Journal on New Biological Reports
JNBR 9(2) 254 – 258 (2020)

ISSN 2319 – 1104 (Online)

Published by
www.researchtrend.net

Can host plant shape fungal symbionts of the leafhopper *Orosius albicinctus*

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| **Received**: 25 May 2020 | **Accepted**: 16 July 2020 |

How to cite: Hemmati C, Nikooei M. 2020. Can host plant shape fungal symbionts of the leafhopper *Orosius albicinctus*. J New Biol Rep 9(2): 254 – 258.

ABSTRACT

Yeasts associate with numerous insects, and they can assist the metabolic processes within their hosts. Although the effect of host plant on bacterial endosymbionts communities was investigated for many insects, no research focused on this effect on insect fungal endosymbionts. To investigate this effect on the fungal endosymbionts communities of *Orosius albicinctus*, a major vector of phytoplasma disease, we analyzed the fungal communities associated with this vector fed on *Suaeda aegyptiacae* and *Citrus aurantifolia*. Insects were collected by a D-Vac and stored at -20 ºC up to the DNA extraction. Total DNA was extracted and PCR was conducted with specific primer sets targeting 18S rRNA and 28S rRNA of the symbionts. Results revealed that this vector fed on *S. aegyptiacae* harbored two yeast symbionts namely Yeast-like Symbiont of *Orosius albicinctus* and *Metschnikowia comanche* with a similarity of (98-99%) to those reported from the other Cicadellids. While, the leafhopper feeding on Mexican lime harbored only yeast-like symbiont of *Orosius albicinctus*. These results revealed the first evidence of fungal endosymbionts of this species and demonstrated that host plant can shape the endosymbionts community.

Key words: Fungal endosymbionts, Host plants, Yeast-Like symbionts, phytoplasma vector.

INTRODUCTION

Over the last century, the discovery of microbial endosymbionts in a wide variety of arthropods has been a significant finding in arthropod biology. For example, the recognition that bacterial endosymbionts were widespread among arthropods and may play important roles like providing essential amino acids, resistance to parasitoids and pathogens and manipulation in sex ratio (McCutcheon & Moran 2010). In contrast, although a number of fungal endosymbionts of insects were previously reported, relatively few were substantiated (Vega & Dowd 2005). However, obligate fungal gut endosymbionts are known in planthoppers, leafhoppers and aphids (Homoptera) and three families of beetles. The fungal endosymbionts all appear to play important roles in insect nutrition, broadening the range of available resources by supplying enzymes for degradation or detoxification of plant material (Stefanini 2018).

Leafhoppers and planthoppers are known to harbor endosymbiotic bacteria and yeasts (Noda et al. 2001; Houghes et al. 2011). *Hishimonus phycitis*, *Laodelphax striatellus* and *Nilaparvata lugens* possess yeasts that are classified within the *Metschnikowia* (*Candida*) genus (Bai et al. 2010; Dong et al. 2011; Hemmati et al. 2017). In other insects, *Metschnikowia* (*Candida*) yeasts have been

identified in the gut, which are presumably involved in nutrient provisioning and are vertically transmitted to their progeny by coating the egg shell (Sung-Oui et al. 2005; Vega & Dowd 2005). Other yeast-like symbionts (YLS) in leafhoppers reside in mycetocytes within the insect, and are maternally transferred. These YLS are not free-living in nature and are impervious to culture, most probably because of the unique and specific environment provided by the insect host (Suh et al. 2001).

Based on recent metagenomic studies, the variation in gut-associated bacterial communities was dependent on the host plants in *Lymantria dispar*, *Helicoverpa armigera*, *Drosophila melanogaster*, *D. simulan*, on the diet in *Anopheles gambiae* and *Bemisia tabaci* (Su et al. 2016). No research has been found that surveyed the possible effect of host plants on fungal endosymbionts of insects. Therefore, we hypothesized that the biotic factors of host plant might affect the diversity of *Orosius albicinctus*-associated fungal endosymbionts. *Orosius albicinctus* is a major agricultural pest, as it is a common and established vector of phytoplasma in Europe and the Middle East, including strains that cause the diseases on different plants (Hemmati et al. 2018, Nikooei & Hemmati 2018). This species is active on a range of host plants like *Suadea aeygyptiacae* (Chenopodiacea), Mexican lime, Sesame, *Petunia*, beet and more (Pakarpour Rayeni et al. 2016). To evaluate the hypothesis, we selected *O. albicinctus* feeding on *S.aegyptiacae* a salt-tolerant species grown in naturally salt affected area and Mexican lime, *Citrus aurantifolia*, a horticultural crop.

