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# Gene cloning and difference analysis of vitellogenin in *Neoseiulus barkeri* (Hughes)

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## Abstract

*Neoseiulus barkeri* (HUGHES) is the natural enemy of spider mites, whiteflies and thrips. Screening for chemically-resistant predatory mites is a practical way to balance the contradiction between the pesticide using and biological control. In this study, the number of eggs laid by fenpropathrin-susceptible and resistant strains of *N. barkeri* was compared. Additionally, we cloned three *N. barkeri* vitellogenin (Vg) genes and used quantitative real-time polymerase chain reaction to quantify Vg expression in susceptible and resistant strains. The total number of eggs significantly increased in the fenpropathrin-resistant strain. The full-length cDNA cloning of three *N. barkeri* Vg genes (*NbVg1*, *NbVg2* and *NbVg3*) revealed that the open reading frames of *NbVg1*, *NbVg2* and *NbVg3* were 5571, 5532 and 4728 bp, encoding 1856, 1843 and 1575 amino acids, respectively. The three *N. barkeri* Vg possessed the Vitellogenin-N domain (or lipoprotein N-terminal domain (LPD_N)), von Willebrand factor type D domain (VWD) and the domain with unknown function 1943 (DUF1943). The *NbVg1* and *NbVg2* expression levels were significantly higher in the resistant strain than in the susceptible strain, while the *NbVg3* expression level was lower in the resistant strain. Thus, we speculate that the increased number of eggs laid by the fenpropathrin-resistant strain of *N. barkeri* may be a consequence of changes in Vg gene expression.

**Keywords:** *Neoseiulus barkeri*, fecundity, fenpropathrin, vitellogenin, gene expression

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## Introduction

*Neoseiulus barkeri* belongs to the superorder Acari, Phytoseiidae family, and preys on spider mites, thrips, whiteflies, tarsonemids, as well as gall mites (Hessein & Parrella, 1991; Momen, 1995; Grafton-Cardwell *et al.*, 1999; Nomikou *et al.*, 2001; Furtado *et al.*, 2007; Bjrnson, 2008; Tuovinen & Lindqvist, 2010; Jafari *et al.*, 2012; Yao *et al.*, 2014). Currently, *N. barkeri* is regarded as one of the most valuable and promising commercial biological control agents for mites and trips because of its irreplaceable biological traits, such as wide distribution, polyphagy, short life span, ease of culture and high fecundity (Fernando *et al.*, 2010). However, due to the complexity of pest species and their population dynamics, predatory mites cannot prey on all pests and pest management still heavily relies on a wide spectrum of chemical pesticides. Specially, the pyrethroid insecticide fenpropathrin is widely used in orange orchards, which inevitably causes a large number of natural enemies to die. As a result, the control effect of predation mites is weakened, even leads to pest outbreaks (Fournier *et al.*, 1985; Bonafos *et al.*, 2007). Actually, the seed

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selection of predatory mite resistance to pesticides can be regarded as a considerable way to mitigate the contradiction between the release of predatory mites and the application of chemical pesticides (Auger *et al.*, 2005; Salman & Ay, 2013). To reduce fenpropathrin toxicity on predatory mites, we generated a new *N. barkeri* strain with ~620 times resistant to fenpropathrin compared with earlier strains (Lin *et al.*, 2016).

After acquiring resistance to chemical pesticides, insects often appear to reduce the fitness of themselves, such as decrease in fertility, increase in development duration, etc. For example, Lee *et al.* (1996) reported a decrease in fertility, a shorter period of laying egg, a shorter lifespan, and an increase in larval development, including a longer incubation period, in resistant strains compared with susceptible strains of the *German cockroach*. There was also a negative correlation between reproduction and organophosphorus pesticide resistance in *Drosophila melanogaster* and *Culex quinquefasciatus* (Ferrari & Georghiou, 1981; El-Khatib & Georghiou, 1985; Miyo & Oguma, 2002). It is worth noting that several investigators have found increase in fitness of insects due to their insecticide resistance. For instance, the fenpropathrin-resistant strain of *Tetranychus cinnabarinus* exhibited increased fecundity (Liu *et al.*, 2016), the egg-laying rate in the malathion-resistant strain of *Tribolium castaneum* was remarkably higher than in the susceptible strain (Haubruge & Arnaud, 2001; Arnaud & Haubruge, 2002). In addition, Banks and Needham (1970) obtained that the number of eggs laid by female adults was higher in the *Myzus persicae* dimethoate-resistant strain than in the susceptible strain during the first 5 days of egg laying. Similarly, the reproductive capacity of *Blattella germanica* pyrethroid-resistant strains were increased (Roses, 1991).

