Genetic basis for tetrodotoxin (TTX) resistance in Nassarius

Shanmei Zou[1] and Yuxiao Hua[1]

[1]Nanjing Agricultural University

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Abstract

Understanding the genetic basis in animals possessing toxin could provide us significant insights into the adaptive evolution in toxin resistance. The reason of toxin resistance may refer to the mutation in sodium channels or the toxin-binding proteins. Sea snail Nassarius can accumulate tetrodotoxin (TTX) in their body and has caused serious people poisoning and death incident. However, the genetic basis of Nassarius including genetic expression and mutation has not been researched yet. Here we investigated the genetic basis from two Nassarius species (Nassarius succinctus and Nassarius variciferus) for TTX resistance with transcriptome. The genetic express pattern for both species was clearly revealed. The cellular and metabolic process, and binding and catalytic activity accounted for the top classification categories. The toxic samples generally produced more up-regulated genes than non-toxic samples. Some genes which possibly produce TTX-binding proteins were flagged. One sodium channel gene was identified from N. succinctus where one mutation cite was found as being different from all other animals included. The genetic basis of Nassarius revealed in this study would contribute to the further TTX-resistance mechanism research.

1 | INTRODUCTION

Understanding how organisms could be adaptive to environments around would greatly contribute to reveal the mechanisms of evolutionary diversification. Some organisms could accumulate chemicals risk self-intoxication in their body since they can resist and defend the chemical toxins through compartmentalization, metabolic detoxification, or target site insensitivity (Venkatesh et al., 2005; Yotsu-Yamashita,Yamaki,Okoshi, & Araki, 2010; Tarvin et al., 2017).

Saxitoxin (STX) and Tetrodotoxin (TTX) are both potent neurotoxin that specially blocks voltage-gated sodium channel (Narahashi,Moore, & Poston, 1967; Kao 1982; Soong, & Venkatesh, 2006) and have been detected in various organisms of different phylogenic classes, such as*Charonia sauliae* (Narita et al., 1981), xanthid crab*Atergatis floridus* (Noguchi et al., 1983), blue-ringed octopus*Hapalochlaena maculosa* (Sheumack,Howden, & Spence, 1984), and pufferfish and frogs (Mosher,Fuhrman,Buchwald, & Fischer, 1964; Noguchi, & Hashimoto, 1973; Kim,Brown, & Mosher, 1975; Tarvin et al., 2017). These toxins specifically bind to the voltagegated sodium channels by sharing one binding site with high affinities. It has been reported that the toxin resistance in animals may be due to some mutation sites in the sodium channel genes (McGlothlin et al., 2014) where the mutation could block the binding of toxin and thus produce resistance, like the puffer fish, snakes, frogs and shellfish (Tarvin et al., 2017; Venkatesh et al., 2005; Geffeney et al., 2005; Jost et al., 2008; Hanifin, & Gilly, 2015). However, the mutation mechanism in sodium channels for resistance is undetermined. The toxin-binding protein may also could produce resistance by transferring or excluding toxin (Yotsu-Yamashita,Yamaki,Okoshi, & Araki, 2010; Yotsu-Yamashita et al., 2013; Yotsu-Yamashita et al., 2000). For example, in pufferfish, the mRNA levels of many transcripts in toxic specimens were higher than that in the non-toxic specimens (Feroudj et al., 2013) which included genes encoding forming structural filaments (keratins) and genes related to vitamin D metabolism, immunity and Saxitoxin and Tetrodotoxin binding protein (PSTBP) (Yotsu-Yamashita,Yamaki,Okoshi, & Araki, 2010; Yotsu-Yamashita et al., 2013;

Casewell et al., 2013). Thus, the resistance to STX and TTX in animals could be the reason of mutations taking place in sodium channel genes or toxin-binding proteins.

Nassarius , a species-rich genus of Nassariinae, is distributed throughout worldwide oceans and is important in maintaining the balance of marine ecological system. For a long time, *Nassarius* is consumed as popular food in Asian countries as economic species (Zou,Li, & Kong, 2012). However, eating nassariids of *Nassarius* have caused hundreds of food poisoning and death incidents in the last several years, especially in China. Previous studies detected that the food poisoning incident was due to TTX accumulated in *Nassarius*sp's body (Zou,Song,Wang, & Wang, 2019). Currently the genetic basis involving TTX resistance of *Nassarius* is not studied.

