Identification of Two Species of Yeast-like Symbiotes in the Brown Planthopper, *Nilaparvata lugens*

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Abstract To determine the species of the yeast-like symbionts (YLS) in the brown planthoppers (BPH), *Nilaparvata lugens*, YLS were first isolated and purified by ultracentrifugation from the fat bodies of BPH, and then 18S rDNA and internal transcribed spacer (ITS)–5.8S rDNA sequences of YLS were amplified with the different general primers for fungi. The results showed that the two different 18S and ITS–5.8S rDNA sequences of YLS were obtained. One 2291-bp DNA sequence, which contained 18S and ITS–5.8S rDNA, showed the high similarity to *Cryptococcus* and was named *Cryp*-Like symbiotes. Another 1248-bp DNA sequence, which contained a part of 18S and ITS–5.8S rDNA, showed the high similarity to *Pichia guilliermondii* and was named *Pichia*-Like symbiotes. It was further proved that *Cryp*- and *Pichia*-Like symbiotes existed in BPH through nested PCR with specific primers for two symbiotes and in situ hybridization analysis using digoxigenin-labeled probes. Our results showed that BPH harbored more than one species of eukaryotic YLS, which suggested that diversity of fungal endosymbiotes may be occurred in planthoppers, just like bacterial endosymbiotes.

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Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is a serious insect pest in paddy fields in the East and South China. BPH harbors yeast-like symbiotes (YLS), especially in mycetocytes formed by fat body cells of abdomen [6]. YLS are intimately associated with every developmental stage of its host, and are vertically transmitted to the next generation by the transovarilal infection and proliferated by asexual budding [8]. YLS supplied its host with proteins for the normal embryonic and postembryonic development [15] and played an important physiological role in uric acid metabolism [11]. YLS are dominant endosymbionts in BPH, though it was reported there existed several bacterial symbionts [23]. The two morphologically different YLS were detected in egg and fat bodies from the abdomen of nymph and adult of BPH [17]. Seven morphologically different YLS were also isolated and cultured from the eggs of BPH, and further determined their taxonomic affiliation by PCR amplifications of 18S rDNA and restriction endonucleases [13]. In our previous studies [7], four morphologically different YLS were also found in adult BPH female adults. Therefore, it was proposed that there existed more than one species of YLS in BPH on the base of morphological traits. However, only one species of YLS in BPH was identified through 18S rDNA sequencing and phylogenetic analysis, which was supposed to be derived from within the filamentous ascomycetes (Euascomycetes) [19, 22].

In recent years, the application of molecular techniques to elucidate classical issues on the taxonomy and diversity

of populations of yeast species are becoming an important tool for rapid yeast identification based on similarity or dissimilarity of DNA [10, 25]. These include the technique of conserved DNA sequencing, such as ribosomal RNA (rRNA) genes (rDNA), ITS (internal transcribed spacer) regions (non-coding and variable), and the 5.8S rRNA gene (coding and conserved). The complex ITS–5.8S rDNA are useful in measuring close fungus genealogical relationships since they exhibit far greater interspecific differences than the 18S and 28S rRNA genes [3, 5]. Because ribosomal regions evolve in a concerted fashion, they have been proved very useful for the classification of *Saccharomyces* [25], *Kluyveromyces* species [2] and for the identification of yeast species [10].

In this study, to determine the species of the YLS in BPH, YLS were first isolated and purified from the fat bodies of BPH, and then 18S rDNA and 5.8S rDNA–ITS sequences of YLS were amplified with the general primers for fungi. Based on 18S rDNA and 5.8S rDNA–ITS sequence, two species of YLS were presumed, which were then identified by nested PCR and in situ hybridization (ISH).

Materials and Methods

Insects and YLS

The field BPH populations were collected from rice fields in Hangzhou, Zhejiang, China, and maintained in an incubator at $26 \pm 1°C$, humidity 70–80% and 16 h light/ 8 h dark photoperiod on TN1 plants. The YLS were isolated from adult insects according to the method described by Sasaki et al. [21]. The insects were sterilized by immersion in 75% ethanol for 3 min, and fat bodies were collected from surface sterilized BPH and homogenized in 0.02 M phosphate-buffered saline (PBS) at pH 7.4. Percoll (Pharmacia, Sweden) was added to the homogenate to give a final concentration of 30%, and then centrifuged at $2000g$ for 10 min. The pellet was suspended in PBS containing 250 mM sucrose and 75% Percoll, and centrifuged at $100,000g$ for 20 min. The YLS were collected from the 65–85% region of Percoll gradient, and the purity of YLS was detected on the microscope and number of YLS was calculated according to the formula stimulated by Chen et al. [6].

