INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20F–005/2020/24–3–398–404
DOI: 10.17957/IJAB/15.1451
http://www.fspublishers.org

*Full Length Article*

# Molecular Detection of Species and Resistance to Neonicotinoid Insecticides in Aphids of Hunan Province, China

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*Received 16 January 2020; Accepted 21 February 2020; Published 11 July 2020*

## Abstract

The purpose of this study was to establish methods for the rapid and accurate identification of allied aphid species, and for the effective detection of neonicotinoid insecticide resistance in aphids. In this study, polymerase chain reaction (PCR) and sequencing analysis were performed for the amplification and sequencing of mitochondrial cytochrome oxidase I (COI) gene fragments (709 bp) from 8 aphid populations collected from 7 types of host plants in 2 regions of Hunan Province. Restriction digestion of PCR products was performed using four restriction endonucleases, namely DraI, HinfI, TaqI, and SspI and allied aphid species were identified via restriction fragment length polymorphism (RFLP) analysis and comparisons between nucleic acid sequences and reference NCBI sequences. Next, PCR amplification and sequencing were performed on an acetylcholine receptor gene (nAChR) $\beta$ subunit fragment (200 bp) containing the R81T mutation, which confers neonicotinoid resistance, on the 8 sampled aphid populations. Mutation analysis was performed by restriction digestion of the amplification products using BcoDI and HincII. Neonicotinoid resistance in aphids was determined based on RFLP patterns of digested PCR products and toxicity tests. We found that the aphid species collected from 7 types of host plants include *Myzus persicae* (green peach aphid), *Aphis nerii* (oleander aphid) and *A. craccivora* (cowpea aphid); none of these aphid species were detected resistance to the neonicotinoid insecticide acetamiprid. Thus, the findings of this study provide a theoretical basis for the rapid and accurate identification of allied aphid species as well as the effective detection of neonicotinoid insecticide resistance in aphids. © 2020 Friends Science Publishers

**Keywords:** Aphids; Species identification; Neonicotinoid resistance; DNA sequencing; PCR-RFLP

## Introduction

Aphids are common and serious pests for a wide range of vegetables and crops. There are many aphid species, the most common of which are *Myzus persicae* (green peach aphid), *Lipaphis erysimi* (turnip aphid) and *Brevicoryne brassicae* (cabbage aphid). As most aphids possess distinct morphological features, and the methods of morphological analysis are simple, intuitive, and easy to use, morphological classification methods have dominated the identification of aphid species for a long time (Zhao *et al.* 2013). However, aphids are among the fastest-evolving insects within Hemiptera. Because of their abundant populations, the multidirectionality of evolution, and the directional nature of natural selection, the morphologies of certain allied aphids can be very similar. Therefore, it can be extremely difficult to distinguish allied aphid species simply based on external morphology and morphologic degeneration led to fewer features can be used for taxonomy and phylogenetic analysis (Huang and Qiao 2006). Many researchers have investigated the classification of insects through mitochondrial DNA (mtDNA) using techniques, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplification of polymorphic DNA (RAPD-PCR) and DNA probes. These studies provided effective means for the identification of infraspecific taxa as well as the identification and discrimination of allied species (Lu and Gu 1995; Wei *et al.* 2010). In particular, RAPD-PCR was widely applied in studies on the classification of aphid populations (Black *et al.* 1992; Cenis *et al.* 1993) and the RAPD-PCR marker technique has previously played a major role in promoting the development of molecular ecology. However, this technique is limited in its ability to differentiate between heterozygous and homozygous genotypes, and thus, is ineffective in distinguishing heterozygous genotypes. In addition, RAPD reactions are readily influenced by various external factors and have poor repeatability, limitations which have hampered the scope of its applications (Zhang *et al.* 2000). In fact, journals such as *Molecular Ecology* no longer endorse the use of RAPD as a tool for scientific research (Wang *et al.* 2007a). On the other hand, PCR-

RFLP is widely used in research because of several strengths, including the fact that it does not require the addition of markers or probes in samples, it is highly sensitive, low-cost, exhibits high reproducibility, and is very robust. Furthermore, it is not affected by external factors, such as age and sex and gene products, and the targets of RFLP are widely distributed across the genome (Faten *et al.* 2002; Raboudi *et al.* 2005; Valenzuela *et al.* 2007).

