Phylogenetic position of the yeast-like symbiotes of Tagosodes orizicolus (Homoptera: Delphacidae) based on 18S ribosomal DNA partial sequences

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Abstract: Tagosodes orizicolus Muir (Homoptera: Delphacidae), the endemic delphacid species of tropical America carries yeast-like symbiotes (YLS) in the abdominal fat bodies and the ovarial tissues, like other rice planthoppers of Asia. These YLS are obligate symbiotes, which are transmitted transovarially, and maintain a mutualistic relationship with the insect host. This characteristic has made in vitro culture and classification of YLS rather difficult using conventional methods. Nevertheless, microorganisms of similar characteristics have been successfully classified by using molecular taxonomy. In the present work, the YLS of Tagosodes orizicolus (YLSTo) were purified on Percoll® gradients, and specific segments of 18S rDNA were amplified by PCR, cloned and sequenced. Sequences were aligned by means of the CLUSTAL V (DNASTAR) program; phylogenetic trees were constructed with the Phylogeny Inference Package (PHYLIP), showing that YLSTo belong to the fungi class Pyrenomycetes, phylum Ascomycota. Similarities between 98% and 100% were observed among YLS of the rice delphacids Tagosodes orizicolus, Nilaparvata lugens, Laodelphax striatellus and Sogatella furcifera, and between 89.8% and 90.8% when comparing the above to YLS of the aphid Hamiltonaphis styraci. These comparisons revealed that delphacid YLS are a highly conserved monophyletic group within the Pyrenomycetes and are closely related to Hypomyces chrysospermus. Rev. Biol. Trop. 52(3): 777-785. Epub 2004 Dic 15.

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Homoptera have also been studied through histology, production of aposymbiotic insects, transovarial infection and the effect on insect nutrition (Noda 1974, Noda and Saito 1979a and 1979b, Kusumi et al. 1979 and 1980, Houck 1980, Chen et al. 1981a,b, Nasu 1981, Frölich 1989 and Sasaki et al. 1996). Delphacids have microbial symbiotes in their bodies, probably contributing to the lack of proteinases in their salivary glands, like aphids and other insects that are phloem feeders (Frölich 1989). It has been observed that a decrease in the YLS populations in the insect produces deleterious effects, such as reduction in nymph emergence, ecdisis inhibition, decrease in insect size and reproductive failure (Chen et al. 1981b, Fredenhagen et al. 1987 and Frölich 1989). It is also known that the main source of esterol (24-methylenecholesterol) is provided by the YLS, since the lack of these symbiotes affects the insect esterol metabolism, a precursor of ecdisone (Noda and Saito 1979b, Fredenhagen et al. 1987, Frölich 1989).

YLS are transmitted vertically by an intra-ovarian process, and later infect the fat tissues of the insect’s abdomen (Mitsuhashi 1975, Kusumi et al. 1979, Chen et al. 1981a, Frölich 1989), as it occurs with the delphacids Laodelphax striatellus and Nilaparvata lugens. In the case of Tagosodes orizicolus, YLS inhabit the intracellular spaces in the fat bodies of the insect’s abdomen, in diffuse patterns associated with syncitia, but without forming mycetomes (Espinoza et al. 2004).

Purification and isolation of YLS have been performed on eggs and adult delphacids; however, maintenance of cultures has not been successful because these microorganisms undergo autocatalytic processes (Mitsuhashi 1975, Kusumi et al. 1979, Nasu et al. 1981, Chen et al. 1981a). This situation has limited the application of conventional classification and identification methods that require in vitro culture of the YLS.

An alternative method for the classification of these YLSs was developed by Noda et al. (1995), based on the partial 18S rDNA sequences. They determined the phylogenetic relationships of the YLS of three rice delphacids: Nilaparvata lugens (YLSNl), Sogatella furcifera (YLSf) and Laodelphax striatellus (YLSl). These were classified among the Pyrenomycetes in the subphylum Ascomycota. These symbiotes had a monophyletic origin and close genetic distances. Aphids also have symbiotic microorganisms; however, these are mainly prokaryotes. One exception is found in the aphid Hamiltonaphis styraci, which, like delphacids, contains YLS belonging to the Pyrenomycetes class (Buchner 1965, Houk and Griffiths 1980, Fukatsu and Ishikawa 1992, 1996, Fukatsu 1994, Fukatsu and Ishikawa 1996). The beetles Stegobium panicum and Lasioderma serricorne also have yeast-like symbiotes. These microorganisms are transmitted from generation to generation when the newly emerged larvae feed on the yeast-covered eggshell. These symbiotes form a monophyletic group in the Discomycetes class (Noda and Kodama 1996).

