Symbiont Diversity in Imidacloprid-Resistant and Imidacloprid-Susceptible Populations of *Nilaparvata lugens* (Hemiptera: Delphacidae)[1]

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Abstract Imidacloprid, a neonicotinoid insecticide, is efficacious against hemipterans, including the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae). However, frequent use of this insecticide has resulted in the development of high levels of resistance among brown planthopper populations. Endosymbionts of insects have contributed to host physiology and evolution and play a role in resistance to chemical toxins. In this study, polymerase chain reaction–denaturing gradient gel electrophoresis was used to analyze the bacterial and yeast-like symbiont communities of imidacloprid-resistant and -susceptible brown planthopper populations. The Shannon-Weaver diversity index and the evenness index indicated no differences in the richness and the expression of overall species distribution of the symbiotic communities of resistant and susceptible populations. The similarity coefficients of 0.53 and 0.56 for bacterial and yeast-like symbionts, respectively, indicated the main types of differences among microorganisms in resistant and susceptible populations. Sequence comparison analysis indicated the bacterial species in the susceptible population were members of the Enterobacteriaceae and Moraxellaceae, and those in the resistant population were members of the Enterobacteriaceae, Oxalobacteriaceae, Rhodobacteriaceae, and Sphingomonadaceae. Differences also were found in the composition of yeast-like symbionts of the two populations; *Cryptococcus luteolus, Pseudozyma aphidis,* and *Pseudozyma antarctica* were detected in the susceptible population, and *Cladosporium perangustum* was detected in the resistant population.

Key Words imidacloprid, *Nilaparvata lugens*, PCR-DGGE, insecticide resistance, neonicotinoids

The brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is an economically important pest that damages Asian rice, *Oryza sativa* L., crops by direct injury and by transmitting rice virus disease. The brown planthopper is distributed over approximately half of the rice-growing area of China and causes annual losses of approximately 1–1.5 million tons of rice (Lou and Cheng 2011).

Effective control of the brown planthopper has been seriously compromised in recent years by the widespread appearance of resistance to imidacloprid, a neonicotinoid insecticide that has been used as the primary control of brown planthopper infestations in eastern and southeastern Asia since the mid-1990s. Imidacloprid resistance was first reported in Thailand but has since been reported in other countries, including

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China (Lou and Cheng 2011, Matsumura et al. 2008, Wang et al. 2008). The detoxification mechanisms of insects in response to neonicotinoid insecticides mainly include the up-regulation of P450 detoxification enzyme activity and the enhancement of transport metabolism ability.

Microbial symbionts benefit their insect hosts (Gibson and Hunter 2010) by impacting nutrition (Douglas 2009, Hosokawa et al. 2010), reproduction (Wilkinson et al. 2001), virulence regulation (Lu et al. 2004), and detoxification (Dowd and Shen 1990, Xu et al. 2009), with detoxification indicating possible links between insect gut symbionts and resistance to chemical toxins. Cheng et al. (2017) found that the gut symbiont *Citrobacter* sp. plays a key role in the degradation of trichlorfon. DNA sequencing revealed that members of the Lactobacillales or other scarcer taxa play a role in conferring insecticide resistance to *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Xia et al. 2013). The susceptibility of *Myzus persicae* Sulzer (Hemiptera: Aphididae) to nine insecticides was determined after treatment with ampicillin; the susceptibility of *M. persicae* to imidacloprid, cyantraniliprole, and clothianidin decreased significantly (3.4 times, 2.2 times, and 2.0 times, respectively) (Yang et al. 2020). Kikuchi et al. (2012) found that fenitrothion-degrading *Burkholderia* strains established a symbiosis with the hemipteran *Riptortus pedestris* (F.), conferring resistance to fenitrothion.