Vitellogenesis is important for growth and reproduction of oocyte in ovipara (Boldbaatar *et al.*, 2010). Vitellogenin (Vg) is the precursor of vitellin (Veerana *et al.*, 2014; Tran *et al.*, 2016; Trapp *et al.*, 2016), which provides substrates and energy for the development of embryo and ovary. Recent studies have demonstrated that there exists correlation between the level of Vg and the number of eggs laid by various insects. Liu *et al.* (2016) suggested that overexpression of Vg and its receptor may be the reason for the increase in fecundity of cinnabar spider mite's fenpropathrin-resistant strain. Liu *et al.* (2015) and Zhai *et al.* (2015) found a decrease in fecundity after deleting the Vg or related gene, indicating that Vg can affect egg laying.

In this study, the fecundity of fenpropathrin-resistant strain for *N. barkeri* was determined to understand the effects of pesticide resistance on reproductive capacity. Specifically, the Vg mRNA levels in fenpropathrin-susceptible and -resistant strains were quantified to explore the relationship between the number of eggs laid and the expression of *N. barkeri* Vg gene. We also reported the cloning and bioinformatics analysis of the *N. barkeri* Vg gene for the first time.

## Results

### *Determining the number of eggs laid by* N. barkeri *susceptible and resistant strains*

The total number of eggs laid by females of the *N. barkeri* resistant strain was significantly higher (33.07 ± 2.58 heads) than in females of the susceptible strain (27.6 ± 2.59 heads) (table 1). There were no significant differences in the number of eggs laid daily and the time of egg laid by each female between *N. barkeri* susceptible and resistant strains.

Table 1. Fecundity in the susceptible strain (Ss) and fenpropathrin-resistance strain (Rs) of *N. barkeri.*

| Strains | No. eggs/female/day | No. eggs/female | Oviposition period (day) |
|---|---|---|---|
| Ss | 1.51 ± 0.13a | 27.6 ± 2.59b | 18.93 ± 0.88a |
| Rs | 1.57 ± 0.51a | 33.07 ± 2.58a | 20.87 ± 0.74a |

Values are means ± SD (standard deviation). Means in a row followed by different letters are significantly different ($P < 0.05$).

### *Cloning of* N. barkeri *Vg genes*

The three *N. barkeri* Vg genes *NbVg1*, *NbVg2*, and *NbVg3* (GenBank accession number: KX620366, KX620367 and KX620368) were cloned. The open reading frames (ORFs) for *NbVg1*, *NbVg2*, and *NbVg3* were 5571, 5532 and 4728 bp, encoding 1856, 1843, and 1575 amino acids, respectively.

### *The prediction of conserved domain, subcellular localization, and signal peptides of* N. barkeri *Vg*

Conserved Domain Database (CDD) was used to identify conserved domains of NbVg1, NbVg2 and NbVg3 (fig. 1). NbVg1, NbVg2 and NbVg3 all have two Vg-conserved domains, namely, a domain with unclear function 1943 (DUF1943) and a von-Willebrand factor type D domain located in the C-terminus. NbVg1 and NbVg3 possessed a Vg_N domain at the N-terminus, while NbVg2 possessed a lipoprotein N-terminal domain (LPD_N). PSORT II Prediction software was used to predict the subcellular localization of Vg in *N. barkeri*. NbVg1 and NbVg2 were predicted to mainly reside in the outside of the cell, while NbVg3 was predicted to locate in the endoplasmic reticulum membrane, plasma membrane, and Golgi apparatus with certainty of 0.685, 0.640, and 0.46, respectively (table 2). SignalP 4.1 was used to predict the presence of signal peptide (fig. 1). NbVg1, NbVg2, and NbVg3 all possessed signal peptide. The signal peptides for NbVg1 and NbVg2 were cleaved between the 16th and 17th amino acids, while for NbVg3 between the 30th and 31th amino acids.

### *The prediction of primary, secondary and tertiary structure of* N. barkeri *Vg*

ProtParam was used to predict the physical and chemical properties of the amino acids encoded by *NbVg1*, *NbVg2*, and *NbVg3* (table 3). The molecular weights of NbVg1, NbVg2, and NbVg3 were 212, 211 and 179 kDa, and the isoelectric points were 8.61, 8.98, and 6.88, respectively. The three proteins were hydrophobic. SOPMA was used to predict the secondary structures of NbVg1, NbVg2, and NbVg3 (table 4). The most common secondary structure manifestation was the α-helix, and next the random coil, extended strand and β-pleated sheet (<10%). The SWISS-MODEL server, which is available within ExPASy, was used to predict the tertiary structures of NbVg1, NbVg2, and NbVg3 (fig. 2). They were 16.16, 17.61, 14.12% homologous with lipovitellin, respectively.