The purpose of this research is to determine the phylogenetic relationships of the yeast-like symbiotes of the delphacid Tagosodes orizicolus with other ascomycetes fungi, as well as to determine the nucleotide similarity of the YLS of T. orizicolus with the YLSs of Nilaparvata lugens, Sogatella furcifera, Laodelphax striatellus and Hamiltonaphis styraci, using partial sequences of the 18S rDNA.

MATERIALS AND METHODS

Sample collection: Tagosodes orizicolus was collected from sixty day-old-plants in rice fields in the Costa Rican rice growing regions of Guanacaste (N 10°29'04.9” ; W 085°24'23.7”) and Parrita (N 09°34'15.3” ; W 084°33'23.3”). Capture was performed with a 32cc motor pump (Craftzman, model 358-797920), modified by placing a mesh in the suction duct. Insects were transported on ice and stored at -20°C. Adult selection was performed according to male and female genitalia (Mora et al. 2002).
Purification of yeast-like symbiotes: Purification was performed on Percoll® gradients, based on the protocol described by Noda and Omura (1992). One gram of adult insects was homogenized on a porcelain mortar with 10 volumes of 0.85% NaCl. Each sample was filtered on cotton cloth and centrifuged for 5 min at 3500 rpm on a clinic IEC centrifuge. The pellet was washed twice, re-suspended in 0.85% NaCl and mixed with four volumes of Percoll (Pharmacia LKB, Sweden). The mixture was centrifuged at 26000 rpm for forty minutes in a Beckman, SW50.1 rotor and a second gradient was performed to remove tissue debris. A standard was prepared by placing 5 ml of each of the floating density markers re-suspended in 1 ml 0.85% NaCl and adding 4 volumes of Percoll. The standard was centrifuged under the same conditions as the samples. Each fraction of the gradient was observed under a light microscope (10X and 40X), confirming the presence of YLS.

DNA extraction: Yeast DNA was extracted by modifying the methods described by Cenis (1992) and Noda et al. (1995). YLSTo were re-suspended in 50 mM EDTA, pH 8, 1/3 volume of 1% litiace (Sigma) on 0.01 M sodium phosphate pH 7.5 and 50% glycerol. Spheroplasts were obtained by incubation at 37°C during 45-60 minutes, followed by centrifugation. The pellet was homogenized in 300 l buffer (200 mM Tris.HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS and glass beads of 212-300 microns). Samples were then treated with 150 l of 3M sodium acetate and incubated at -30°C for one hour, followed by centrifugation at 13 000 rpm for ten minutes. The supernatant was recovered and DNA was obtained by precipitation with isopropanol. It was then re-suspended in distilled water and quantified by UV spectrophotometry at 260 nm.

PCR amplification: Partial sequences of 18S rDNA were amplified from total DNA. The PCR protocol and the primers used were those described by Noda and collaborators (1995). Approximately 40 ng of DNA were used for each reaction and amplification products were analyzed in 0.7% agarose gels. The bands obtained were cut and the products were extracted according to the QIAquick gel extraction protocol (QIAGEN 1997). Ligation and transformation were performed according to the pGEM-T Vector System I (Promega 1997) protocol. The samples were incubated on Luria Bertani (LB) broth for four hours with agitation, and the DNA preparation for sequencing was made by following the protocol of Sambrook et al. (1989). The clones were stored in 30% glycerol at -70°C.

RESULTS

The symbiotes were located in densities between 1.087 g/ml to 1.119 g/ml on Percoll gradients, densities similar to those previously reported for the YLS of other delphacids. The YLSTo were thus exhaustively purified for DNA extraction in order to avoid contamination by host DNA. The sequence of the dominant species of YLSTo was obtained through this procedure, because T. orizicolus, as well as other delphacids, could host other species of symbiotes among their microbiota.
Table 2 shows the partial sequences of the 18S rDNA of YLS *Tagosodes orizicolus* as compared to the NS1-2 region, positions 46 to 531 of *Saccharomyces cerevisiae* 18S rDNA. The analysis also included sequences from YLS of the delphacids *Nilaparvata lugens*, *Laodelphax striatellus* and *Sogatella furcifera*.