Here we performed transcriptional analysis for both toxic and non-toxic specimens in two species *N.succinctus* and *N.variciferus* , aiming to reveal the genetic basis which refers to gene expression pattern and mutation sites in sodium channel genes for TTX resistance study.

2 | MATERIALS AND METHODS

2.1 | Sample collection, treatment and toxicity test.

The samples used in this study were from our previous research (Zou,Song,Wang, & Wang, 2019) which collected the various toxic samples from the China coast, treated the samples and tested their toxicity. Based on the results of the toxicity test, *N. succinctus* and*N. variciferus* which both included toxic and non-toxic populations with TTX were selected for genetic basis study here. The liver and pancreas were treated for RNA extraction and sequencing since they contain more toxin. For RNA-seq protocol, the toxic and non-toxic samples of two species were sequenced as treatment group and control group. Three toxic samples and three non-toxic samples were as triple repeat respectively for each species. The detailed sample collection was shown in Fig.1.

2.2 | RNA library preparation, transcriptome and gene assembly.

The total RNA was extracted and treated with DNase I, and then the Oligo(dT) were used to isolate mRNA. The mRNA mixed with fragmentation buffer were fragmented and the cDNA was then synthesized using the mRNA fragments as templates. Short fragments were purified and resolved with EB buffer for end reparation and were connected with adapters. The fragments with suitable length were selected for the PCR amplification. During the QC steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System was used in quantification and qualification of the sample library. Then the library was sequenced using Illumina HiSeq 4000 (Conesa et al., 2005).

After obtaining the "Clean Reads" by filtering out raw reads the Trinity (v2.0.6) (Grabherr et al., 2011) was used to perform de novo assembly with clean reads. N50 and ortholog hit ratios (OHR) were used to compare and evaluate assembly quality and completeness between the normalized data subset and the whole dataset. Paired reads passing the filter were then concatenated using Concatenate datasets (version 1.0.0) in both the right and left direction. And Transcript abundancies were calculated using RSEM version 1.1.1754 (Haas et al., 2013; Dewey, & Bo, 2011) with default settings using the pool of non-normalized reads.

2.3 | Differentially expressed gene patterns.

The databases of NT, NR, GO, COG, KEGG, SwissProt and InterPro were used for functional annotation. The final assembly was submitted to these databases using BLASTX with the threshold of E-value [?] 10-5. Blast2GO software (v2.8) (Conesa et al. 2005). The NR annotation was used to get the GO annotation, and InterProScan5 was used to get the InterPro annotation. We got Unigenes from mapping clean reads using Bowtie2 (Langmead, & Salzberg, 2012), and then calculated gene expression level with RSEM. The PCA analysis was performed with all samples using princomp, a function of R. We detected DEGs (Differentially Expressed Genes) with Possion Dis as requested. PossionDis was based on the poisson distribution, performed as described at Audic (1997) .

2.4 | Q-PCR confirmation.

Using the cDNA synthetized above real-time PCR was performed with the SG Fast qPCR Master Mix(High Rox)and using the ABI Stepone plus system. Cycle threshold values were normalized to the gene 18S. A total of 18 DEGs closely related with coding toxin-binding protein were selected as target Q-PCR functional genes. All these selected genes were from both toxic and non-toxic *N. succinctus* and *N. variciferus*samples. Fold changes were determined with the relative expression software tool.

2.5 | Flagging mutations in sodium channels genes.

Based on the transcript Unigenes of both species, we tried to retrieve the **s** odium channels genes to which the TTX binds. After firstly selecting the target genes from Unigenes abundant of **s** odium channels genes from closely related species with *Nassarius* were downloaded and clustered together as reference database to blast all Unigenes for confirmation. While no matched sequences were flagged the clean reads of each sample were further used to retrieve. After getting the target genes, we designed specific primers to PCR each sample and sequence for confirmation.

3 | RESULTS

3.1 | Quality of sequencing data and assembly.