### *Phylogenetic analysis*

Mega 5.04 software was used to generate a neighbor-joining (NJ) phylogenetic tree. The reliability of the branching was tested using a bootstrap of 1000. Cluster analysis was performed with Vg amino acid sequences from *N. barkeri* and

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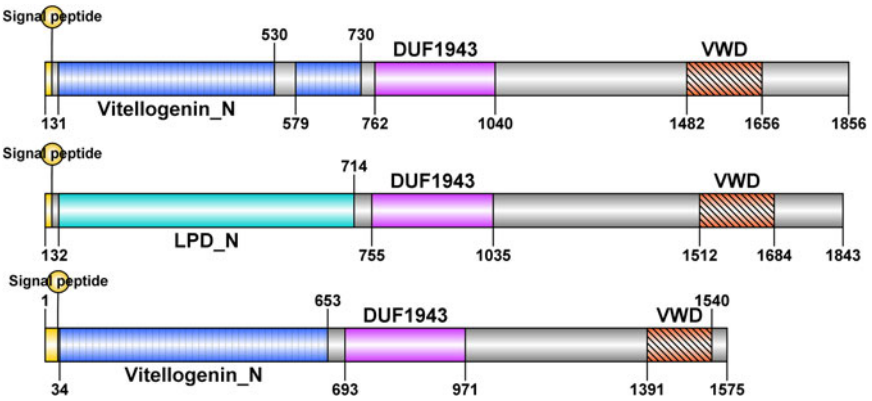


Fig. 1. Conserved domain prediction of NbVg1, NbVg2 and NbVg3 in *N.* barker, where the conserved domains (Vitellogenin_N, LPD_N, DUF 1943, VWD) of VG are colored and the putative signal peptides are highlighted.

Table 2. Subcellular localization of NbVg1, NbVg2, and NbVg3 in *N. barkeri.*

| Protein | Subcellular localization | Certainty |
|---|---|---|
| NbVg1 | Outside | 0.657 |
| | Lysosome (lumen) | 0.190 |
| | Microbody (peroxisome) | 0.107 |
| | Endoplasmic reticulum (membrane) | 0.100 |
| NbVg2 | Outside | 0.542 |
| | Lysosome (lumen) | 0.190 |
| | Endoplasmic reticulum (membrane) | 0.100 |
| | Endoplasmic reticulum (lumen) | 0.100 |
| NbVg3 | Endoplasmic reticulum (membrane) | 0.685 |
| | Plasma membrane | 0.640 |
| | Golgi body | 0.460 |
| | Endoplasmic reticulum (lumen) | 0.100 |

other insects deposited in the NCBI database (fig. 3). NbVg1 and *Amblyseius cucumeris* Vg-1 were found on one branch, NbVg2, NbVg3 were most closely related to *Bactrocera tau* and *Aphis medicaginis*, respectively.

### Vg *expression in* N. barkeri *susceptible and resistant strains*

*NbVg1* and *NbVg2* mRNA levels in the *N. barkeri* resistant strain were higher than in the susceptible strain ($P < 0.05$), while the *NbVg3* mRNA level was lower in the resistant strain than in the susceptible strain ($P < 0.05$) (fig. 4).

## Discussion

The vast majority of previous studies reported the decrease in fecundity in pesticide-resistant insects and mites, however, there also exists some studies gave the different findings (Miyo & Oguma, 2002; Nicastro *et al.*, 2011; Stocco *et al.*, 2016). For example, the increased fecundity of esbiothrin and organic phosphorus-resistant insects and mites (Pathan *et al.*, 2010), the egg-laying amount in the *T. cinnabrinus* fenpropathrin-resistant strain was higher than that of the susceptible strain (Liu *et al.*, 2016). For natural enemies, the increase in fecundity after gaining resistance, will not only reduce the toxicity of chemicals to natural enemies but also benefit the breeding of natural enemies and enhance field application. In this study, the total number of eggs laid by the fenpropathrin-resistant strain was higher than susceptible strain, suggesting that increased egg laying can broaden the field application of *N. barkeri.*