Bacterial and yeast-like symbiotes are important symbionts of the brown planthopper that provide their hosts with cholesterol, vitamins, and essential amino acids that the host cannot produce (Sasaki et al. 1996). Exposure of neonatal planthoppers to high temperature, antibiotics, lysozyme via injections, and insecticides reduced yeast-like symbiont abundance and thus influenced host growth and development (Raguraman et al. 1988, Shankar and Baskaran 1988). These symbionts also play crucial roles in changes in the virulence of brown planthopper populations for resistant rice varieties (Lu et al. 2004) because the rate of change in endosymbiotic genes is much more rapid than that in host genes (Campbell 1990, Ishikawa et al. 1986). The symbiont-related mechanisms underlying high resistance to imidacloprid in the brown planthopper have received relatively little attention. Analysis of strains revealed a single point mutation (Y151S) in two nAChR subunits that were associated with a dramatic reduction in binding to imidacloprid (Liu et al. 2005). Enhanced detoxification of imidacloprid by carboxyesterase, glutathione-*S*-transferase, and cytochrome P450 monooxygenase appears to be the predominant mechanism of resistance in field-selected populations (Puinean et al. 2010, Wen et al. 2009).

Zhang et al. (2013) used polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) to study the mid-gut bacterial communities of the larvae of the striped rice stem borer (*Chilo suppressalis* (Walker)). Hou et al. (2013) analyzed yeast-like symbiont diversity in *N. lugens* with PCR-DGGE and found several previously detected, undetected, and uncultured fungi. Xu et al. (2014) used DGGE to elucidate the structures of bacterial communities in *N. lugens* from different geographic and resistant virulent populations. In this study, we used PCR-DGGE to assess bacterial and yeast-like symbiont communities in imidacloprid-resistant and imidacloprid-susceptible brown planthopper populations, with the goal of revealing the possible functions of these endosymbionts in the development of imidacloprid resistance.

Table 1. PCR primers used in this study.

Primer type	Designation	Sequence (5′–3′)
Bacteria specific	49f	GAGTTTGATCCTGGCTCAG
	1525r	AGAAAGGAGGTGATCCAGCC
Archaea specific	341f-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCG GGG GCACGGGGGGCCTAGGGGAGGCAG CAG
	534r	ATTACC GCG GCT GCT GG
Yeast specific	GCNL-1	GCATATCAATAAGCGGAGGAAAAG
	NL-4	GGTCCGTGTTTCAAGACGG
	LS2	ATTAAACAACTCGACTC

Materials and Methods

Rice varieties, including TN1 (brown planthopper susceptible), were planted in clay pots (15 cm in diameter) in the greenhouse of the Zhejiang Academy of Agricultural Sciences, Hangzhou, China. The experiments described below were conducted with 45-d-old rice plants.

The susceptible *N. lugens* population, provided by the Zhejiang Research Institute of Chemical Industry, had not been exposed to any insecticide for at least 10 yr before the study. The resistant *N. lugens* population was selected by spraying imidacloprid (at the LC_{50}) for more than 50 generations at the Zhejiang Academy of Agricultural Science. The resistance ratio of the resistant *N. lugens* population was nearly 400 times greater than that of the susceptible population. Female adult *N. lugens* from the imidacloprid-susceptible and imidacloprid-resistant populations were collected and used for tests.

Fifty adult female brown planthoppers from each population were used to collect total DNA. Each planthopper was surface sterilized with 75% ethanol for 1 min. Genomic DNA was extracted with a bacterial DNA kit (Omega Bio-Tek Company Ltd., Guangzhou, China) or a yeast DNA kit (Omega Bio-Tek). DNA purity and concentration were measured with a protein nucleic acid spectrophotometer (DU800, Beckman Coulter Life Sciences, Indianapolis, IN).

All primers used in this study are shown in Table 1 and were synthesized by Shanghai Shenggong Bioengineering Company, Ltd. (Shanghai, China). For analysis of specific bacterial diversity, PCR amplification of the 16S rRNA gene was conducted using bacteria-specific primers 49f and 1525r (Henckel et al. 1999, Muyzer et al. 1993). For general bacterial diversity analysis, PCR amplification of the 16S rRNA gene was performed using the 341f-GC and 534r primers (Nakagawa and Fukui 2003). For analysis of yeast diversity, PCR amplification of the 26S rRNA gene was performed as described previously (Prakitchaiwattana et al. 2004) with initial amplification of the D1/D2 region with eukaryotic universal primers NL-1 and NL-4 (Taylor et al. 2002) followed by nested PCR using primers GCNL-1 and LS2 (Cocolin et al. 2002). DNA from each sample was subjected to DGGE following PCR amplification with each primer set (Table 1). All PCR amplification was conducted in a final volume of

25 μl containing 0.5 μl (50 ng/μl) of template, 1 μl of template DNA, 0.5 μl of primer NL-1 and primer NL-4 (10 μM), 21.5 μl of Platinum PCR Supermix High Fidelity, and 1.5 μl of sterile double-distilled water. Reactions were performed in a PTC-220 DNA Engine Dyad thermocycler (MJ Research, Saint-Bruno-de-Montarville, Quebec, Canada).