A total of 12 samples were used for transcriptome sequencing, 6 out of which were from *N. succinctus* and the rest were from *N. variciferus.* For each of *N. succinctus* and *N. variciferus* , 3 samples were toxic as treat groups and 3 samples were non-toxic as control groups. A total of 39.96 Gb bases and 40.19 Gb bases were generated for *N. succinctus* and *N. variciferus*respectively after Illumina Hiseq sequencing. The clean reads quality metrics after filtering sequencing reads containing low-quality, adaptor-polluted and high content of unknown base (N) reads were shown in Table 1, and the distribution of base content and quality were shown in Figure S1. These reads quality results indicated that the transcriptome sequencing performed well for biological functional analysis. Accession numbers of 12 samples in Genbank was: SRR10582953, SRR10582959, SRR10582955, SRR10582954, SRR10582956, SRR10582961, SRR10582957, SRR10582958, SRR10582960, SRR10582963, SRR10582963, SRR10582964.

3.2 | Gene functional annotation.

A total of seven functional databases (NR, NT, GO, COG, KEGG, Swissprot and Interpro) were used for functional annotation. For both *N. succinctus* and *N. variciferus* , the Nr and Nt databases got higher annotation proportion than other databases, with more than 30%. The overall annotation proportion for *N. succinctus* and *N. variciferus* is 52.61% and 55.07% respectively. The distribution of annotated species is statisticed with NR annotation (Fig. 2). The main annotated species were generally consistent between *N. succinctus*and *N. variciferus* , which includes *Aplysia californica* ,*Oncorhynchus mykiss* , *Octopus bimaculoides* , *Monosiga brevicollis MX1* , *Lottia gigantea* , *Biomphalaria glabrata* ,*Crassostrea gigas* , *Trichuris suis* , *Exaiptasia pallida* , *Lingula anatine* and *Mus musculus* . Among these species, *Aplysia californica* has the highest annotation proportion. After functional annotation, the CDS was selected from segment of Unigenes that best mapped to functional databases. For some Unigenes which were not annotated, the ESTScan was used to predict the CDS (Iseli et al. 1999). For both *N. succinctus* and *N. variciferus* , the total number of CDS is between 10000-80000 and the mean length is 300-500bp. There is no much difference between the CDS prediction between the two species.

3.3 | Gene expression patterns.

After assembly and mapping clean reads to Unigenes, the gene expression level for each sample was calculated by PCA analysis. As shown in Figure 3, the expression level between the toxic and non-toxic samples were different for both *N. succinctus* and *N. variciferus.* The non-toxic *N. succinctus* samples from Dalian showed more similar gene expression level. Two toxic samples of *N. variciferus* from Lianyungang had different expression from the non-toxic samples from Dalian.

The DEGs indicated up-regulated and down-regulated genes between toxic and non-toxic samples for both species (Fig. 4). Compared with the non-toxic samples (ZD) in *N. variciferus* , each toxic sample (ZL) showed

more up-regulated genes. For *N. succinctus* , the toxic samples (HL) also produced more up-regulated genes in comparison with the non-toxic samples (HQ), especially for HL3. The Gene Ontology (GO) classification and functional enrichment, including three ontologies of molecular function, cellular component and biological process, was performed for DEGs. For both *N. variciferus* and *N. succinctus* , all comparisons of toxic and non-toxic samples indicated coincident patterns of functional enrichment. For both species, the cellular process and metabolic process from biological process, and binding and catalytic activity from molecular function accounted for the top classification categories for the DEGs (Figure. S2).

3.4 | Different expressed genes.

Different expressed genes were selected from the toxic and nontoxic samples within *N. succinctus* and *N. variciferus* . Multiple genes which were mostly differently expressed were found as cytokeratin; cytochrome c oxidase subunit II; K07604 type I keratin, Vitamin D metab..olism Vitamin D 25-hydroxylase; Major histocompatibility complex class I protein; Coagulation factor; and Complement component and fibrinogen-like protein, which were also demonstrated in previous studies. We also found some additional genes which were mostly differently expressed: heat shock protein, RNA-binding protein, Histone-lysine N-methyltransferase, hypothetical and putative protein, flocculation protein related with oligomeric mucus, PH domain and leucine rich repeat protein phosphatase, myosin binding subunit, tissue factor pathway inhibitor, ATP-dependent RNA helicase A, palmitoyltransferase and poly(A) RNA polymerase. Among all the genes, the flocculation protein related with oligomeric mucus were the most different expressed in all toxic and non-toxic samples for both *N. succinctus* and *N. variciferus* .