Accumulating evidence has shown that Vg derives from a multi-gene family, and some species may express two or more Vg genes. For example, *Caenorhabditis elegans* possesses six Vg genes (Blumenthal *et al.*, 1984), *Gallus gallus* expresses three (Schip *et al.*, 1987; Silva and Fischer, 1989) and *Xenopus laevis* expresses four (Wahli *et al.*, 1979). In 1999, Matsubara *et al.* (1999) found *Verasper moseri* possesses two forms of serum Vg protein. In this study, we first reported the cloning of three *N. barkeri* Vg genes. The ORFs for *NbVg1*, *NbVg2II*, and *NbVg3* were 5571, 5532 and 4728 bp, respectively. These sizes were comparable with that of the *Chrysopa septempunctata* Vg at 5433 bp (Liu *et al.*, 2015), but smaller than most insect species at 6–7 kb (Tufail and Takeda, 2005, 2008). Bioinformatics analyses showed that the amino acid sequences of *NbVg1* and *NbVg3* possess the domains that are characteristic of insect Vg, such as the Vg_N domain near the N-terminus, von-Willebrand factor type D domain at the C-terminus and the domain with unclear function 1943 (DUF1943) super family. The first two domains are highly conserved in Vg proteins from both vertebrates and invertebrates (Hayward *et al.*, 2010; Zhang *et al.*, 2010). While NbVg2 contains the lipoprotein domain (LPD_N) at the N-terminus, which is responsible for lipid transport (Smolenaars *et al.*, 2007), the same as *C. septempunctata* Vg (Liu *et al.*, 2015). In most insects, the molecular weights of the Vg are approximately 200 kDa. For example, the *Spodoptera litura* Vg was 198.73 kDa (Shu *et al.*, 2009), while the 215 kDa *Dermacentor variabilis* Vg-2 protein was calculated (Khalil *et al.*, 2011). Our data showed that three Vgs exist in *N. barkeri*, NbVg1, NbVg2, and NbVg3 (table 3) of calculated molecular weights being 212, 211 and 179 kDa, respectively. Amino acid sequences in aforementioned three NbVgs were quite different. SignalP software analysis revealed that the cleavage site of NbVg1 and NbVg2 was identified between 16th and 17th amino acids, while that of NbVg3 occurred between 30th and 31st amino acids.

The site and the process of Vg synthesis are still controversial. Mainly have two kinds: Extra-ovarian sources, namely by the organ beyond ovary synthesis precursor, Vg was considered to be taken into the developing oocytes from the hemolymph by the vitellogenin receptor (VgR) via receptor-mediated endocytosis; Vg synthesis also may be endogenous

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Table 3. Primary structure analysis of NbVg1, NbVg2, and NbVg3 in *N. barkeri*.

| Protein | Amino acids | Formula | Molecular weight | Theoretical pI | Instability index | Asp + Glu | Arg + Lys | Aliphatic index | Grand average of hydropathicity (GRAVY) |
|---|---|---|---|---|---|---|---|---|---|
| NbVg1 | 1856 | $C_{9415}H_{14905}N_{2665}O_{2822}S_{60}$ | 212,508.6 | 8.61 | 43.94 | 223 | 238 | 78.03 | −0.613 |
| NbVg2 | 1843 | $C_{9317}H_{14891}N_{2671}O_{2796}S_{68}$ | 211,242.0 | 8.98 | 44.77 | 209 | 235 | 80.17 | −0.640 |
| NbVg3 | 1575 | $C_{8007}H_{12487}N_{2171}O_{2369}S_{74}$ | 179,441.7 | 6.88 | 41.17 | 189 | 184 | 80.17 | −0.382 |

Table 4. Predicted secondary structures of NbVg1, NbVg2, and NbVg3 in *N. barkeri.*

| Secondary structure | Protein | The number of amino acids | Percentage (%) |
|---|---|---|---|
| Alpha helix | NbVg1 | 753 | 40.57 |
| | NbVg2 | 783 | 42.49 |
| | NbVg3 | 575 | 36.51 |
| Beta turn | NbVg1 | 184 | 9.91 |
| | NbVg2 | 154 | 8.36 |
| | NbVg3 | 133 | 8.44 |
| Extended strand | NbVg1 | 347 | 18.7 |
| | NbVg2 | 332 | 18.01 |
| | NbVg3 | 323 | 20.51 |
| Random coil | NbVg1 | 572 | 30.82 |
| | NbVg2 | 574 | 31.14 |
| | NbVg3 | 544 | 34.54 |

(i.e., auto-synthesis), whereby the oocyte itself produces Vg with participation from relevant organelles (Raikhel & Dhadialla, 1992; Melo *et al.*, 2000; Khalil *et al.*, 2011; Agnese *et al.*, 2013; Ni *et al.*, 2014). Generally, Vg is synthesized in fat body within insects. According to Boldbaatar *et al.* (2010), Vg-2 and Vg-3 of *Haemaphysalis longicornis* were transcribed in the fat body, while the transcription of HlVg-1 was found only existing in the midgut. In this work, subcellular localization analyses showed that NbVg1 and NbVg2 likely localize outside of the cell, and NbVg3 likely localizes to the endoplasmic reticulum membrane and plasma membrane. So, we speculate that the difference between NbVg1, NbVg2, and NbVg3 might be caused by the mode of synthesis and the functions of the three Vg proteins. In future, additional studies are needed to be done for understanding the synthesis and function of *N. barkeri* Vg gene.