MATERIAL AND METHODS

Insect collection

To study the effect of different host plants on fungal endosymbionts, adults of *O. albicinctus* leafhopper were collected from Mexican lime (*Citrus auranitfolia* L.) trees (specimen no. = 64) and *Suaeda aegyptiaca* (specimen no. = 72) from Minab, Hormozgan Province using a D-Vac aspirator. Specimens were preserved in acetone and stored at -20ºC (Fukatsu 1999).

DNA extraction

DNA was extracted from leafhoppers using a CTAB method in accordance with protocol of Reineke et al. (1998) with some modification. Each sample of DNA was dissolved in 50 µL of double distilled water and stored at -20ºC. The quality of the extracted DNA was verified on a 1% agarose gel.

Amplification of fungal rRNA genes and phylogenetic analysis

Specific forward primers (YLS-18S-F and YLS-28S-F) were selected which only amplified DNA of yeasts when used in conjunction with the universal primers (Table 1) (Nishino et al. 2016). Yeasts DNA were amplified by PCR (Dual-PeqLab, Germany) in 25 µL reaction mixture containing 12.5 µL Master Mix, 1 µL of each primer (10 pmol/ µL), 1 µL of extracted DNA and 9.5 µL double-distilled water. Aliquots 5 µL of each PCR product were visualized on a 1% agarose gel stained with Gel Stain dye (SMOBIO, Denmark). All PCR products were directly sequenced with both primers by Macrogen Sequencing Service (South Korea).

Phylogenetic analysis

Phylogenetic analyses of *18S* and *28S rRNA* gene sequences were conducted by neighbor joining (NJ) methods using MEGA 6.0 software (Tamura et al. 2013). To assess statistical support for hypothesized NJ clades, bootstrap analysis was done with 1000 bootstrap replicates. The sequences of *18S* and *28S rRNA* genes were deposited in GenBank (accession number MK296473; AP589637-39) and other sequences used in phylogenetic analysis were downloaded from GenBank.

Table 1. Primers and PCR conditions used in identification of yeast and yeast-like symbionts associated with *Orosius albicinctus* fed on different host plants

Primer	Primer sequence (5'-3')	PCR condition
Y28S	F-GGTCCGTGTTTCAAGACGG R-GGATTGCCCCAGTAACG	94°C: 2 min, followed by 35 cycles: 94°C: 1 min, 55°C: 30 s, and 72°C: 1 min; and 5 min at 72°C
Y18S	F-CACAAGTTATCGTTTATTTGATAGCACCTTAC R-GGCTGCTGGCACCAGACTTGC	94°C: 2 min, followed by 35 cycles: 94°C: 1 min, 64 °C: 30 s, and 72°C: 1 min; and 5 min at 72°C

RESULTS

The *18S* ca. 700bp ribosomal regions was amplified from *O. albicinctus* fed on two species plants. Phylogenetic analysis based on *18S rRNA* gene revealed the presence of an YLS in all *O. albicinctus* fed on two different plant species [herein designated *O. albicinctus*-YLS (Oa-YLS)] and had 99% similarity to YLSs reported from *Hishimonus phycitis*, *Laodelphax striatellus* Fallén, *Nilaparvata lugens* Stål and *Sogatella furcifera* (Horvåth). In phylogenetic tree, the *18S rRNA* gene sequences resulted from *O. albicinctus* fed on two plant species was placed in the clade of YLS supported by 100% statistical support, with allied YLS sequences from *N.lugens* (Fig. 1). Our diagnostic PCR survey of *O. albicinctus*

representing 136 individuals, detected Oa-YLS with 100% infection frequency in all individuals examined. DNA samples subjected to PCR amplification, amplified a 700 bp region of the *28S rRNA* gene using universal primers form only the leafhopper fed on *S. aegyptiaca*. No amplicon was obtained from the all leafhoppers fed on Lime trees. The PCR products were sequenced, edited and compared with GenBank sequences using BLAST algorithms. After conducting BLASTN search, the sequences exhibited the most similarity (98%) with *Metschnikowia picachoensis* (*Candida picachoensis*) reported from *Chrysoperla comanche* gut. This sequence was placed in the *Metschnikowia* clade with 95% statistical support and allied with *M.pichachoensis* sequence isolated from *C. comanche* gut. Infection frequency of *C. picachoensis* was 100% in all individuals fed on S. *aegyptiacae.*

DISCUSSION

To find the possible effect of host plants on fungal endosymbionts of *O. albicinctus*, we examined the presence of yeast and yeast-like endosymbionts of this species fed on *S. aegyptiacae* and *C. aurantifolia*. In