Aphids can damage plants either directly by feeding on plant sap or indirectly via the transmission of viruses and diseases (Shew and Lucas 1991). At present, chemical control remains the primary method of aphid control; however, many aphids have developed differing levels of resistance to a variety of insecticides in many countries (Harlow and Lampert 1990; Gubran *et al.* 1992; Hollingsworth *et al.* 1994; Ahmad *et al.* 2003; Foster *et al.* 2007; Wang *et al.* 2007b), including neonicotinoid insecticides (Panini *et al.* 2014). It appeared that overexpression of one or more P450s was the primary mechanism of neonicotinoid resistance in insect pests (Nauen and Denholm 2005; Karunker *et al.* 2008). Moreover, R81T mutation in the nicotinic acetylcholine receptor (nAChR) $\beta$ subunit has been proved to be associated with resistance of aphids to neonicotinoid pesticide (Bass *et al.* 2011; Puinean *et al.* 2013). This increased resistance leads to increased insecticide use, which in turn raises the cost of pest control and exacerbates environmental pollution. Thus, determination of resistance development and resistance levels in aphids to each class of insecticide is pivotal for the formulation of effective aphid control programs. Currently, the most common resistance detection methods being used are biological and biochemical methods. However, these approaches have several shortcomings requiring substantial time and effort to perform as well as needing large numbers of insect samples. The rapid and effective determination of resistance in pests to certain classes of insecticides at the molecular level is crucial for insect resistance management. Previous studies have shown that detection of the R81T mutation in the acetylcholine receptor gene of the aphid genome is an effective method for determining the resistance of aphids to neonicotinoids (Panini *et al.* 2014; Voudouris *et al.* 2016).

In this study, RFLP and sequencing analyses were performed on an aphid mitochondrial COI gene fragment (709 bp) to classify aphid populations and identify the species found on eight types of host plants in three regions of Hunan Province. The identified aphid species subsequently underwent RFLP analysis of a 200 bp acetylcholine receptor gene fragment containing the R81T mutation, which confers neonicotinoid resistance. We then determined using RFLP analysis and toxicity tests whether the aphids living on the eight aforementioned host plants have neonicotinoid resistance. Our results from this study provide a theoretical basis for using RFLP to rapidly and accurately identify allied aphid species and to effectively detect neonicotinoid resistance in aphids.

## Materials and Methods

### Experimental materials

Aphids were collected in 2015–2016 from various host plants in the Changsha and Yueyang regions of Hunan Province (Table 1). The host plants were tobacco, rapeseed, cabbage, oleander, and radish in Changsha, cabbage, cowpea, and hyacinth bean in Yueyang. Eight of the collected aphids were used for DNA extraction, while the remaining aphids were reared on their corresponding hosts for one generation in our laboratory; the leaf dipping method was then used to conduct toxicity tests. The susceptible population used as a reference for the toxicity tests was a population of *M. persicae* that were reared continuously on tobacco plants in our laboratory without any insecticides. The rearing conditions were a temperature of 25 ± 1°C, a relative humidity of 70±5% and a light/dark photoperiod of 14 h and 10 h, respectively.

### Experimental reagents

DNA extraction was performed using the TIANamp Genomic DNA Kit (Catalog No. DP304, Tiangen Biotech Co., Ltd., Beijing, China) in accordance with the manufacturer's instructions. The Quick Gel Extraction Kit, DNA markers, ExTaq DNA polymerase, 10×buffer and dNTPs were all purchased from Tiangen Biotech (Tiangen Biotech Co., Ltd., Beijing, China). The restriction endonucleases, BcoDI and HincII, and buffers were purchased from New England Biolabs (New Jersey, New York, America) and the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

### Aphid toxicity tests

Distilled water containing 0.05% (v/v) Triton X-100 was used to dilute an acetone-solvated acetamiprid microemulsion (Guizhou Daoyuan Biotechnology Co., Ltd., Daoyuan, China) into a graded series of concentrations. Distilled water containing 0.05% (v/v) Triton X-100 was used as a control.

The leaf-dip method was used to test resistance of wingless aphids larvae to acetamiprid according to Voudouris *et al.* (2016) and revised. A hole punch was used to punch leaf disks with a diameter of 2 cm from fresh, clean leaves of each host plant. The disks were soaked in a pre-diluted insecticide solution for 10s, removed, and dried either in a shaded and well-ventilated area for approximately 30 min or dried through the removal of excess insecticide solution using absorbent paper. Next, the disks were placed in a 12-well ELISA plate pre-coated with 1 mL of 1% agarose gel and then all test disks were placed in incubator at a temperature of were kept at 18°C and 16:8 h L:D. Apterous adult aphids (20 per disk) were carefully placed into the leaf disks using an ink brush. Five insecticide concentrations were used in each treatment, with three replicates for each concentration.

The ELISA plates were placed in an illuminated incubator at a temperature of 22±1°C and the number of dead insects was counted after 24 h. An aphid was considered dead if no leg movement was observed upon gentle contact with a brush. A probit analysis program was used to calculate the $LC_{50}$ value, toxicity regression line (y= a+bx) and 95% confidence interval of each tested insecticide.

**Genomic DNA extraction from single aphids and amplification of mitochondrial COI gene segments**

A single aphid was placed in a 1.5 mL centrifuge tube to which 50 $\mu$L of homogenization buffer for DNA extraction was added. A sterile disposable pestle was used to crush the aphid until the body completely disintegrated into a cell suspension. Aphid genomic DNA was then extracted and Gel electrophoresis was performed. The gels were visualized and photographed using 0.05% ethidium bromide under UV illumination. Gene Ruler 100 bp DNA Ladder Plus (Tiangen Biotech Co., Ltd., Beijing, China) was used as a molecular weight standard and a UV spectrophotometer (Thermo, USA) was used to measure the OD260 and OD260/280 values. Ten single aphid was extracted genomic DNA each time.