Previous studies investigated the correlation between Vg expression and the egg-laying amount. When RNAi was used to identify the genes related with the number of eggs laid by brown planthopper (BPH), Qiu *et al.* (2016) found that 91.21% of the genes relating to the regulation of Vg expression and may influence BPH fecundity. Lu *et al.* (2015) silenced the Vg receptor in *Nilaparvata lugens* by RNAi, and found that the Vg level decreased in the ovary while increased in the hemolymph, besides, *N. lugens* failed to spawn. Shu *et al.* (2011) silenced the Vg receptor in *Spodoptera litura* via a similar approach and arrived at similar results. Zhang *et al.* (2014) stated that *Vg* expression for the *Bacillus thuringiensis* (Bt) toxin resistant strain of *Helicoverpa armigera* was 50% lower than its susceptible strain, indicating that the difference in the egg laying amount caused by Bt resistance may be related with *Vg* expression. Liu *et al.* (2016) found that the overexpression of *Vg* and its receptor in the fenpropathrin-resistant strain of *T. cinnabrinus* was critical for the increase of fecundity. In this study, *NbVg1* and *NbVg2* gene expression was higher in the resistant strain than in the susceptible strain, while *NbVg3* gene expression was lower. We speculated that the expression of *NbVg1*, *NbVg2*, and *NbVg3* might be associated with an increase in the total number of eggs laid by the *N. barkeri* fenpropathrin-resistant strain. But additional studies are needed to substantiate this hypothesis.

## Materials and methods

### *Materials*

In 2009, the predatory mite *N. barkeri* (fenpropathrin-susceptible strain, approximately 500 mites) was collected

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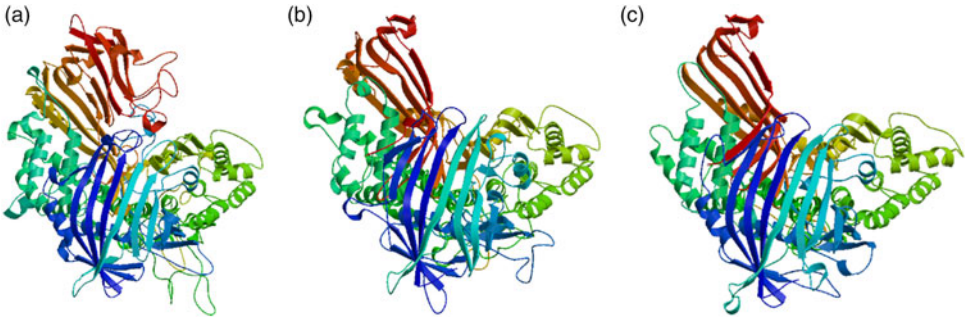


Fig. 2. Putative tertiary structure of *N. barkeri*: (a) NbVg1; (b) NbVg2; (c) NbVg3.

from the leaves of lemon trees at the Citrus Research Institute of Southwest University (Beibei, Chongqing, China; longitude 106_2213311 E/latitude 29_4611411 N), housed indoors, and fed acaroid mites under pesticide-free conditions. To produce the fenpropathrin-resistant strain, the mites were exposed to increasing concentrations of fenpropathrin (10–5000 mg $l^{-1}$). Approximately 10–20% of the original number of mites (50–100 mites) survived exposure; they were defined as the fenpropathrin-resistant strain. The susceptible and resistant strains were housed at a temperature of 25 ± 1 °C, a relative humidity of 80 ± 5%, and a photoperiod of 14 h light:10 h dark.

## Methods

### *Determination of the number of eggs laid by* N. barkeri *fenpropathrin-resistant and susceptible strains*

*N. barkeri* female nymphs were housed in a hollow-glass, single-head feeding room containing a sufficient number of *Panonychus citri* nymphs. After maturation, female mites were allowed to mate with male mites. The fecundity was observed every 24 h and recorded until there were no eggs being laid. The strains were housed in an artificial climate incubator at a temperature of 25 ± 1 °C, a relative humidity of 80 ± 5%, and a photoperiod of 14 h light:10 h dark. This experiment was repeated three times, with a total of 30 heads.

### *Primer design*

The *N. barkeri* transcriptome database was used to identify three unigenes (*NbVg1*, *NbVg2*, and *NbVg3*) corresponding to the Vg gene. Primer Premier 5.0 software was used to design the primers (table 5).

### *Total RNA extraction and cDNA synthesis*

RNA Isolater Total RNA Extraction Reagent (Vazyme, China) was used to extract total RNA from the *N. barkeri* fenpropathrin-resistant and susceptible strains. The quality of the extracted RNA was evaluated through 1% agarose gel electrophoresis, and the concentration was determined with an Nanodrop 2000N spectrophotometer (Thermo Fisher Scientific, USA). The PrimeScript® RT Reagent Kit (TaKaRa Bio, Dalian, China) was used for cDNA synthesis, while the SMARTer™ RACE cDNA Amplification Kit (TaKaRa Bio, Dalian, China) was used for gene cloning.