For confirming the accuracy of the transcript data, multiple DEGs found as functional genes relevant with toxicity resistance were selected for qPCR, from toxic and non-toxic groups of *N, succinctus* and*N. variciferus* . The qPCR of these Unigenes was performed for each pair of toxic and non-toxic groups (as observation and control groups respectively). The 18S was selected as the reference gene. In general, the statistic results indicated that the 2 -([?][?]Ct) and actual expression level for each comparison of observation and control group showed consistent tendency as the Figure 5 showed.

3.5 | Mutation sites in sodium channel genes.

Based on the transcript Unigenes of both species, we tried to retrieve the sodium channel genes for both species. For *N. succinctus,* a total of 3425bp target gene (unigene CL8899.Contig1_All in *N. succinctus*) was obtained which could be matched to the sodium channels genes in reference sequences with high identify score more than 80%. After clustering, the amino acid of the target gene was identified as Domain II and Domain III of sodium channels. Among the mutations which were relevant with TTX resistance, we found one new amino acid 'L' in Domain II (Fig. 6). By PCR confirmation, we got the same sequences of this sodium channels gene from the toxic *N. succinctus* sample. Unfortunately, no matched sodium channels genes were uncovered from the*N. variciferus* Unigenes, even by the way the clean reads of the both species were used to blast with the reference genes.

4 | DISCUSSION

In recent years, studies gave insights that various animal organisms could resist environment by accumulating toxin in their body, e.g. PSP, TTX and acetylcholine (Bricelj et al., 2005; Li et al., 2017). A growing body of investigation has been reporting that the mutation of sodium channel genes may contribute to the resistance of toxin in animals like snakes, frogs, shellfish etc (Venkatesh et al., 2005; Bricelj et al., 2005; Mcglothlin et al., 2016; Li et al., 2017). However, researches also revealed that some toxin binding protein could also takes part in toxin transfer and transport for toxin resistance. It would be meaningful to study the genetic basis from transcribe level between toxic and non-toxic samples to retrieve the genetic expression patterns and potential related toxin-resistance genes. In this study, based on our previous toxin detection research, genetic patterns of both toxic and non-toxic samples from two typical *Nassarius* species were compared.

Firstly of all, *N. succinctus* and *N. variciferus*demonstrated similar DGE patterns. Generally the toxic samples indicated more up-regulated genes than the non-toxic samples. For *N. variciferus* , the up-regulated

genes for each toxic sample was significantly higher than that of the non-toxic samples. For *N. succinctus* , two toxic samples had more up-regulated genes. This suggest that different mechanism activities associated with TTX-resistance may occur in toxic samples. Additionally, despite not much apparent the PCA analysis of gene expression showed that the toxic samples had similar gene expression patterns and the nontoxic samples had similar expression patterns for both species. Furthermore, we flagged the detailed different expression genes between the toxic and nontoxic groups. Some differently expressed genes for coding fibrinogen-like protein between toxic and nontoxic samples were previously indicated as TTX binding protein in toxic pufferfish in Lee et al (2007). We also found some new different expressed genes which were upregulated in toxic individuals, like heat shock protein, RNA-binding protein, Histone-lysine N-methyltransferase, hypothetical and putative protein, and tissue factor pathway inhibitor. Their detailed metabolic mechanism deserved to be further investigated.

Based on all the transcriptome sequences, we tried to retrieve the sodium channel. One mutation locus "L" which was different from all other amino acids in the Domain 2. Unfortunately, the Domain 1 and 4 of sodium channel could not be retrieved by any way of transcript blast or RACE-PCR. We infer the reasons is that the RNA-seq sequences did not cover the sodium channel regions due to RNA degraded. We would employ comprehensive samples and more advanced sequence technologies to explore the complete sodium channel sequences of various toxic and non-toxic samples in different species of *Nassarius* in further studies.

Acknowledgments

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Data Accessibility Statement

Transcriptome sequences of 12 samples in this study were submitted into NCBI. Their Genbank numbers were: SRR10582953, SRR10582959, SRR10582955, SRR10582954, SRR10582956, SRR10582961, SRR10582957, SRR10582958, SRR10582960, SRR10582963, SRR10582963, SRR10582964.

Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

Shanmei Zou designed the project, conducted the experiment, analyzed the data, and wrote the manuscript. Yuxiao Hua helped the samples collection.

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Figure legends

FIGURE 1 Toxic and nontoxic sample of *N. succinctus* and*N. variciferus* collection from China coast.

FIGURE 2 Distribution of annotated species for both *N. succinctus* and *N. variciferus.* Two species show similar annotated patterns. The *Aplysia californa* species take accounted for the top proportion.