To amplify the mitochondrial COI gene fragment in aphids, we used the following primers designed by Valenzuela *et al.* (2007) in their study on the determination of allied aphid species: LCO1490(5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGCTGACCAAAAAATCA-3′). The PCR reaction mixture for amplification of the 709 bp target sequence consisted of 1×reaction buffer (3.5 m*M* $MgCl_2$), 4 m*M* of dNTPs, 10 $\mu$M of both sense and antisense primers, 10ng template DNA, and 0.2 U Taq DNA polymerase (Takara, Japan), with sterile water to a final volume of 25 $\mu$L. The PCR amplification conditions were as follows: 95°C for 6.5 min, followed by 40 cycles of 94°C for30s, 50°C for 30s and 72°C for 40s, with a final extension at 72°C for 3 min. Amplification products were separated by electrophoresis using 1.5% agarose gels, and gels were examined using a gel imaging system.

**mtDNA sequencing and PCR-RFLP**

Amplified COI gene segments were sequenced and MEGA version 7.0 was used for sequence analysis and construction of the phylogenetic tree. All sequences were submitted to GenBank, and the New England Biolabs (NEB) cutter tool (NEBcutter, https://www.neb.com/tools-and-resources/interactive-tools) was used to determine the restriction endonuclease cutting sites of the sequences obtained from each sample (Table 2). For restriction digestions, 20U *Dra*I, 10U *Hinf*I, and 20U *Ssp*I, were added separately to centrifuge tubes containing 4ng of the DNA, which were incubated overnight at 37°C. Similarly, 20U *Taq*I was also added to a centrifuge tube containing 4ng of DNA followed by overnight digestion at 65°C. Digestion products were separated by electrophoresis using 2% agarose gels, which were examined using a gel imaging system.

**Detection of the R81T mutation using PCR-RFLP**

The primers designed by Voudouris *et al.* (2016) in their study on the molecular detection of neonicotinoid resistance in aphids (ApF: 5′-TCTAATTATGGGGTTAATTTATAGTCG-3′ and ApR: 5′-ACAGGCGGTCAGGAAGTGTA-3′) were used for PCR amplification of the 280 bp target sequence. The PCR reaction mixture consisted of 1×reaction buffer (1.8 m*M* $MgCl_2$), 0.8 m*M* of dNTPs, 0.3 $\mu$M of the sense and antisense primer, 10ng template DNA, and 0.8 U of Taq DNA polymerase. Sterile water was added to a final volume of 25 $\mu$L. PCR thermocycling conditions were 94°C for 30s, 53°C for 30s and 68°C for 20 s, followed by 29 cycles. Amplification were separated by electrophoresis using 1.5% agarose gels, and the gels were evaluated using a gel imaging system. Next, a second (nested) PCR was performed on the previously amplified 280 bp sequence using the primers by Voudouris *et al.* (2016) to generate a 200 bp target segment containing the R81T mutation (nAChRF: 5′-CCTGCAGCTATTAAAATATCCA-3′and nAChRR: 5′-ACGTTAGAAAGGAAACTGTTTA-3′). The PCR reaction mixture consisted of 1×reaction buffer (1.8 m*M* $MgCl_2$), 0.8 m*M* of each dNTPs, 0.3 $\mu$M of each of the sense and antisense primers, 10ng template DNA, and 0.8 U Taq DNA polymerase, with sterile water to a final volume of 25 $\mu$L. The PCR amplification conditions were followed by 29 cycles of 94°C for 30s, 58°C for 15s, and 68°C for 15s. For restriction digestion, 10U *Bco*DI, and 10U *Hinc*II were separately added to centrifuge tubes containing 8ng of DNA, which were incubated overnight at 37°C. Digestion products were separated by electrophoresis using 2.0% agarose gels and the gels were examined using a gel imaging system.

## Results

**Identifying aphid species**

**PCR amplification and sequencing of mtCOI in aphids:** Using PCR amplification, we obtained approximately 700 bp bands from genomic DNA extracted from aphids corresponding to the eight types of host plant in three regions of Hunan Province (Fig. 1). We found following sequencing and analysis of the PCR fragment that the nucleotide segment was 709 bp in length, with a shared sequence similarity of 92.38% among aphid samples.

**RFLP identification of mtCOI in aphids:** Restriction digestion of the target COI gene fragments derived from aphid DNA samples was performed using the four restriction endonucleases, *Dra*I, *Hinf*I, *Taq*I, and *Ssp*I.