### *Gene cloning*

For gene cloning, the 25 µl PCR reaction system included: 2.5 µl of 10× PCR Buffer ($Mg^{2+}$ free), 2.5 µl of $MgCl_2$ (25 µmol·$l^{-1}$), 2.0 µl of dNTPs (2.5 mmol·$l^{-1}$), 1.0 µl each of forward and reverse primers (10 µmol·$l^{-1}$) (table 1), 1.0 µl of the cDNA template, 0.25 µl of *Taq* enzyme (2.5 U·µ$l^{-1}$), and 14.75 µl of sterile $H_2O$ (TaKaRa Bio). The PCR reactions were conducted under the following conditions: predenaturation for 3 min at 94 °C, followed by 33 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52–58 °C, extension for 2 min at 72 °C, and a final extension for 10 min at 72 °C. For 5′ (3′) end cloning, PCR reaction system was set up as follows: 10.0 µl of 2× *Taq* Plus Master Mix (Vazyme, China), 1.0 µl of the primer from the first PCR (10 µmol·$l^{-1}$), 1.0 µl of 10× Universal Primer A Mix, 2.0 µl of the 5′ (3′) RACE cDNA template, and 6.0 µl of sterile $H_2O$. The product from the first PCR served as the cDNA template for the second PCR. The PCR reactions were conducted under the following conditions: 3 min at 94 °C, 33 cycles of 30 s at 94 °C, 30 s at 60–75 °C, 2 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR products were analyzed by agarose gel electrophoresis, and the fragments of interest were recovered from the agarose gel using a Gel Extraction Kit (TaKaRa Bio). The PCR products were cloned into the pMD19-T vector (TaKaRa Bio) according to the manufacturer's instructions. The vectors were transformed into competent *Escherichia coli* cells, and the recombinant bacteria were identified by the blue–white screening method, and PCR-positive clones were selected for sequencing (Invitrogen, Shanghai, China).

### *Bioinformatics analysis*

BioXM 2.7 software was used to analyze the nucleotide sequence and to deduce the amino acid sequence. ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to determine the ORFs, and identify the conserved domains, as well as align *NbVg1*, *NbVg2* and *NbVg3* sequences. PSORT II Prediction (http://psort.hgc.jp/form.html) and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) were used to determine the protein subcellular localizations and to predict the presence of signal peptides. ProtParam (http://web.expasy.org/protparam), SOMPA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_sompa.html) and SWISS-MODEL (http://swissmodel.expasy.org/) were used to determine the physical and chemical properties of the

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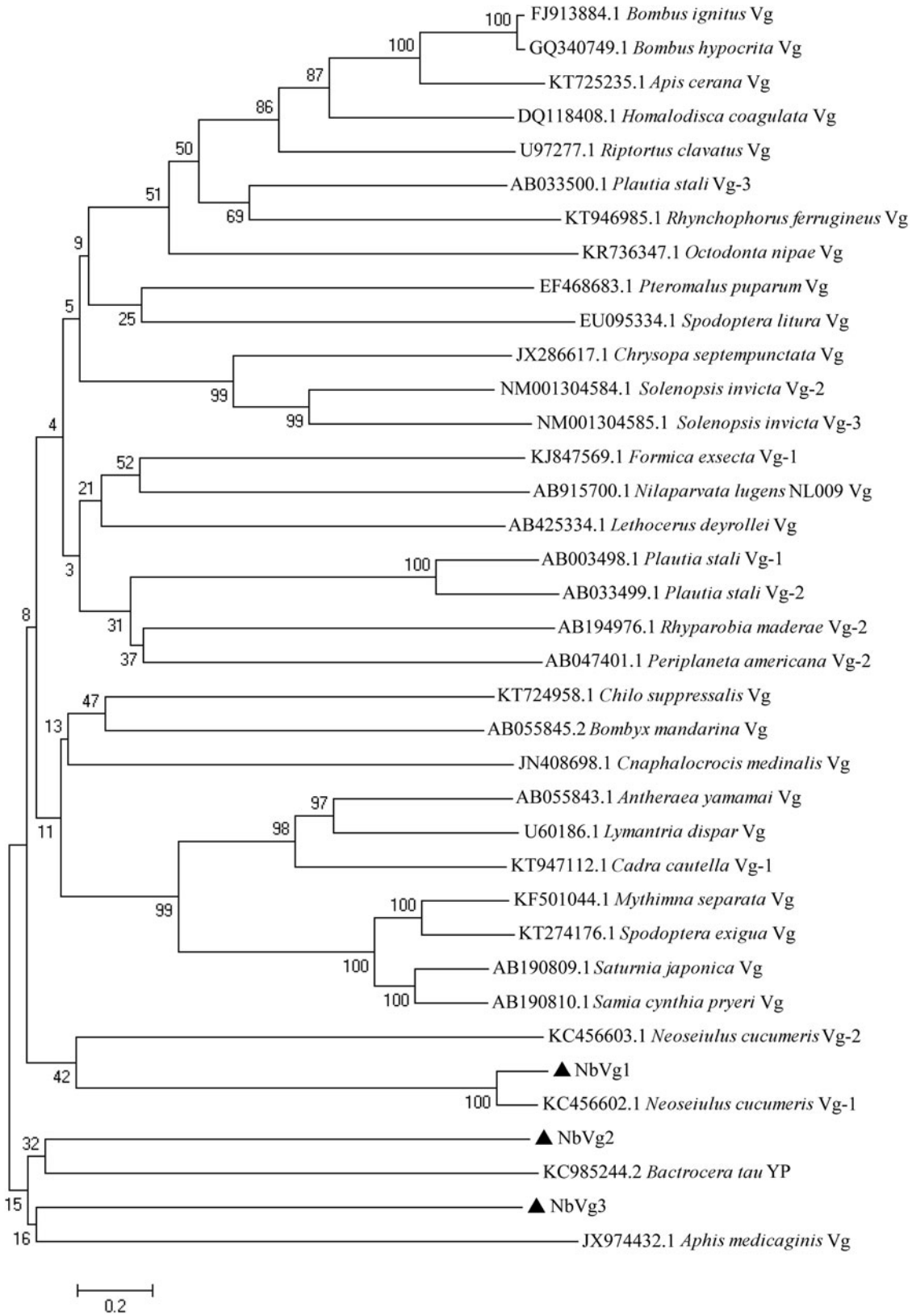