FIGURE 3 PCA plot for difference of expression level between the toxic and non-toxic samples (ZD & HD: nontoxic samples, ZL & HL: toxic samples). It indicated that generally the toxic and nontoxic samples showed similar expression patterns.

FIGURE 4 DEGs patterns between toxic and nontoxic samples of*N. succinctus* and *N. variciferus.* HD in*N.succinctus* and ZD *N.variciferus* (nontoxic samples) were set as control group and HL and ZL were set as observed group.

FIGURE 5 RT-PCR confirmation: 2 -([?][?]Ct) and actual expression level for each comparison of observation (transcript expression) and control groups (RT-PCR). Six potential DEGs related with TTX resistance was included.

FIGURE 6 Amino acids mutations related with TTX resistance on sodium channel Nav1 identified by us and other studies. The site indicated in yellow is the only amino acid identified in *N. succinctus*.

FIGURE S1 Distribution of base content and quality for all samples studied.

FIGURE S2 The top classification categories for the DEGs for both species.

TABLE S1 Functional annotation for both *Nassarius*species from 7 databases

Table 1 . The clean reads quality metrics after Quality control.

Sample	Total Raw Reads(Mb)	Total Clean Reads(Mb)	Total Clean Bases(Gb)	Clean Reads Q20(%)	Clean Reads
HL1	58.37	44.66	6.7	98.09	94.47
HL2	59.9	44.32	6.65	97.88	93.98
HL3	55	44.12	6.62	98.27	94.7
HD1	60.43	44.31	6.65	98.19	94.59
HD2	60.55	44.24	6.64	98.42	95.42
HD3	62.14	44.67	6.7	98.45	95.53
ZL1	63.16	44.59	6.69	96.85	90.95
ZL2	63.16	44.47	6.67	96.75	90.71
ZL3	63.16	44.86	6.73	96.74	90.65
ZD1	59.92	44.65	6.7	96.88	91.04
ZD2	59.92	44.91	6.74	96.86	91.05
ZD3	61.54	44.41	6.66	96.85	91.03