DGGE was performed using the D Code Universal Mutation System (Bio-Rad Laboratories, Hercules, CA) for separation of PCR products. PCR products were applied directly onto 8% (w/v) polyacrylamide gels in a running buffer containing 1× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 8.3) and a denaturing gradient of 30–60% urea and formamide (for bacteria and yeast) or 35–55% urea and formamide (for Archaea, where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 80 V for 14 h at a constant temperature of 60°C. After electrophoresis, the gels were stained with SYBR Green I nucleic acid stain (Thermo Fisher Scientific, Waltham, MA) and photographed under ultraviolet transillumination. Sterile blades were used to excise bands from the gels, and each band was mixed with 20 μl of 0.1× TE buffer solution, incubated overnight at 4°C, and used for PCR amplification with the appropriate primer set.

DGGE profiles were analyzed with quantity BIO-1D software (Bio-Rad Laboratories) to determine the position and intensity of each band. The Shannon-Weaver index (H), an expression of the proportional abundance of species in a community, was calculated using the formula:

$$H = -\sum_{i=1}^{s} p_i \ln p_i$$

where p_i is the ratio of the DNA quantity of the ith band to the total DNA quantity of all the bands of the sample and S is the number of DGGE bands in the sample.

The evenness index (E), an expression of overall distribution of species y in an environment, was calculated using the formula:

$$E = \frac{H}{\ln S}$$

where S is the total number of species in the sample.

Sorenson's pairwise similarity coefficient (C_S) is used to compare the presence or absence of species in different populations and was calculated using the formula:

$$C_S = 2j/(a+b)$$

where a and b are the number of bands in the DNA DGGE lanes of two different samples and j is the number of same bands in the two DGGE lanes.

The significance of differences between the two insect populations was analyzed with a one-way analysis of variance with a significance threshold of $P < 0.05$.

Results

The Shannon-Weaver diversity index values for both bacterial and yeast-like symbionts of the resistant population (Table 2) were close to that of those values for the susceptible population, thus suggesting that the richness and diversity of symbiotic microorganisms of the two populations were similar. The evenness index values for

Table 2. Shannon-Weaver index and evenness index values and similarity coefficients for symbiotic microorganisms in imidacloprid-susceptible and imidacloprid-resistant populations of *N. lugens*.

	Bacterial symbionts			Yeast-like symbionts		
Population	Shannon-Wiener	Evenness	Similarity Coefficient	Shannon-Wiener Index	Evenness Index	Similarity Coefficient
Susceptible	2.37	0.87	0.53	2.27	0.90	0.56
Resistant	2.35	0.87		2.23	0.87	