Fig. 3. Phylogenetic analysis of NbVg1, NbVg2, and NbVg3 in *N. barkeri*.

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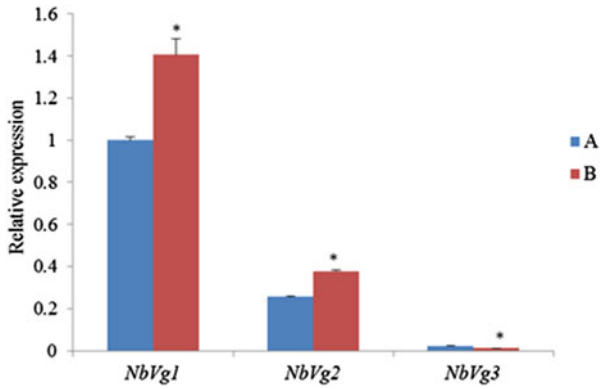


Fig. 4. Relative expression of *NbVg1*, *NbVg2 and NbVg3* in *N. barkeri*, where A denotes the susceptible strain and B represents the resistance strain.

primary, secondary, and tertiary structures. The phylogenic tree was constructed with MEGA 5.04 software using the NJ method. The reliability of the branching was tested using a bootstrap of 1000.

*Differential Expression Analysis of NbVg1, NbVg2, and NbVg3 in N. barkeri susceptible and resistant strains*

*NbVg1*, *NbVg2*, and *NbVg3* expression in *N. barkeri* fenpropathrin-susceptible and -resistant strains was measured by quantitative polymerase chain reaction (qPCR). The 20 µl reaction mixture contained 10.0 µl of 2× Go*Taq*® qPCR Master Mix (Promega, USA), 0.4 µl each of forward and reverse primers (10 µmol·l$^{-1}$), 2.0 µl of the cDNA template, and 7.2 µl of sterile $H_2O$. PCR reaction conditions were as follows: predenaturation for 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 1 min at 60 °C. β-Actin (ACTB) and ubiquitin-conjugating enzyme served as reference genes. The reaction was carried out on

Table 5. Primers used in this study.