DNA Extraction

Yeast genomic DNA were extracted with a Yeast DNA mini kit (Tiangen, Beijing, China), which is based on enzymic lysis of yeast cells using recombinant Lyticase (Sigma, St. Louis, MO, USA), followed by spin-column purification. The DNA concentrations were measured by absorbance at 260 nm, and the purities were measured by calculating the ratio of absorbance at 260–280 nm with a spectrophotometer DU-600 (Beckman, Fullerton, CA).

PCR Amplification

To amplify different species of YLS in BPH, different primer sets were used (Table 1). The reaction system (25 μl) was composed of 10× buffer 2.5 μl, dNTP 0.8 μM, $MgCl_2$ 1.5 mM, primers 0.5 μM each, Taq polymerase 1.25 U, template DNA. PCR was performed with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were recovered, ligated into pGEM-T easy vector, and transformed into competent cells. The inserted sequences were sequenced and aligned by using a BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). Sequences used in the phylogenetic study included ITS sequences of *Brettanomyces bruxellensis* (AF043508), *Candida tropicalis* (AB534606), *Cryptococcus sp_* HLS105 (FJ770078), *Debaryomyces hansenii* (HM368665), *Hanseniaspora valbyensis* (AY046197),

Table 1 Primers and probes used in the PCR and in situ hybridization

Procedure and target organism	Sequence (5′ → 3′)
Fungal universal primers[a]	
NS1	GTAGTCATATGCTTGTCTC
NS5	AACTTAAAGGAATTGACGGAAG
NS8	TCCGCAGGTTCACCTACGGA
ITS4	TCCTCCGCTTATTGATATGC
Nest PCR	
Pichia-Like 1 forward	CAAGGTTTCCGTAGGTGA
Pichia-Like 1 reverse	CAGAAATATCCCGCCACA
Pichia-Like 2 forward	TTGGTGGAGTGATTTGTC
Pichia-Like 2 reverse	GAGATCCGTTGTTGAAAG
Cryp-Like 1 forward	TTTCACAAGATTACCCAGTCC
Cryp-Like 1 reverse	GAAAGCATTTGCCAAGGATG
Cryp-Like 2 forward	CGTTTCGCTGCGTTCTTCAT
Cryp-Like 2 reverse	TGGTTGGTGGCTGGCTTCTT
In situ hybridization	
Ascomycetes[b]	GCCCCTCCCTCTGTGGAACCCC
Cryp-Like	TCCCTCTAAGAAGCCAGCCACC
Pichia-Like	CACAATTTAATTATTTTTACAGT

[a] Position refers to the specific locations of the primers in the YLS 18S sequences reported by Noda et al. [17]

[b] Sequences adapted from White et al. [23]

Kluyveromyces marxianus (AY046214), *Lipomyces starkeyi* (U82459), *Pichia guilliermondii* isolate CNRMA 200600032 (EU568967), *Rhodotorula glutinis* (HM190198), *Saccharomyces cerevisiae* (FJ231430), *Saccharomycodes ludwigii* (AY046204), and *Schizosaccharomyces pombe* (AB054041). Sequence comparisons were performed using Clustal W program [24], and a molecular phylogenetic tree (neighbor-joining) was constructed with 1000 bootstrap replicates using the MEGA version 3.1 [14].

Nested PCR

A first amplification step used the yeast universal primer pair NS1/NS8 for *Pichia*-Like symbiotes and NS1/ITS4 for *Cryp*-Like symbiotes. Diluted amplicons of the first PCR steps were used as template for a second amplification with the specific primers for *Pichia*- and *Cryp*-Like symbiotes as listed in Table 1. The reaction was performed under the conditions described above, except for the annealing temperatures, which were specific for each pair of inner primers. Amplicons were analyzed by agarose gel electrophoresis (1%), ethidium bromide staining, and UV light.