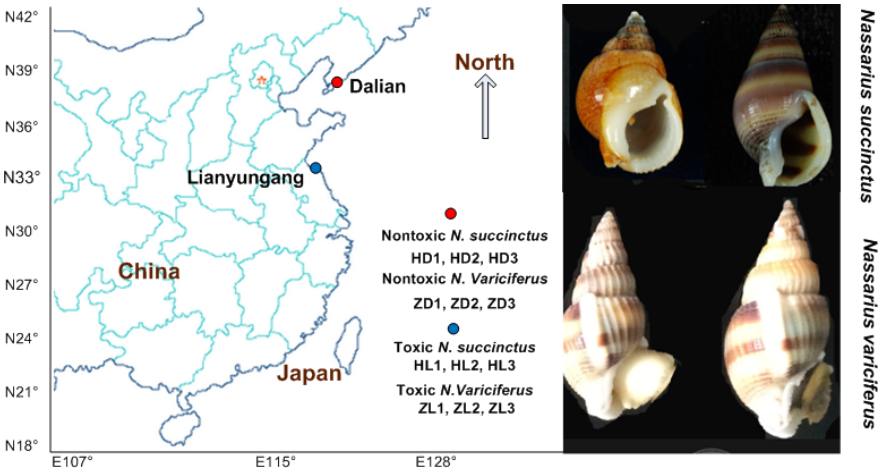


Fig. 1

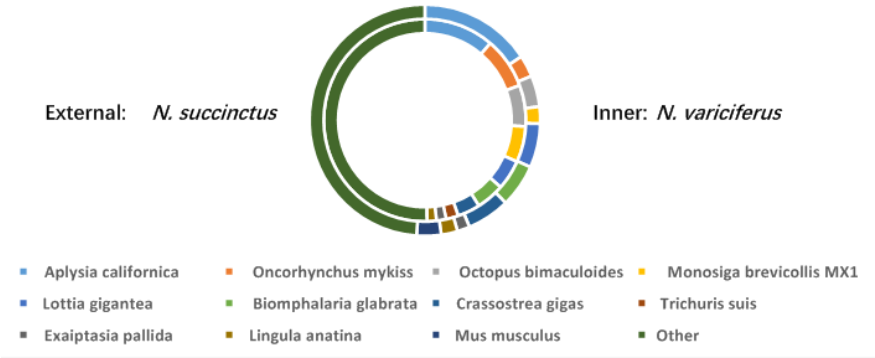


Fig. 2

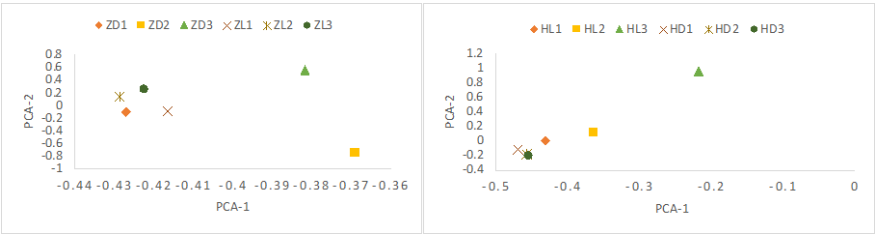


Fig. 3

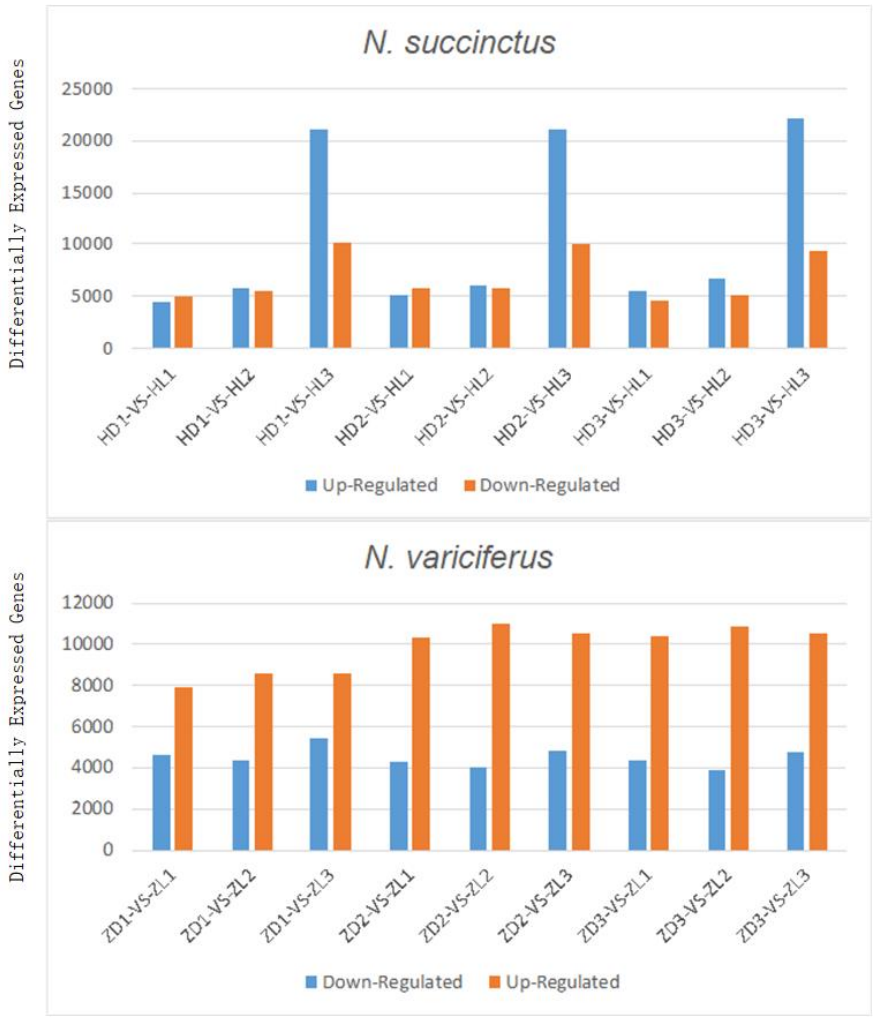
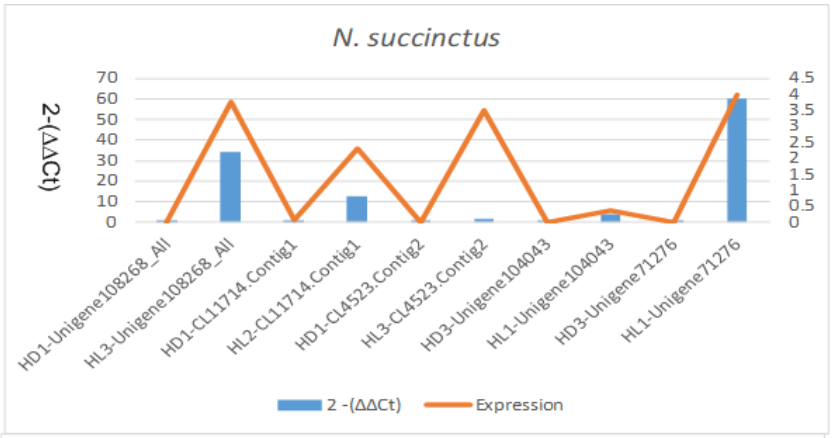
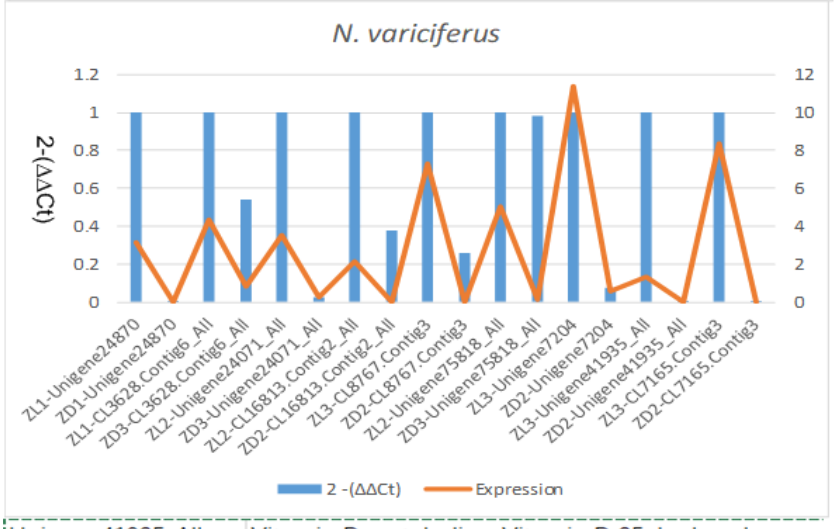