Following *Dra*I digestion and sequencing, we found that the mtCOI PCR products of aphids from cowpea and hyacinth bean plants in Yueyang, cabbage, rapeseed, radish, and tobacco plants in Changsha were cleaved into two

**Table 1:** Sample collection details of the 7 aphid populations examined in this study

| Aphid population | Collection site | GPS latitude/longitude | Collection date | Host plant | Temperature | Intensity |
|---|---|---|---|---|---|---|
| 1 | Changsha | 113.08,28.19 | 4th Apr., 2016 | Tobacco | 14-17°C | ~20 per leave |
| 2 | Changsha | 113.08,28.19 | 4th Apr., 2016 | Rapeseed | 14-17°C | ~10 per leave |
| 3 | Changsha | 113.08,28.12 | 26th Oct., 2016 | Cabbage | 14-17°C | ~20 per leave |
| 4 | Changsha | 113.09,28.19 | 18th May, 2016 | Oleander | 18-24°C | ~10 per leave |
| 5 | Changsha | 113.08,28.19 | 10th Oct., 2016 | Radish | 17-24°C | ~15 per leave |
| 6 | Yueyang | 112.75,28.68 | 15th Nov., 2016 | Cabbage | 16-18°C | ~20 per leave |
| 7 | Yueyang | 112.83,28.65 | 15th Nov., 2016 | Cowpea | 16-18°C | ~15 per leave |
| 8 | Yueyang | 112.83,28.65 | 15th Nov., 2016 | Hyacinth bean | 16-18°C | ~15 per leave |

**Table 2:** RFLP fragment patterns (sizes in bp) using four restriction endonucleases of a 709bp region of the mtCOI gene for identification of aphid species

| DraI | HinfI | TaqI | SspI | Host plant (Region) |
|---|---|---|---|---|
| 87, 622 | 709[a] | 91, 93, 525 | 108, 275, 326 | Hyacinth bean and cowpea (Yueyang) |
| 87, 268, 354 | 709 | 91, 93, 525 | 108, 275, 326 | Cabbage (Yueyang) |
| 87, 622 | 82, 201, 426 | 709 | 15, 108, 203, 383 | Cabbage, rapeseed, radish, and tobacco (Changsha) |
| 709 | 709 | 91, 93, 525 | 275, 434 | Oleander (Changsha) |

[a] Undigested PCR product

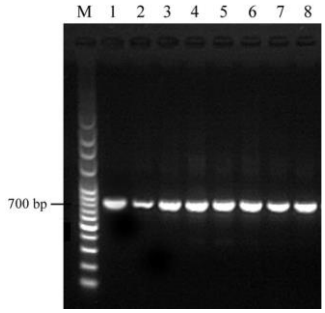


**Fig. 1:** PCR amplification of a 709bp region of the mtCOI gene in aphids from seven types of host plants in two regions of Hunan Province. M: Gene Ruler 100bp DNA Ladder Plus; Lanes 1-5: aphids collected from tobacco, rapeseed, cabbage, oleander, and radish plants in Changsha, respectively; Lanes 6-8: aphids collected from cabbage, cowpea and hyacinth bean plants in Yueyang, respectively

fragments, 87 and 622 bp in size. The mtCOI target segment of aphids from cabbage plants in Yueyang was enzymatically cut into 87, 268 and 354 bp fragments, while the target segment of aphids from oleander plants show an undigested 709 bp product, indicating a lack of *Dra*I restriction sites (Fig. 2a).

After enzymatic digestion with *Hinf*I and sequencing, we found that the mtCOI PCR products of aphids from cabbage, rapeseed, radish and tobacco plants in Changsha were cleaved into three fragments, 82, 201 and 426 bp, while those of aphids from hyacinth bean, cowpea, and cabbage plants in Yueyang, and oleander plants in Changsha were not digested (Fig. 2b).

Next, we found following *Taq*I digestion and sequencing that the mtCOI target segments of aphids from hyacinth bean, cowpea, and cabbage plants in Yueyang as well as aphids from oleander plants in Changsha were digested into 91, 93 and 525 bp fragments. In contrast, no *Taq*I digestion sites were found in the target PCR product of aphids from cabbage, rapeseed, radish, and tobacco plants in Changsha (Fig. 2c).

After *Ssp*I restriction digestion and sequencing, we found that mtCOI PCR products of aphids from hyacinth bean, cowpea and cabbage plants in Yueyang generated 108, 275 and 326 bp fragments. Further, the target PCR products of aphids from cabbage, rapeseed, radish, and tobacco plants in Changsha were enzymatically cut into 15, 108, 203 and 383 bp fragments. We also found that the mtCOI-derived amplification of aphids from oleander plants in Changsha contained a single *Ssp*I restriction site resulting in 275 and 434 bp fragments (Fig. 2d).

Nucleotide sequences of the PCR-amplified 709 bp mtCOI region of aphids collected in this study were compared to the corresponding 709 bp sequence region of various aphid samples in the NCBI database using BLAST. A sample was deemed to belong to a corresponding species in the database when the nucleotide sequence of a sample had 100% similarity with a sequence of a particular species in the database. In particular, the sequences obtained for aphids from hyacinth bean, cowpea, and cabbage plantsin Yueyang were identical to the reference NCBI sequence for *Aphis craccivora* (KX447141.1), while the sequences obtained for aphids from cabbage, rapeseed, radish, and tobacco plants in Changsha matched that found for *M. persicae* (KU236024.1).

