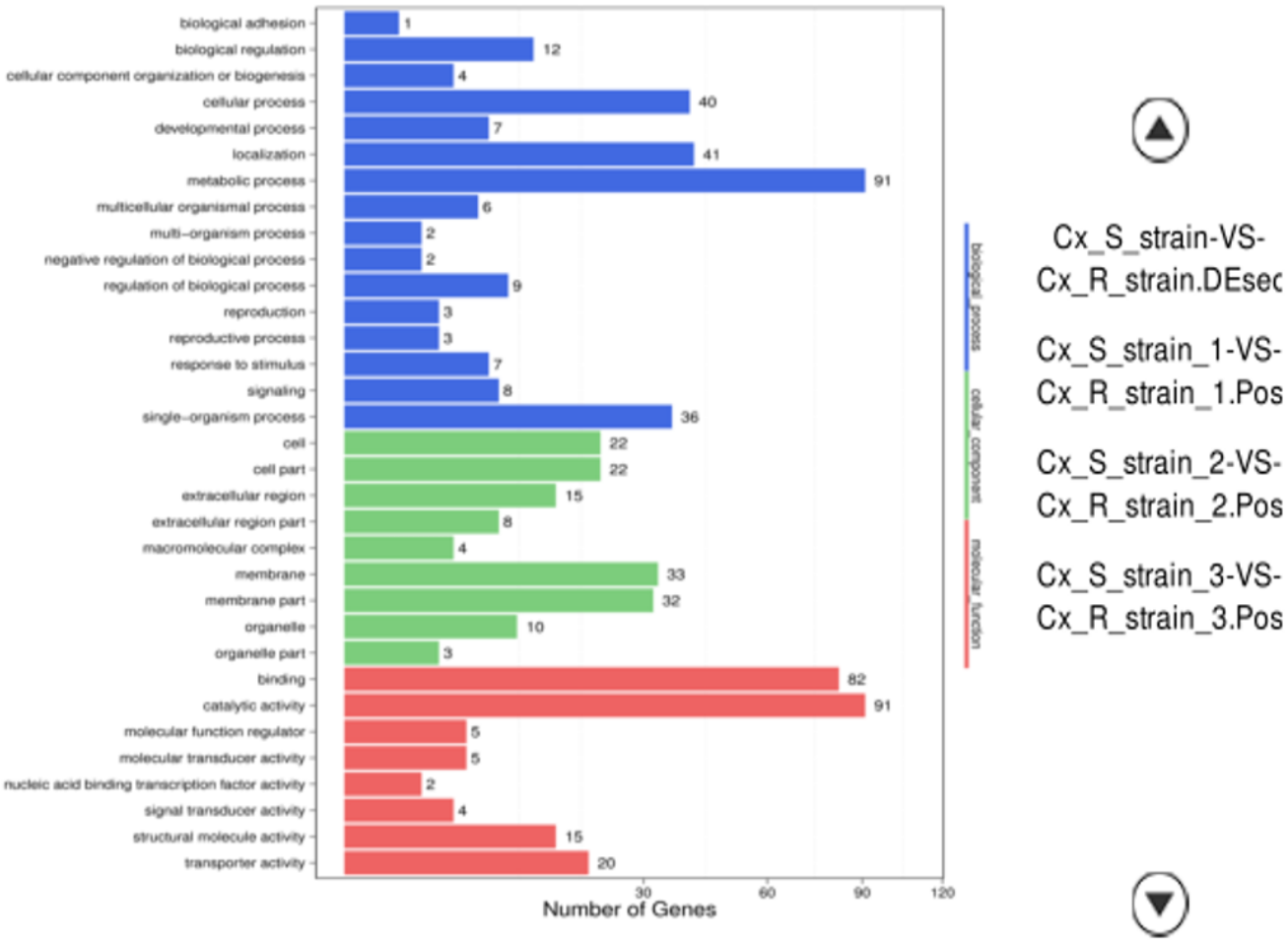

Figure 4

GO function classification map of DEGs. The X axis represents the number of DEGs, and the Y axis represents GO function classification.