Fig. 4





Unigene41935_All	Vitamin D metabolism Vitamin D 25-hydroxylase
CL3628.Contig6_All	Coagulation factor
Unigene75818_All	Histone-lysine N-methyltransferase
Unigene108268_All	flocculation protein related with oligomeric mucus
Unigene24071_All	myosin binding subunit
CL16813.Contig2_All	palmitoyltransferase and poly(A) RNA polymerase

Fig. 5

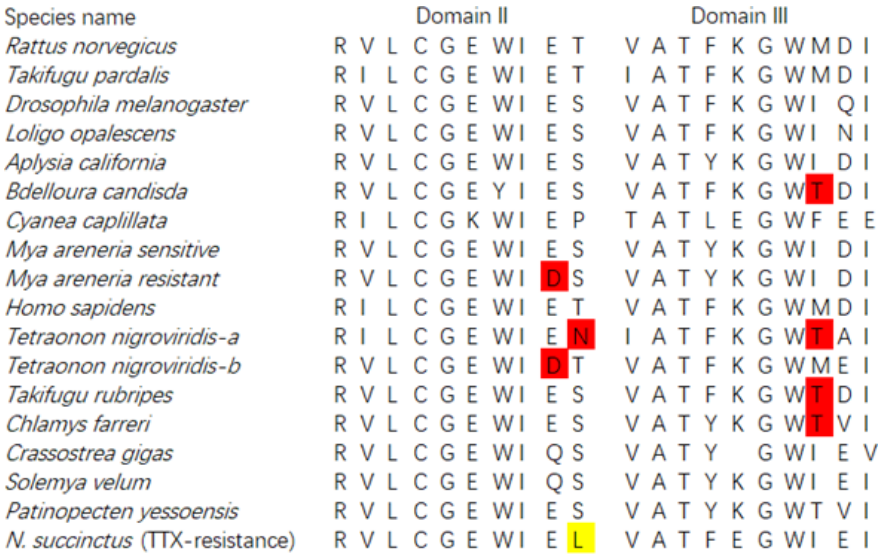


Fig. 6