Upon comparing the YLS sequences of *T. orizicolus* with those of the YLS from *N. lugens*, *S. furcifera* and *L. striatellus*, ten variable positions among the delphacid symbiotes were found (Table 2). Six additional variable positions were obtained when the YLSs of the aphid *H. styraci* were included (data not shown). As observed, YLSSf and YLSLs showed the same nucleotide changes along the whole region analyzed in relation to YLSNI. In the mean time, YLSTo varied in the positions 389, 423 and 437 in relation to the former three. The most variable sequence among the symbiotes of rice delphacids was that of YLSNI.

Diversity in the previously described positions was also analyzed by similarity percentages among the sequences (Table 3).

In this sense, YLSTo are more similar to other delphacid YLSs, such as YLSLs and YLSSf, showing 99.4% similarity, while the
similarity with YLS was of 98%. In addition, YLS exhibits a 90.2% similarity with those from the aphid Hamiltonaphis styraci. In other words, the YLS have diverged less in relation to YLS and YLSSf (0.4%), at an intermediate level as compared to YLSNl (1.2%) and to a higher degree with respect to YLSHs (1.6%) (Table 3).

The position of YLS within the phylum Ascomycota was determined after aligning the complete sequence of the NS1-6 (1438 nt) clone with that of eleven ascomycete fungi: Arcoesphaera apis, Aspergillus fumigatus, Coccidioides immitis (Class Plectomycetes); Hypomyces chrysospermus, Ophiostoma ulmi, Podospora anserina (Class Pyrenomycetes); Lecanora dispersa, Spathularia flavida, Spaenophorus globosus (Class Discomycetes); Candida albicans, Saccharomyces cerevisiae (Order Saccharomycetales) and Ustilago maydis (Class Basidiomycetes) as an outgroup (Fig. 1). The analyses revealed that YLSTo grouped with H. chrysospermus, O. ulmi and P. anserina in the class Pyrenomycetes. Furthermore, the conformation of three other subgroups was observed. According to the classic taxonomic division (morphologic characteristics), these three subgroups correspond to the fungi Discomycetes, Plectomycetes and Saccharomycetales of the phylum Ascomycota (Fig. 1).

The relationship between YLSTo with the Pyrenomycetes was determined by comparing the symbiote 1386 nt 18S rDNA segment with that from nine fungi of this class: H. chrysospermus, O. ulmi, P. anserina, Glomerella cingulata, Microsac us cirrosus, Ophiostoma ulm i, Podospora anserina, Candida albicans, Saccharomyces cerevisiae (Order Saccharomycetales) and Ustilago maydis (Class Basidiomycetes) as an outgroup (Fig. 1). The analyses revealed that YLSTo grouped with H. chrysospermus, O. ulmi and P. anserina in the class Pyrenomycetes. Furthermore, the conformation of three other subgroups was observed. According to the classic taxonomic division (morphologic characteristics), these three subgroups correspond to the fungi Discomycetes, Plectomycetes and Saccharomycetales of the phylum Ascomycota (Fig. 1).

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Chaetomium elatum, Leucostoma persoonii, Ophiostoma schenckii and Sordaria fimicola. They conformed a monophyletic group along with H. Chrysospermus, G. cingulata and M. cirrosus. S. flavida (Discomycete) was included as an external group (Fig. 2).

The analysis of the 18S rDNA partial sequences (1 a 531 nt) revealed very close relationships among the YLS\textit{To} and other delphacid and aphid YLS (Fig. 3). The dendrogram showed a monophyletic origin of the delphacid YLS and very short genetic distances between YLS\textit{To} and YLS\textit{Ls}, YLS\textit{Sf} and YLS\textit{Nl}, but slightly larger distances with YLS\textit{Hs}. The symbiotes of the beetle \textit{Lasioderma serricorne} (YLS\textit{Lse}) were placed outside the class Pyrenomycetes and grouped with S. flavida, both Discomycetes.
DISCUSSION

The yeast-like symbiotes of *Tagosodes orizicolus* clustered with members of the class Pyrenomycetes when compared to other members of the phylum Ascomycota, like Plec-tomycetes, Discomycetes, Saccharomycetales and Basidiomycetes (Fig. 1). The clustering of YLSTo, along with *G. cingulata, M. cirrosus*, and *H. chrysospermus*, within the Pyrenomycetes, supported the above observation (Fig. 2). YLSTo and other delphacid and aphid yeast-like symbiotes formed a separate cluster, when compared to other members of the Pyrenomycetes and Discomycetes (Fig. 3). In contrast, the beetle YLSSLs showed affinity to *Spathularia flavida*, a Discomycetes, suggesting that different insect orders may have been colonized by YLS belonging to different groups within the phylum Ascomycota.