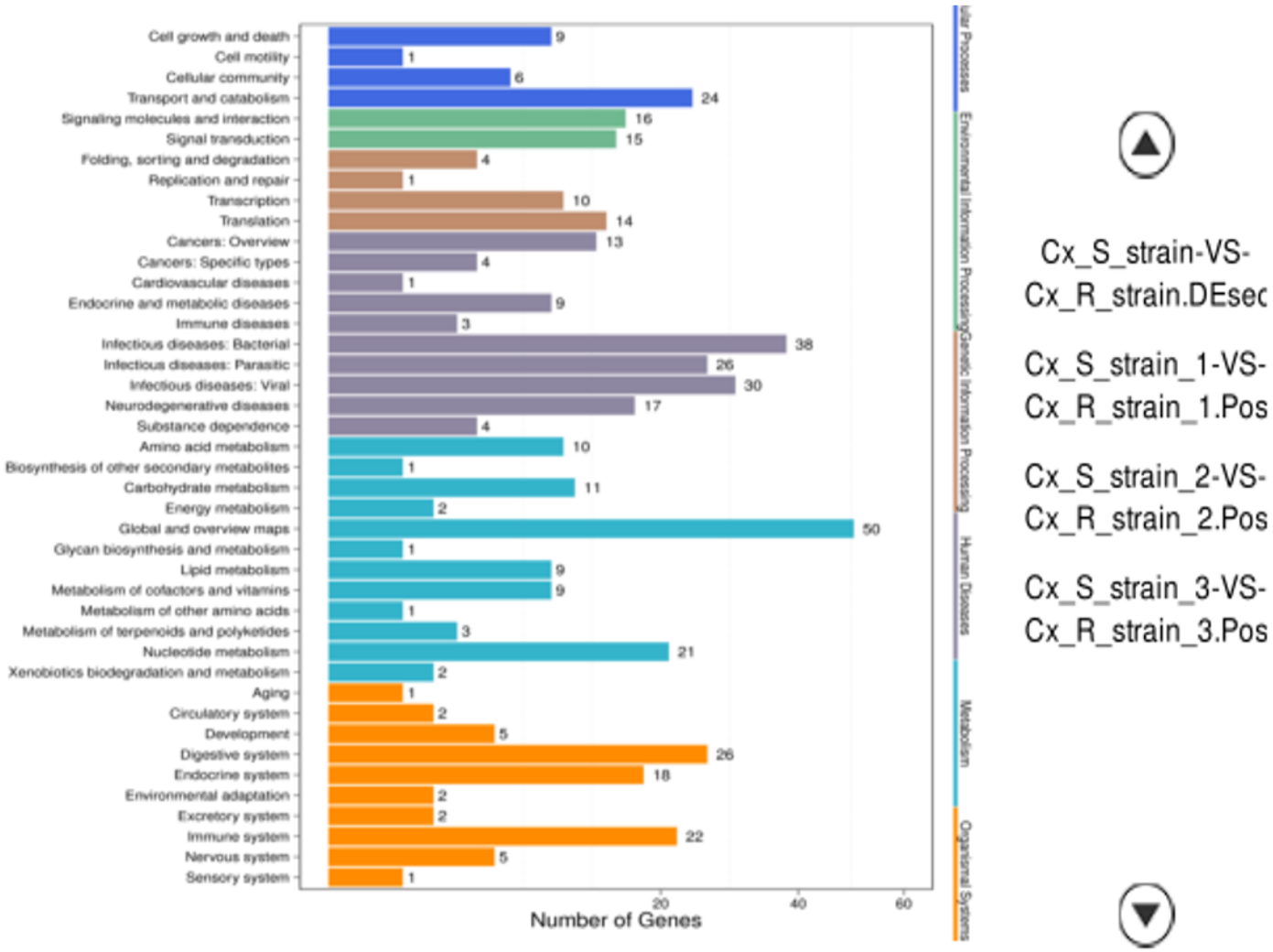

Figure 5

Pathway classification of DEGs. The X axis represents the number of DEGs, and the Y axis represents the KEGG pathway.