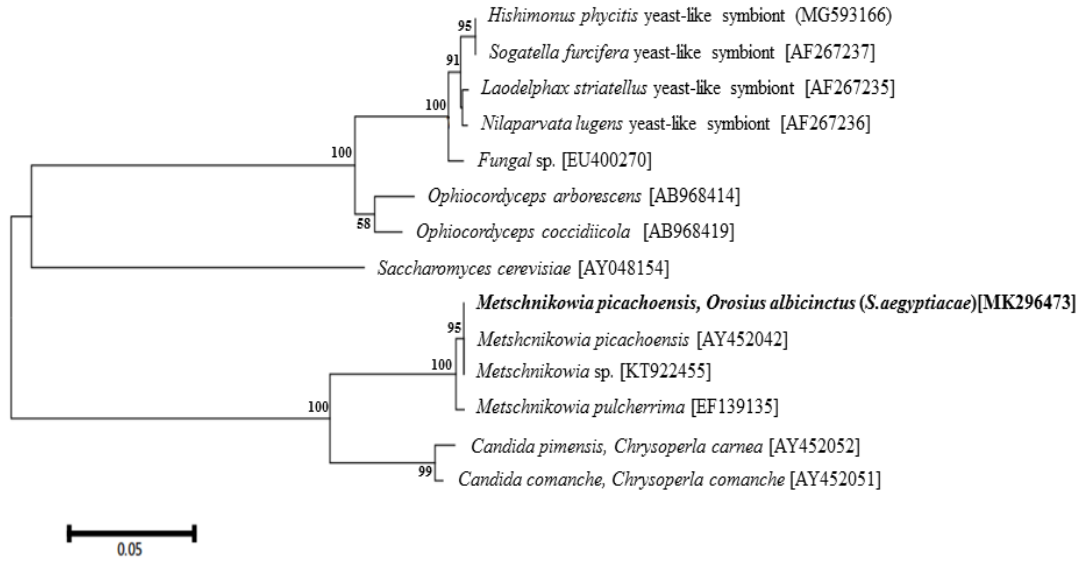

Fig. 1. Phylogenetic relationship of YLS endosymbionts of *O. albicinctus* to YLS and some entomoparasitic fungi of other hemipteran insects on the basis of 18S rRNA gene sequences. A neighbour-joining (NJ) phylogeny inferred from 700 aligned nucleotide sites is shown. Bootstrap probabilities for the NJ analysis at 50% or higher are shown at the nodes. The sequences obtained from the leafhoppers in this study are highlighted by boldface type, wherein insect species and origin, insect family in parentheses, and nucleotide sequence accession number in brackets are indicated. Scale bar shows branch length in terms of number of nucleotide substitutions per site.

addition to providing the answer of the question of this study, this work provided the first evidence of presence of two yeast and yeast-like endosymbionts of the leafhopper. Iasur-Kruh and co-workers (2013) conducted a survey on diversity of bacterial endosymbionts of this species and reported that this species harbored five bacterial endosymbionts including *Sulcia*, *Nasuia*, *Wolbachia*, *Arsenophonus* and *Ricketsiella*. The fungal endosymbiont found in this species was in accordance with the findings of other researchers who found that leafhoppers and plant hoppers harbor two fungal symbionts. For example, Hemmati et al. (2017) reported the two fungal endosymbionts of the leafhopper *Hishimonus phycitis* and Houghes et al. (2012) showed that planhoppers *L. striatellus*, *N. lugens* and *Perkinsiella saccharicida* were the host of yeas-like symbionts and *Metschnikowia pimensis* (*Candida pimensis*) (Houghes et al. 2011; Hemmati et al. 2017).

This study showed that the host plant played an important role in shaping the composition of the fungal community associated with *O. albicinctus*. We found that *O. albicinctus* fed on *S. aegypticae* had a yeast symbiont namely *Metschnikowia* than counterparts fed on lime. There is no document reporting the effect of host plants on fungal microbiome composition in insects. So we compare our results with the literature conducted on bacterial endosymbionts. Our results can also be supported by Pan et al. (2013) that host plant can affect the relative amount of symbionts such as *Portiera*, *Cardinium*, *Rickettsia*, and *Hamiltonella* in *B. tabaci*. In addition, Anderson et al. (2012) also found that highly similar bacterial communities were shared among related and trophically similar herbivorous ant species.