**Phylogenetic tree of aphid samples:** The phylogenetic tree of the aphids examined in our study was constructed using MEGA version 7.0, based on the topological structure of the mtCOI gene (Fig. 3). Because aphids from cabbage, hyacinth bean and cowpea plants in Yueyang and the known *A. craccivora* and *A. glycines* species clustered in the same group, these species all likely belong to the same genus. Similarly, aphids from radish, cabbage, tobacco, and rapeseed plants in Changsha clustered in the same group and thus, may belong to the same genus, while aphids from oleander plants in Changsha belong to a unique genus.

**Molecular detection of insecticide resistance in aphids**

**Toxicity tests on aphids:** In this study, we conducted

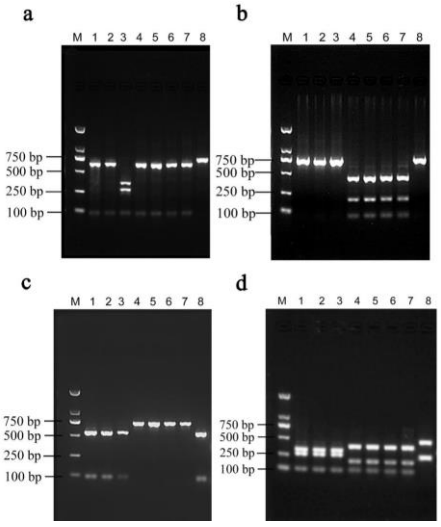


**Fig. 2:** Identification of aphids from seven types of host plants in two regions of Hunan Province by restriction digestion using DraI(a), HinfI(b), TaqI(c), SspI(d). M: D2000 DNA ladder; Lanes 1-8: target fragment patterns of aphids collected from hyacinth bean, cowpea, and cabbage plants in Yueyang, cabbage, rapeseed, radish, tobacco and oleander plants in Changsha, respectively

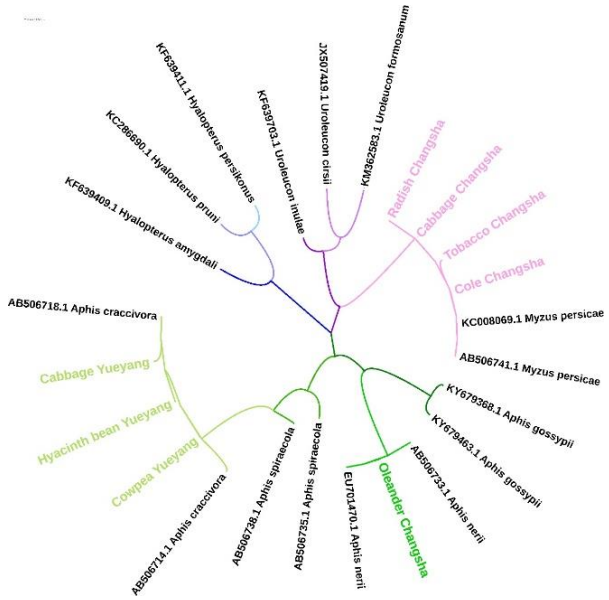


**Fig. 3:** Phylogenetic tree of the aphid samples characterized in this study. (Maximum Likelihood Tree，2000 replications)

relative acetamiprid toxicity tests on aphids collected from different regions of Hunan Province using an insecticide-sensitive strain as a reference, and determined toxicity regression equations and median lethal concentrations ($LC_{50}$) for each aphid sample (Table 3).

Based on the results of the toxicity tests, we found that the $LC_{50}$ value of acetamiprid for aphids from all host plants were very low, and thus, indicates that the aphids collected in this study have minimal resistance to acetamiprid.

**Genotyping of the R81T mutation in aphid samples:** The 280 bp DNA region of Myzus persicae from Changsha was PCR amplified using the ApF and ApR primers (Fig. 4).

We then performed nested PCR on the previously amplified 280 bp sequences using the nAChRF and nAChRR primers obtaining 200 bp target amplification, which contain the R81T mutation (Fig. 5).

**Identification of neonicotinoids resistance in aphids:** Sequencing analysis of the 200 bp amplified nested-PCR gene segments, which harbor the R81T mutation, was performed for all the various aphid samples collected in this study. Nested PCR amplification was successful from all samples, which shared 100% sequence similarity.

Restriction digestion of nested PCR products was performed using BcoDI and HincII, followed by RFLP analysis and sequencing. It was found that the R81T-containing PCR amplicon from all aphid samples contained a BcoDI restriction site resulting in 93 and 107 bp fragments, while there was no digestion following HincII incubation because of the absence of restriction sites in the PCR product (Fig. 6).

## Discussion

Currently, morphological analysis is the primary method for classification of aphids in China. In 1999, Qiao *et al.* (1999) classified gallnut aphids into 14 species (including 4 subspecies) in 5 genera according to the morphological features of a late viviparous female aphids, the shape of the gallnuts, and differences in their summer hosts. However, identification based on morphological analysis is heavily reliant on the professional knowledge and experience of taxonomists, and requires specimens with largely intact external morphological features. In fact, it is often difficult to satisfy these requirements during actual aphid classification processes. In addition, the morphologies of allied species and allied genera of aphids are highly similar making it extremely difficult to distinguish allied species through morphological identification methods.