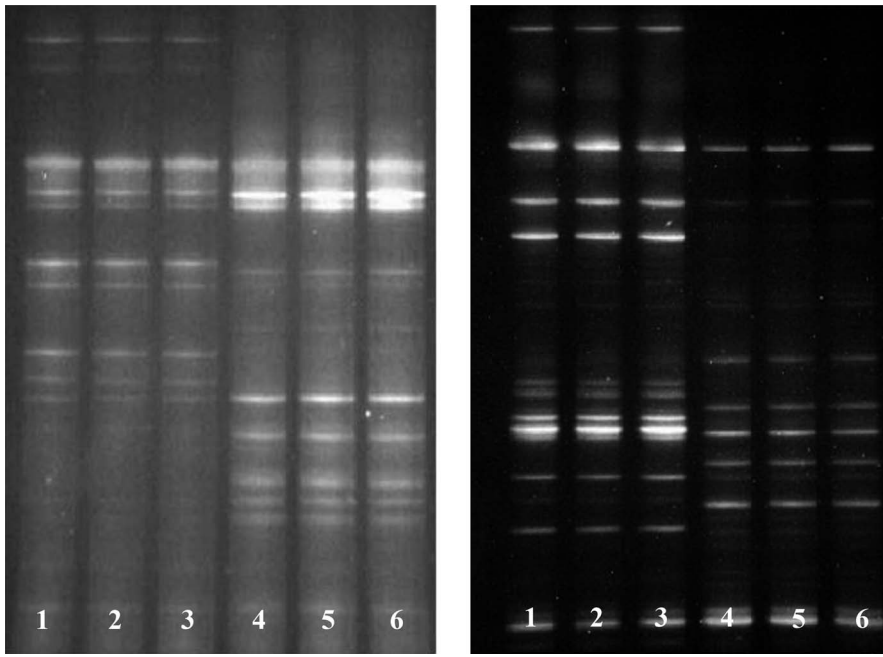

Fig. 1. DGGE profile of bacterial (left) and yeast-like (right) symbionts of *N. lugens.* Lanes 1-3 are from imidacloprid-resistant populations; lanes 4–6 are from imidacloprid-susceptible populations.

both bacterial and yeast-like symbionts of the two populations were all close to 1, meaning the relative abundance of microbial species in imidacloprid-resistant and imidacloprid-susceptible *N. lugens* populations were even (e.g., no difference in the evenness of resistant and susceptible microbial community). The similarity coefficients comparing the imidacloprid-resistant and imidacloprid-susceptible populations were 0.53 and 0.56 for the bacterial and yeast-like symbiont communities, respectively (Table 2), indicating differences in the main types of microorganisms in the two populations. Fifteen bacterial bands and 12 yeast-like bands from gels were selected for partial sequencing (Fig. 1). Sequence comparison analysis was conducted based on sequences in the National Center for Biotechnology Information GenBank database. All selected clones were closely related (≥97% sequence identity) to the species reported in GenBank. The number of bacterial species in the imidacloprid-susceptible and -resistant populations were not significantly different. The bacteria in the susceptible population were members of the Enterobacteriaceae and Moraxellaceae families. The bacteria in the resistant population belonged to more families: Oxalobacteriaceae, Rhodobacteriaceae, Sphingomonadaceae, and Enterobacteriaceae (Table 3). Yeast-like fungi *Cryptococcus luteolus, Pseudozyma aphidis, Pseudozyma antarctica,* Capnodiales species, and *Cladosporium perangustum* were identified for the first time in *N. lugens*. The imidacloprid-susceptible population contained more yeast-like symbiont species than did the imidacloprid-resistant population. *Cryptococcus luteolus, P. aphidis,* and *P. antarctica* were detected in

Table 3. Names and registration numbers of GenBank organisms closest to the bacterial symbionts in imidacloprid-susceptible and imidacloprid-resistant populations of *N. lugens*.

Group	Most Closely Related Hit in GenBank	Identity				GenBank Accession Number
		Resistant Population		Susceptible Population		
		N	%	*N*	%	
Enterobacteriaceae	*Arsenophonus nasoniae*			1	99	JN035221.1
	Enterobacteriaceae bacterial endosymbiont of *N. lugens*			1	99	GU124504.1
	Enterobacter asburiae	1	99	3	98	JN033555.1
	Klebsiella oxytoca			1	99	JN001159.1
	Pantoea stewartii			1	99	JF819695.1
	Proteobacterial symbiont of *N. lugens*	1	98	1	99	FJ774960.1
	Enterobacter sp.	1	98	1	98	JN680698.1
Moraxellaceae	*Acinetobacter berezinae*			1	98	HQ396909.1
Oxalobacteriaceae	*Herbaspirillum* sp.	1	97			FN386764.1
Rhodobacteriaceae	*Amaricoccus* sp.	1	97			JF957136.1
Sphingomonadaceae	Uncultured *Sphingomonas* sp.	1	98			JN710165.1
Unknown	Uncultured bacterium	1	98			GU185657.1
	Uncultured bacterial clone	1	98			EF552043.1

only the susceptible population, *C. perangustum* was detected in only the resistant population, and Capnodiales species and some unknown species existed in both populations (Table 4).