In situ Hybridization

The oligonucleotide probes specific for *Ascomycetes*, *Pichia*- and *Cryp*-Like symbiotes were designed from variable regions of the 18S rDNA or ITS genes (Table 1). All probes were 3′ tailed with digoxigenin-dUTP and then diluted to a final concentration of 2.0 ng/μl in hybridization buffer. The female abdomens of adult BPH were sectioned on a freezing microtome at a thickness of 4 μm and thaw-mounted onto polylysine-coated glass slides. Then, the slides were baked at 37°C for 2 h. Sections were rinsed in 0.1 mM PBS at pH 7.4, incubated with 4% paraformaldehyde, and then treated with 0.3% Triton X-100. After three washings of PBS, sections were treated with 1% H_2O_2/methanol, treated with 0.2 M HCl for 20 min, and digested with 1 μg/ml proteinase K (Sigma, St. Louis, MO, USA), followed by acetylation for 5 min with freshly prepared 0.6% acetic anhydride in 0.1 M triethanolamine (pH 8.0), and then rinsed twice in 2× standard saline citrate (SSC). They were then incubated with a hybridization solution containing 2 ng/μl digoxigenin-labeled DNA probes for each YLS, or no probe for negative control. Slides were coverslipped, heat treated at 95°C for 5 min, cooled on ice for 1 min, and hybridized in a humid environment for at least 3 h at 42°C. After posthybridization washing, digoxigenin-labeled probes were detected according to the manufacturer's instructions (DIG detection kit; Boster, Wuhan, China). The stained hybridization slide was dried and mounted with a coverglass for observation by light microscopy.

Results

18S rDNA and ITS–5.8S rDNA Sequences of Two YLS Species

The PCR products amplified by the primer sets NSl and NS8, NS5 and ITS4, and NS1 and ITS4, were sequenced. The nucleotide sequences amplified with the primers NS1 and NS8 were the same with the 18S rDNA sequence reported by Noda et al. [19], which was named *Ascomycetes* symbiotes. With the primers NS5 and ITS4, a 1248-bp DNA sequence (containing a part of 18S rDNA and

Fig. 1 Phylogenetic relationships of two species of YLS in *Nilaparvata lugens* to 13 selected species of yeasts. The phylogenetic tree (neighbor-joining) was constructed using the MEGA version 3.1 on the ITS sequences. Numbers in the *nodes* correspond to bootstrap values in 1000 replicates
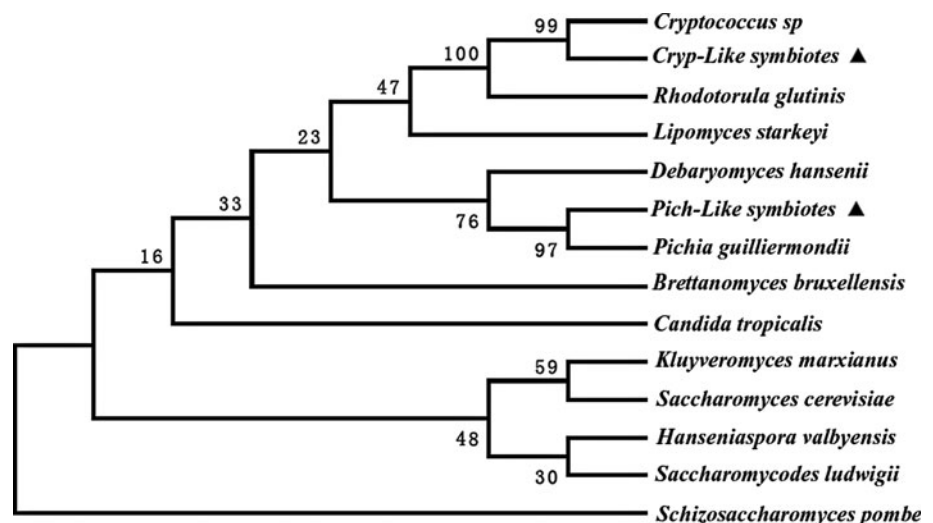
ITS–5.8S rDNA) were amplified, which showed the high similarity (about 99.6%) to *Pichia guilliermondii* (EU568967) based on a NCBI blast. A phylogenetic analysis of ITS sequences also showed that the amplified sequence belonged to *Pichia* (Fig. 1). These results suggested that there existed one species of *Pichia*-like symbiotes in *N. lugens*, which was named *Pichia*-Like symbiotes.

A 2291-bp DNA sequence (containing 18S rDNA and ITS–5.8S rDNA) was amplified by the primers NS1 and ITS4. The initial 1781 bp, containing 18S rDNA sequence, showed the high similarity (99.5%) to *Cryptococcus peneaus* (GenBank No: AB085799), and only 9 bp were different. The posterior part of sequence (510 bp), containing 5.8S–ITS sequence showed the high similarity (94.1%) to *Cryptococcus sp.* HLS105 (GenBank No: FJ770078). A phylogenetic analysis of ITS sequences also showed that the amplified sequence belonged to *Cryptococcus* (Fig. 1). These results suggested that there existed another species of *Cryptococcus*-like symbiotes in *N. lugens*, which was named *Cryp*-Like symbiotes.