The techniques that are currently available for the molecular classification of aphids include isoenzyme analysis, PCR-RFLP, and RAPD-PCR. The PCR-RFLP approach for mitochondrial DNA analysis overcomes limitations associated with RAPD such as its susceptibility to external factors, and the successful application of PCR-RFLP in the identification of infraspecific taxa of insects have been reported in the literature (Bogdanowicz *et al.* 1993; Cognate *et al.* 1999; Schroeder *et al.* 2003; Schroeder and Scholz 2005). Valenzuela *et al.* (2007) performed PCR-RFLP analysis on mitochondrial COI gene fragments (709 bp) of aphids, in which four restriction endonucleases (*Dra*I, *Hinf*I, *Taq*I and *Ssp*I) were used for leading to a single taxon. Based on analyses on the restriction digestion patterns of the cleaved fragments, they classified 26 aphid haplotypes into 25 species. In this study, we PCR amplified mitochondrial COI gene fragments (709 bp) of 8 aphid populations collected from seven types of host plants in two regions of Hunan Province. Based on the restriction digestion patterns

**Table 3:** Acetamiprid sensitivity of aphid samples from different regions of Hunan Province

| Population | Region | Toxicity regression equation | $LC_{50}$ (mg/L) | 95% confidence interval (CI (95%)) |
|---|---|---|---|---|
| Tobacco | Changsha | *Y*=4.89+1.05x | 1.26 | 0.69-2.30 |
| Rapeseed | Changsha | *Y*=4.70+1.07x | 1.92 | 1.05-3.48 |
| Oleander | Changsha | *Y*=5.07+0.49x | 0.84 | 0.41-1.70 |
| Radish | Changsha | *Y*=4.76+0.94x | 1.79 | 0.93-3.45 |
| Cabbage | Changsha | *Y*=4.70+0.95x | 2.05 | 1.07-3.94 |
| Hyacinth bean | Yueyang | *Y*=5.17+0.80x | 0.61 | 0.29-1.31 |
| Cowpea | Yueyang | *Y*=5.19+0.81x | 0.59 | 0.28-1.25 |
| Cabbage | Yueyang | *Y*=5.26+0.86x | 0.50 | 0.25-1.03 |

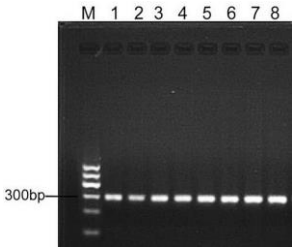


**Fig. 4:** Identification of a 280bp gene fragment containing the R81T mutation in aphids samples. M: Marker I (DNA ladder); Lanes1-8: target bands of aphids collected from hyacinth bean and cowpea plants in Yueyang, cabbage plants in Changsha, cabbage plants in Yueyang, rapeseed and radish plants in Changsha, and oleander and tobacco plants in Changsha, respectively

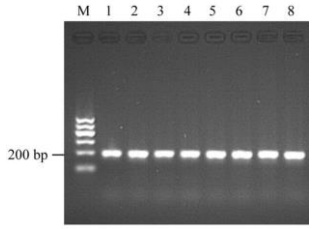


**Fig. 5:** Identification of a 200bp nested PCR product containing the R81T mutation in aphids collected from seven types of host plants in two regions of Hunan Province. M: Marker I (DNA ladder); Lanes1-8: target bands of aphids collected from hyacinth bean and cowpea plants in Yueyang, cabbage plants in Changsha, cabbage plants in Yueyang, rapeseed and radish plants in Changsha, and oleander and tobacco plants in Changsha, respectively

using the four restriction enzymes, *Dra*I, *Hinf*I, *Taq*I, and *Ssp*I, we found that the aphids from oleander plants in Changsha were identical to those in the study of Valenzuela *et al.* (2007), as both aphid populations belong to the same species, *A. nerii*. Furthermore, the nucleotide sequences obtained for these aphids were also identical to the reference sequence for *A. nerii* in the NCBI database (KU236024.1). However, aphids from hyacinth bean, cowpea, and cabbage plants in Yueyang were identified as *A. craccivora*, while aphids from cabbage, rapeseed, radish, and tobacco plants in Changsha were identified as *Myzus persicae*. The differences between our study may be attributed to the directional nature of natural selection during aphid evolution (Feng *et al.* 2004) as well as the fact that aphid mutations are induced by different climates, geographical environments, and hosts (Liu *et al.* 2009).

Biochemical detection methods are the most prevalent

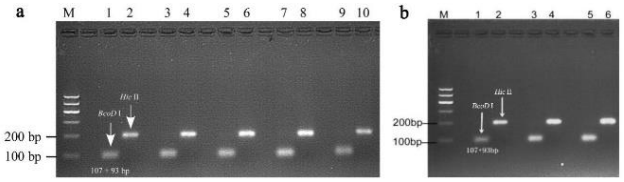


**Fig. 6:** RFLP patterns following BcoDI and HincII digestion of R81T 200 bp target fragments from the aphid samples collected from host plants in Yueyang and Changsha. M: DNA ladder; (a) Lanes1-10: cabbage, rapeseed, radish, oleander and tobacco plants in Changsha, respectively. (b) Lanes1-6: hyacinth bean, cabbage and cowpea plants in Yueyang

approaches for resistance detection in aphids, including the residual film method and the leaf dipping method (Pan *et al.* 2000; Yu *et al.* 2016). We used the leaf dipping method to conduct toxicity tests on the aphids collected in this study, and found that all tested aphids were sensitive to acetamiprid, with $LC_{50}$ values between 0.50–2.05 mg/L (Table 3). However, biochemical detection methods are tedious, require a large number of samples, and involve a number of uncontrollable factors (Wang and Xia 2004).