Discussion

Insects harbor symbionts that enhance fitness by contributing to digestion, nutrition, reproduction, and resistance to xenobiotics (Douglas 2011). However, few studies on the contributions of symbionts to insecticide resistance have been performed because of the difficulty of separating symbionts from host insects. The evolution of insecticide resistance is accompanied by a series of physiological changes in the host (Klepzig et al. 2009), which alter the structure and function of the microorganism community (Gimonneau et al. 2014). In this study, the Shannon-Weaver diversity and evenness index values of the imidacloprid-susceptible and -resistant populations were not significantly different. These findings could indicate that the development of insecticide resistance by *N. lugens* is not closely related to the richness and evenness of the symbiotic microorganism population; instead, insecticide resistance seems to be related to the types of symbiotic microorganisms.

Sequencing analysis revealed that common bacteria (*Arsenophonus nasoniae* and *Enterobacter asburiae*) existed in both the imidacloprid-resistant and -susceptible populations, whereas *Herbaspirillum* sp., *Sphingomonas* sp., and *Amaricoccus* sp. were detected in only the resistant population. We speculated that aromatic compound degradation (Bacosa et al. 2010, Baraniecki et al. 2002, Lafortune et al. 2009, Singleton et al. 2008) resulted in these species transitioning from secondary bacterial species to dominant species under imidacloprid exposure, perhaps enhancing imidacloprid resistance. *Herbaspirillum* sp. and *Amaricoccus* sp. also function in nitrogen fixation (Elbeltagy et al. 2001, Valverde et al. 2003) and intracellular storage of synthesized polymers (Falvo et al. 2001, Lemos et al. 2008) and could contribute to raw material storage by synthesizing amino acids and proteins.

In this study, the yeasts *C. luteolus, P. aphidis, P. antarctica,* Capnodiales species, and *C. perangustum* were found in *N. lugens* for the first time. The yeast-like symbiont population of *N. lugens* was a mixture of many types of yeasts, indicating that the microbial species in imidacloprid-susceptible and imidacloprid-resistant populations is variable. Capnodiales species was detected in both *N. lugens* populations and might have been carried into the body when these insects fed on infected rice plants. *Cladosporium perangustum* was detected in the resistant population, whereas the other three yeasts (*C. luteolus, P. aphidis,* and *P. antarctica*) were found in only the susceptible population.

Cladosporium perangustum is abundant in the air, from which this organism can be absorbed by insects (Hsu et al. 2012). The basidiomycetous yeast *C. luteolus* produces polysaccharides (Vorotynskaya et al. 1992), and *P. antarctica* is an excellent source of edible single-cell protein and facilitates utilization of waste glycerol (Morita et al. 2007). *Pseudozyma aphidis* has biocontrol activity and provides a natural barrier against some plant pathogens (Avis and Bélanger 2001, Urquhart and Punja 2002). The manner in which differences in the microorganism distributions in the populations are related to insecticide resistance merits further study.

Table 4. Names and registration numbers of GenBank organisms closest to the yeast-like symbionts in imidacloprid-susceptible and imidacloprid-resistant populations of *N. lugens*.

Group	Most Closely Related Hit in GenBank	Identity				GenBank Accession Number
		Resistant Population		Susceptible Population		
		N	%	*N*	%	
Tremellaceae	*Cryptococcus luteolus*			1	98	FJ743611.1
Ustilaginaceae	*Pseudozyma aphidis*			1	98	HQ647298.1
	Pseudozyma antarctica			1	99	AB566344.1
Capnodiaceae	Capnodiales species	2	98	2	97	HQ207047.1
Davidiellaceae	*Cladosporium perangustum* strain	1	98			JF499855.1
Unknown	Yeast-like symbiont of *N. lugens*	3	99	2	97	AF267236.1
	Fungal sp.	3	99	1	99	HM123598.1

The results reported here highlight the significant differences in the microbial symbiont communities of imidacloprid-susceptible and imidacloprid-resistant brown planthopper populations. More evidence is required to assess whether changes in microbial community structure are related to insecticide resistance.

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