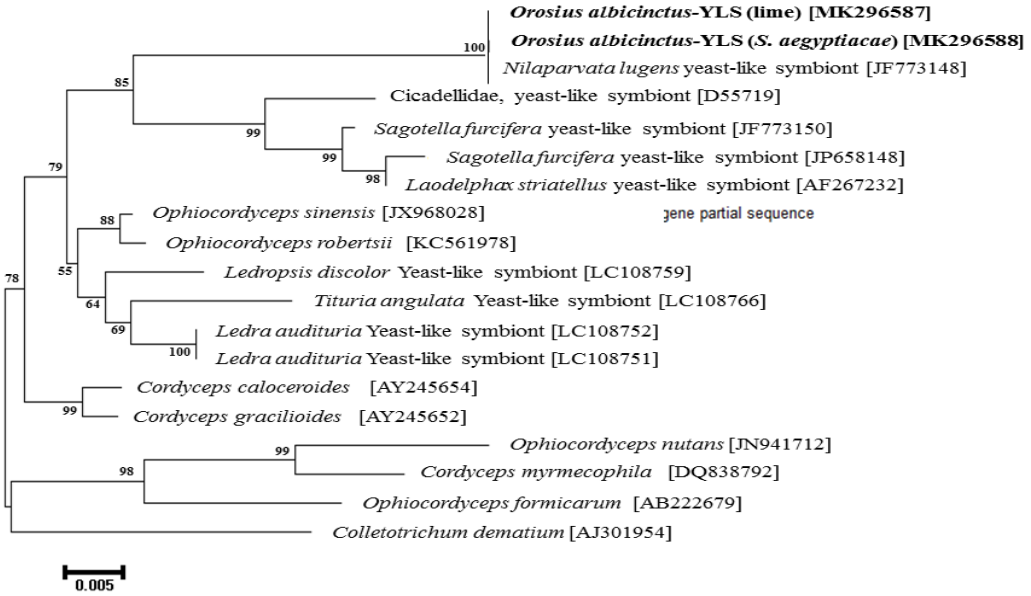

Fig. 2. Phylogenetic relationship of YLS and *Metschnikowia comanche* of *Orosius albicinctus* to YLS and fungi of other hemipteran insects on the basis of 28S rRNA gene sequences. A neighbour-joining (NJ) phylogeny inferred from 700 aligned nucleotide sites is shown. Bootstrap probabilities for the NJ analysis at 50% or higher are shown at the nodes. The sequences obtained from the leafhoppers in this study are highlighted by boldface type, wherein insect species and origin, insect family in parentheses, and nucleotide sequence accession number in brackets are indicated. Scale bar shows branch length in terms of number of nucleotide substitutions per site.

Yeast and yeast-like fungi associated with insects play several roles, the most important ones is a nutritional role in which yeasts supply enzymes for digestion resulted in improved nutritional quality, essential amino acids, sterols and vitamins. Yeasts also cooperate to detoxify toxic plant metabolites in the host's diet (Vega & Dowd 2005). *Suaeda aegyptiaca* is a succulent, annual halophyte plant, which is inhabited and consider native to saline soils of arid and semiarid regions of Iran and widely distributed in Iraq, UAE, Pakistan and North Africa (Askari et al. 2006). This differences in fungal composition may be because of the fact that different plant species harbor different phytotoxin or secondary metabolites which should be detoxified or transformed to another compounds in *O. albicinctus* feeding on the halophyte species. To find the role of this yeast in the insect host, further research is needed. Biological functions of the fungal symbiont in *O. albicinctus* are currently unknown. Diversity of bacterial endosymbionts of this insect distributed in Israel studied by Iasur-Kruh et al. (2013) who reported that this species harbored two obligate endosymbionts namely *Sulcia* and *Nasuia*. Genomics of *Sulcia* and co-symbionts have suggested that these bacterial symbionts cooperatively provide essential amino acids and other nutrients for leafhoppers and other hemipteran insect host (Bennet & Moran 2015). It should be noted that the diversity of bacterial endosymbions of Iranian population should be investigated to find if this population is the host of these bacterial symbionts. Considering the much larger genome size and consequent broader metabolic capability of the fungal symbiont in comparison with the tiny-genome bacterial symbionts (McCutcheon 2010; Moran and Bennett 2014), it is conceivable, although speculative, that the fungal symbiont may play additional biological roles in the hemipteran hosts. Genomics of the fungal symbionts (Vogel & Moran 2013) and physiological studies on normal and fungus-deprived insect hosts (Sasaki et al. 1996) are needed for deeper understanding of functional aspects of the insect-fungus symbiotic association.

In conclusion, we found that host plants can shape the composition of fungal endosymbionts of *O. albicinctus*. We revealed that O. *albicinctus* fed on a halophyte species need more yeast than counterparts fed on lime. The role of this yeast in O. *albicinctus* requires further research. In some cases, the bacterial endosymbionts was replaced by a yeast in some insects. To find this possibility in this insect, further research is needed to study the bacterial endosymbionts of the leafhoppers feeding on different species.

Compliance with Ethical Standards:

Funding: This research has not received any funding.

Conflict of interests: all authors declare that they have no conflict of interest.

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