Assessment of the knockdown resistance (kdr) and R81T mutation status of individual *M. persicae* was performed and described in detail by Panini *et al.* (2014). Also based on the results of the PCR product (200 bp fragment of the acetylcholine receptor gene containing the R81T mutation) from wild-type genotypes (SS) after digestion by *Bco*DI, Voudouris *et al.* (2016) demonstrated that this technique was effective in determining the presence of strong neonicotinoid resistance in aphids. If an aphid has developed resistance to neonicotinoids, *Hinc*II will be able to enzymatically cut the 200 bp R81T-containing gene fragment into 98 and 102 bp fragments, while *Bco*DI would not enzymatically cut the target fragment. In contrast, if an aphid has not developed resistance to neonicotinoids, *Bco*DI will be able to enzymatically cut the 200 bp R81T gene fragment into two fragments, 95 and 105 bp in size, while *Hinc*II would not cleave the PCR product because it lacks *Hinc*II-sensitive restriction sites. Using PCR-RFLP analysis on the 200 bp R81T gene products, we found that the products (do not contain a *Hinc*II restriction site) of all aphids were only digested by *Bco*DI into 93 and 107 bp fragments, but not by *Hinc*II, which may indicate a lack of neonicotinoid resistance, though the products digested by *Bco*DI differed from 95 and 105 bp in size.

In addition, the results of toxicity tests indicate that all

aphids in this study have not developed a strong resistance to the neonicotinoid insecticide acetamiprid. Compared to that found in other countries, the application of neonicotinoids in Hunan Province occurred considerably later.

## Conclusion

Based on these factors as well as our findings in this study, it may be deduced that the aphids of Hunan Province have yet to develop strong levels of resistance to acetamiprid.

## Acknowledgments

Thanks to all members of the Pest Insecticide and Control Group for their helpful discussion and assistance in the experiments. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## References

Ahmad M, MI Arif, I Denholm (2003). High resistance of field populations of the cotton aphid *Aphis gossypii* Glover (Homoptera: Aphididae) to pyrethroid insecticides in Pakistan. *J Econ Entomol* 96:875–878

Bass C, AM Puinean, M Andrews, P Cutler, M Daniels, J Elias, VL Paul, AJ Crossthwaite, I Denholm, LM Field, SP Foster, R Lind, MS Williamson, R Slater (2011). Mutation of a nicotinic acetylcholine receptor $\beta$ subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. *BMC Neurosci* 12; Article 51

Black WC, NM DuTeau, GJ Puterka, JR Nechols, JM Pettorini (1992). Use of the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids (Homoptera: Aphididae). *Bull Entomol Res* 2:151–159

Bogdanowicz SM, WE Wallner, J Bell, TM Odell, RG Harrison (1993). Asian gypsy moths (Lepidoptera: Lymantriidae) in North America: evidence from molecular data. *Ann Entomol Soc Amer* 86:710–715

Cenis JL, P Perez, A Fereres (1993). Identification of aphid (Homoptera: Aphididae) species and clones by random amplified polymorphic DNA. *Ann Entomol Soc Amer* 5:545–550

Cognate AI, SJ Seybold, FAH Sperling (1999). Incomplete barriers to mitochondrial gene flow between pheromone races of the North American pine engraver, Ipspini (Say) (Coleoptera, Scolytidae). *Proc Bio Sci* 266:1843–1850

Faten R, M Mohamed, M Mohamed (2002). Polymerase chain reaction-restriction fragment length polymorphism of ribosomal internal transcribed spacer region analysis on polyacrylamide gel electrophoresis reveals two haplotypes coexisting in *Myzus persicae*. *Electrophoresis* 2:186–188

Feng L, XL Huang, GX Qiao (2004). A study on diversity of aphids in Fujian province, China. *Acta Zootaxon Sinica* 4:666–674

Foster SP, G Devine, AL Devonshire (2007). Insecticide resistance. *In: Aphids as Crop Pests*, pp: 261–285. Emden H, R Harrington (Eds). CAB International, Wallingford, UK

Gubran EME, R Delorme, D Augé, JP Moreau (1992). Insecticide resistance in cotton aphid *Aphis gossypii* (Glov.) in the Sudan Gezira. *Pestic Sci* 35:101–107

Harlow CD, EP Lampert (1990). Resistance mechanisms in two color forms of the tobacco aphid (Homoptera: Aphididae). *J Econ Entomol* 83:2130–2135