Identification of *Pichia*-Like and *Cryp*-Like Symbiotes by Nested PCR

For identification of *Pichia*-Like and *Cryp*-like symbiotes in *N. lugens*, the nested PCR was developed according to specific primers for *Pichia*-Like and *Cryp*-Like symbiotes, respectively. For *Pichia*-like symbiotes, a fragment of about 1200 bp was first amplified with general primers, and then two amplicons with 500 and 700 bp were produced in the second round PCR with the specific primers (Fig. 2). For *Cryp*-Like symbiotes, a fragment of about 1800 bp was first amplified with general primers, and then two amplicons with 600 and 700 bp were produced in the second round PCR with the specific primers (Fig. 2). The results of nested PCR suggested that BPH harbored *Pichia*-Like and *Cryp*-like symbiotes.
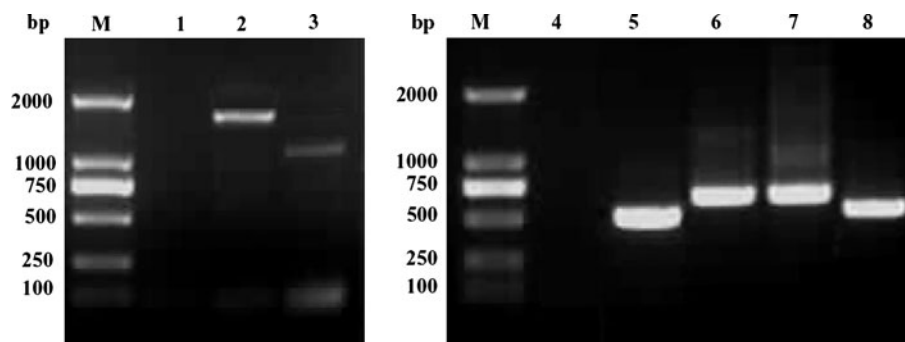
In Situ Hybridization Detection of *Ascomycetes*, *Pichia*-Like and *Cryp*-Like Symbiotes

In situ hybridization detection, conducted using the specific probes for *Ascomycetes*, *Pichia*-Like and *Cryp*-Like symbiotes, revealed clear and distinct staining of some YLS cells in the sections of fat bodies of *N. lugens*, respectively (Fig. 3b, c, d). Moreover, the negative control without probes showed no specific staining of YLS cells (Fig. 3a). These results further proved that there existed *Pichia*-Like and *Cryp*-Like symbiotes in *N. lugens*.

Discussion

The diversity of endosymbiotes or multiple endosymbiosis had been well studied in several host insects [12, 16]. Such as in aphids, almost all aphids possessed a primary bacteria endosymbiont, *Buchnera*, which compensates for dietary deficiencies; many also contain secondary symbionts, such as *Hamiltonella defensa* [4]. In some aphids, several species of "*Candidatus* Serratia symbiotica" was also detected [4, 16]. In the leafhopper *Scaphoideus titanus*, bacteria *Cardinium* symbionts and YLS were detected in the ovary and fat bodies, which were transovarially transmitted to the offspring [20]. In the planthopper *N. lugens*, the prokaryotic and eukaryotic symbiotes were also detected [18, 23]. As far as bacterial symbiotes, a total of 18 operational taxonomic units (OTUs) representing four phyla were identified by sequencing and analyzing 16S rRNA gene libraries in *N. lugens* [23]. However, only one species of YLS were identified through 18S rDNA sequencing [18], which belonged to class Pyrenomycetes in the subphylum Ascomycotina, although several different morphology of YLS were detected in BPH eggs and fat bodies [13, 17]. In this study, we identified another two species of YLS (*Pichia*-Like and *Cryp*-Like symbiotes) in *N. lugens* by nested PCR and in situ hybridization. One species of *Pichia*-Like symbiotes was also identified in the fat bodies