The close similarity (more than 90%) observed between the delphacid and aphid symbiotes suggests that an ancestral Pyrenomycete may have parasitized a homopteran common ancestor before these two groups diverged. This ancestral Pyrenomycete may have developed a mutualist symbiotic relationship, a role that yeast-like symbiotes presently have. The phylogenetic distribution of the symbiotes in both plant hoppers and aphids is complex (bacterioid and yeast-like type), which may indicate that some events, such as symbiote acquisition and replacement, could have sprung either from the evolution from a common ancestor or from horizontal transfer (Fukatsu *et al.*, 1994, Moran and Baumann 1994, Fukatsu and Ishikawa 1996).

It is interesting to emphasize the high degree of similarity (over 98%) observed in the 18S rDNA partial sequences among the YLSs of Asian and American rice delphacid, especially considering that the YLS might have been present in a common ancestor. The high similarity observed might be reasonable, considering the crucial role of the symbiotes in the physiology, development and reproduction of their delphacid hosts, especially since significant genetic variations on the YLS could directly affect the host fitness and reproductive success. In addition, it is important to mention that the 18S rDNA partial sequences used in this study are likely to be highly conserved, due to the important role of rDNA in protein synthesis, and that analyzing other sequences in the YLS genome could result in higher levels of diversity.

The phylogenetic relationships of the YLSs of three Asian rice-feeding delphacids (*N. lugens, S. furcifera* and *L. striatellus*) were previously described by Noda and collaborators (1995), and were compared to twenty species of other Ascomycetes. It was observed that the YLSTo showed high degree of similarity to those of Asian delphacids; although, *T. orizicolus* is strictly confined to tropical America. Therefore, the colonization of a delphacid YLS ancestor may have occurred before these species diverged, possibly before the continental drift. It is important to recall that delphacids maintain very close relationships with their plant hosts. For example, *T. orizicolus* is only capable of feeding and reproducing on rice (*Oryza sativa*) and in *O. glumaepatula* (Hernández and Espinoza, unpublished results), an endemic wild *Oryza* species of tropical America. *O. glumaepatula* is related to other Asian and African *Oryza* species of the *O. sativa* complex, sharing the AA type genome (Akimoto 1999). If cultivated rice was introduced to America less than 500 years ago (Cabezas and Espinoza 2001), it is probable that *O. glumaepatula* may have been the original host of *T. orizicolus* and that, after the introduction of rice in America, this insect may have adapted to the new host.

This first report of yeast-like symbiotes in the American delphacid *Tagosodes orizicolus* offers interesting results concerning the relationship of this organism as compared to other delphacid YLS, as well as other fungi from the phylum Ascomycota. However, in order to understand the acquisition patterns and the evolutionary origin of yeast-like symbiotes, it is still necessary to perform more detailed molecular phylogenetic studies on the symbiotes as well as on their homopteran hosts.
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RESUMEN

Tagosodes orizicolus Muir (Homoptera: Delphacidae) es una especie endémica de América tropical que al igual que otros saltahojas de Asia, tiene simbiontes levaduriformes (YLS, por sus siglas en inglés) en los cuerpos grasos del abdomen y en los tejidos de los ovarios. Los YLS son simbiontes obligados que se transmiten transversalmente y que mantienen relaciones mutualísticas con el insecto hospedero. Esta característica ha hecho muy difícil su cultivo in vitro y por ende su clasificación utilizando métodos convencionales. Sin embargo, otros microorganismos de características similares se han clasificado con éxito utilizando taxonomía molecular. El presente trabajo tiene como objetivo caracterizar los YLS del delfácido Tagosodes orizicolus (YLSTo). Para ello se purificaron los YLSTo en gradientes de Percoll® y el ADN extraído se amplificó por PCR utilizando iniciadores específicos para secuencias parciales del ADN ribosomal 18S. Dichos fragmentos se clonaron y secuenciaron posteriormente. Las secuencias se alinearon mediante el programa CLUSTAL V y posteriormente se compararon con los YLS del áfido Nilaparvata lugens, Laodelphax striatellus y Sogatella furcifera (todos delfácidos de arroz). Se obtuvieron índices de similaridad entre 98% y 100% entre los YLS de Tagosodes orizicolor, Nilaparvata lugens, Laodelphax striatellus y Sogatella furcifera (todos delfácidos de arroz), e índices de similaridad entre 89.8% y 90.8% al compararse con los YLS del áfido Hamiltonaphis styraci. Estas comparaciones revelaron que los YLS de los delfácidos constituyen un grupo monofilético altamente conservado dentro de los Pyrenomyctes y que se relacionan cercanamente con Hypomyces chrysospermus.

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