Hollingsworth RG, BE Tabashnik, DE Ullman, MW Johnson, R Messing (1994). Resistance of *Aphis gossypii* (Homoptera: Aphididae) to insecticides in Hawaii: spatial patterns and relation to insecticide use. *J Econ Entomol* 87:293–300

Huang XL, GX Qiao (2006). Research status and trends in Aphidology. *Acta Entomol Sin* 6:1017–1026

Karunker I, J Benting, B Lueke, T Ponge, R Nauen, E Roditakis, J Vontas, K Gorman, I Denholm, S Morin (2008). Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochem Mol Biol* 38:634–644

Liu Z, XL Huang, LY Jiang, GX Qiao (2009). The species diversity and geographical distribution of aphids in china (Hemiptera, Aphidoidea). *Acta Zootaxon Sin* 2:277–291

Lu L, H Gu (1995). The peculiarity of rapdand its application to insect taxonomy. *Acta Entomol Sin* 1:117–122

Nauen R, I Denholm (2005). Resistance of insect pests to neonicotinoid insecticides: Current status and future prospects. *Arch Insect Biochem Physiol* 58:200–215

Pan WL, ZH Dang, ZL Gao, HM Jia, KJ Zhang (2000). Studies on resistance of 3 species of aphids to imidacloprid. *Chin J Pestic Sci* 4:85–87

Panini M, D Dradi, G Marani, A Butturini, E Mazzoni (2014). Detecting the presence of target-site resistance to neonicotinoids and pyrethroids in Italian populations of *Myzus persicae*. *Pest Manage Sci* 70:931–938

Puinean AM, J Elias, R Slater, A Warren, LM Field, MS Williamson, C Bass (2013). Development of a high-throughput real-time PCR assay for the detection of the R81T mutation in the nicotinic acetylcholine receptor of neonicotinoid-resistant *Myzus persicae*. *Pest Manag Sci* 69:195–199

Qiao GX, GX Zhang, F Zhao (1999). Study on Di Prociphilus gen. nov. from china, with the description of a new species (HOMOPTERA: PEMPHIGIDAE). *Acta Zootaxon Sin* 4:393–396

Raboudi F, M Mezghani, H Makni, M Marrakchi, JD Rouault, M Makni (2005). Aphid species identification using cuticular hydrocarbons and cytochrome b gene sequences. *J Appl Entomol* 2:75–80

Schroeder H, H Klotzbach, S Elias, C Augustin, K Püschel (2003). Use of PCR–RFLP for differentiation of calliphorid larvae (Diptera, Calliphoridae) on human corpses. *Forensic Sci Intl* 132:76–81

Schroeder H, F Scholz (2005). Identification of PCR-RFLP haplotypes for assessing genetic variation in the green oak leaf roller *Tortrix viridana* L. (Lepidoptera, Tortricidae). *Silvae Genet* 54:17–23

Shew HD, GB Lucas (1991). *Compendium of Tobacco Diseases.* American Phytopathological Society, St. Paul, Minnesota, USA

Valenzuela I, AA Hoffmann, MB Malipatil, PM Ridland, AR Weeks (2007). Identification of aphid species (Hemiptera: Aphididae: Aphidinae) using a rapid polymerase chain reaction restriction fragment length polymorphism method based on the cytochrome oxidase subunit I gene. *Aust J Entomol* 4:305–312

Voudouris CC, AN Kati, E Sadikoglou, M Williamson, PJ Skouras, O Dimotsiou, S Georgiou, B Fenton, G Skavdis, JT Margaritopoulos (2016). Insecticide resistance status of *Myzus persicae* in Greece: long term surveys and new diagnostics for resistance mechanisms. *Pest Manage Sci* 72:671–683

Wang QL, JH Xia (2004). Advances in insecticide resistance of insects and its management. *Plant Prot* 6:15–18

Wang KY, QL Guo, XM Xia, HY Wang, TX Liu (2007a). Resistance of *Aphis gossypii* (Homoptera: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China. *J Pestic Sci* 32:372–378

Wang YM, ZR Shen, LW Gao (2007b). Microsatellite markers and their application in aphid population biology. *Acta Entomol Sin* 6:621–627

Wei XT, HJ Xiao, H Bai, JX Zhang, Y Li, YC Wang, FS Xue (2010). Identification and genetic diversity of four geographic populations of *Colaphellus bowringi Baly* (Coleoptera: Chrysomelidae) in China by PCR-RFLP. *Acta Entomol Sin* 2:209–215

Yu XQ, S Zhang, SE Song, QR Zhu, CF Xie, J Ji, XW Gao (2016). Resistance of wheat aphids to six insecticides and assessment of their field efficacy. *Acta Entomol Sin* 11:1206–1212

Zhang SF, XW Yang, JS Ma (2000). A study on genetic distance among different taxa of aphids (Homoptera: Aphidoidea). *Insect Sci* 3:235–242

Zhao C, XF Wang, D Chen, XW Wang, D Xue, GW Ren (2013). Morphological variations of *Myzus persicae* (Hemiptera: Aphididae) from different geographical populations in China. *Acta Entomol Sin* 12:1452–1463