| Gene | Primer | Forward primer (5′ to 3′) | Reverse primer (5′ to 3′) |
|---|---|---|---|
| *NbVg1* | 434-1 | GTTCGGTTGACCATAGTGTTCT | CGTAGTCAGTCTGTTCCTCGTC |
| | 434-2 | CGTCACCTCAAGGATGTTCACC | GCCTGAGGGATGGTCTTCTTGA |
| | 434-3 | ACTCAAGAAGACCATCCCTCAG | CTTGAAGTTGTTGGACTGACGG |
| | 434-4 | CCAAGGGATGCGACTCCAAGGT | GTGATGACCTTCCAGATACCGC |
| | 434-5 | GCGGTATCTGGAAGGTCATCAC | GCGTGGTGCTTCATACCCTTGT |
| | 434-6 | GGCCGGTATGATGATCCGTATC | TGGGGTAGAACTGGAACTTGAC |
| | 434-7 | GTCAAGTTCCAGTTCTACCCCA | GTTGTCGTTGTTGCCACAGATA |
| | 434-8 | CGGAAAGTTCGCCGTTCTCTAC | TGCTTATCCTGGCTTTGGCTCT |
| | 434-9 | GAATACCAACACGCTAATGAAT | ATCGAATAGAATTTTATTAAAT |
| | 434-5R-1 | | TGGTGGTCTCGTATTCGTCGGACTCGGG |
| | 434-5R-2 | | CACGGAAGATGTTGAGGGACCACTGCTG |
| | 434-3R-1 | GCGACCAGACTCAAGGACCCACCGATTT | |
| | 434-3R-2 | TGAAGGAGTTCCATCAGGACAGCCAGCA | |
| | 434-qPCR | TCCGACGAATACGAGACCACC | GTTGTTGGACTGACGGAGGG |
| *NbVg2* | 048-1 | CACACGGATACACATTTCACCA | CATCGGGACCCATCTGCTTCAT |
| | 048-2 | TCACCAAGACCAGGAACTACAT | TGGAGTTCAGTAGCCGACTTTT |
| | 048-3 | CTCTCGCTCAGGTCATCGTTTC | CGACCTGACGGATGAACTGAAC |
| | 048-4 | CACTCTGCCAAGAACTTCGCTC | GTGGTGGATGAAGTTGTAGAGG |
| | 048-5 | GACTCTTTACCACCACCTCACC | TGAGCGTAGCGAGTGTTCTGGA |
| | 048-6 | ATCAACCCTCTCCAGAACACTC | TGACGGACTTCTTCTCACCCTT |
| | 048-7 | CCAGGAACTTCAGCAGTCTCTC | CTGTTCGGAGTAGACGAGTTCA |
| | 048-8 | CTCGTGAACGCTTCCCACCAGA | GAGAGCGAAAGAGCGGACAAAG |
| | 048-9 | GAAGTTCAGCCCAAGAAGACCG | TTTTTGGGTGGTTCACGACGAC |
| | 048-5R-1 | | CTGAGCGAAGTTCTTGGCAGAGTGGAGC |
| | 048-5R-2 | | CGGTAGATGTTGATGACCTCTTCGGGCA |
| | 048-3R-1 | TTTCCGCCTACACCAAGACTCCCCAGCC | |
| | 048-3R-2 | CATCATCGCTCGTGACAACCGCCAGAAG | |
| | 048-qPCR | TACCGTTCTCTTGTTGCCACTG | CTGCTGCTCGTTGACCTGAA |
| *NbVg3* | 573-1 | TCTCCGCTATGGCATCCTTTAA | TGATATCGTGCAAGCCGGTATG |
| | 573-2 | ACATTTTCAAGGCAGTAGTCGC | AAGAGGCTCAACCGAGAGTCAT |
| | 573-3 | TTGGATAGAAGTGAAGATGCCC | AGAGTCCGCCAAATCTTCTTTG |
| | 573-4 | AGCACAAAGAAGATTTGGCGGA | ATGATTCACTGTTCTGGGAGCC |
| | 573-5 | ATCTACCTGTTTCGTGGGGAGC | TTTTATTGCTCTCATAGTTCGC |
| | 573-5R-1 | | TGAGGGTGATATCGTGCAAGCCGGTATG |
| | 573-5R-2 | | TGCCTCGTTAGCCTGAGAGCCTTAGCCG |
| | 573-3R-1 | GAACAAGCAAAACGCGAGTTCATGGCCT | |
| | 573-3R-2 | TCAGCGACGAAGAGCCGAAAGTCACCAA | |
| | 573-qPCR | CGAGGTTGCGAAGAAGGACA | TCGGCAGGACTCGCATCAAC |
| | ACTB | TACGACCAGAAGCGTACAGC | CCAACCGTGAAAAGATGACC |
| | UBC | ATGAAACCCCGCCCTACCTG | TTCCCATAGGCCGTCACTCG |

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an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA).

*Statistical analysis*

The Vg gene expression level in *N. barkeri* fenpropathrin-susceptible and -resistant strains was calculated by the $2^{-\triangle\triangle ct}$ method. The Duncan new multiple range method was used to analyze the real-time PCR data. IBM SPSS Statistics 20 software was used for statistical analysis. $P < 0.05$ was considered significantly different.

## Conclusions

In this study, we reported for the first time the sequences of three *N. barkeri* Vg genes and conducted bioinformatics analysis. The egg-laying amount was significantly higher in the *N. barkeri* resistant strain than in the susceptible strain. By quantitative real-time PCR, *NbVg1* and *NbVg2* expression increased significantly, while *NbVg3* expression decreased significantly. Our results may provide new insights into the relationship between the Vg gene and pesticide resistance.

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