Fig. 2 The amplification products of nested PCR for *Pichia*-Like and *Cryp*-Like symbiotes 18S rDNA in *Nilaparvata lugens*. *Lane M* DL 2000 Marker; *lanes 1* and *4* negative controls of distilled water; *lane 2*

first round PCR for *Cryp*-Like symbiotes; *lane 3* first round PCR for *Pichia*-Like symbiotes; *lanes 5* and *6*, nested PCR for *Pichia*-Like symbiotes; *lanes 7* and *8*, nested PCR for *Cryp*-Like symbiotes

Fig. 3 In situ hybridization detection of *Ascomycetes*, *Pichia*-Like, and *Cryp*-Like symbiotes in fat bodies of *Nilaparvata lugens*. **a** Negative control; **b** probe specific for *Ascomycetes* symbiotes; **c** probe specific for *Pichia*-Like symbiotes; **d** probe specific for *Cryp*-Like symbiotes. *Arrows* indicate the specific stained yeast-like symbiotes. The *bars* represent 0.03 mm
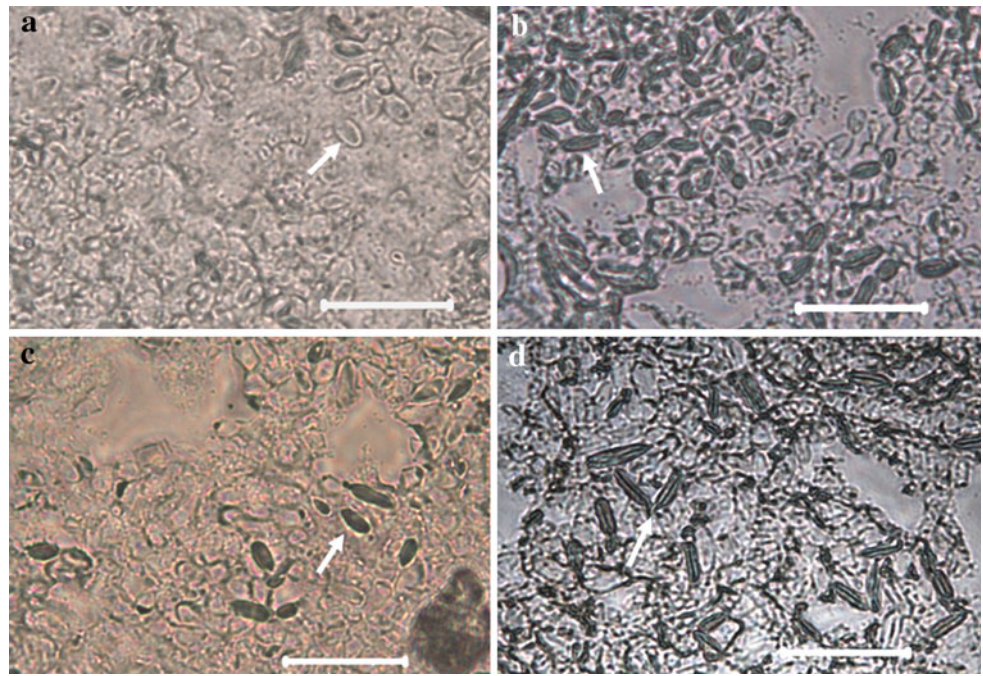
and eggs in the small brown planthopper, *Laodelphax striatellus* [1]. Therefore, we concluded that planthoppers harbored more than one species of eukaryotic YLS, which suggested that diversity of fungal endosymbiotes may be occurred in planthoppers, just like bacterial endosymbiotes. However, the origin of these three fungal endosymbionts and their presence in one *N. lugens* needs to be further studied.

The PCR amplification of 18S rRNA gene had been selected for identification of eukaryotic endosymbiotes in several insects, including rice planthoppers [19], the aphid *Hamiltonaphis styraci* [9] and the three anobiid beetles [18]. However, the ITS regions, which are much less evolutionarily conserved than the rRNA coding genes [4], appear to be more useful in detecting genetic variability among species, which is valuable for taxonomic purposes and also for species identification. In this case, we selected ITS and 5.8S rRNA gene to differentiate among the YLS characteristic for each species in *N. lugens*. When amplified with universal primers for 18S rDNA, the nucleotide sequences of fragments was the same with the 18S rDNA sequence reported by Noda et al. [19]. However, *Pichia*-Like and *Cryp*-Like symbiotes were amplified when used with the former 18S rDNA primers and latter ITS universal primers. We speculated that the reason *Ascomycetes* symbiotes can be only amplified when used with general primers for 18S rDNA was because *Ascomycetes* symbiotes may be the dominant YLS.

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