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Phylogenetic Position of Yeast-like Symbiotes of Rice Planthoppers Based on Partial 18S rDNA Sequences*

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The rice planthoppers, *Nilaparvata lugens*, *Sogatella furcifera*, and *Laodelphax striatellus*, harbor intracellular yeast-like symbiotes (YLS) in the fat body. The YLS are obligated endosymbiotes and are transovarially inherited. Nucleotide sequences of the 18S rRNA genes from the YLS were analyzed by direct sequencing. The YLS of the three species of planthoppers were monophyletic and their taxonomic positions were in the class Pyrenomycetes in the subphylum Ascomycotina.

Nilaparvata lugens *Sogatella furcifera* *Laodelphax striatellus* Planthopper Yeast-like symbiote Endosymbiosis 18S ribosomal RNA 18S rDNA Molecular phylogeny Pyrenomycetes Ascomycotina

INTRODUCTION

Intracellular mutualism is common in blood-sucking and plant-sucking insects and in specialized feeders (Buchner, 1965; Brooks, 1985; Tanada and Kaya, 1993). The number of species of endosymbiotes in the insects, therefore, is large, but the origins of the endosymbiotes are quite enigmatical. Moreover, knowledge on classification and a technical method of identifying the endosymbiotes is still limited. This is partly due to the difficulty of culturing the symbiotes.

The rice planthoppers, *Nilaparvata lugens*, *Sogatella furcifera*, and *Laodelphax striatellus*, harbor yeast-like symbiotes (YLS) in the fat body, and transmit them to the next generation through the ovary (Nasu, 1963; Noda, 1977). The YLS of the planthoppers are one of the largest endosymbiotes in insects, the length being 10–15 μm. The numbers of YLS in the planthoppers is well regulated (Noda, 1974) and their elimination by heat has a deleterious effect on the host planthoppers (Noda and Saito, 1979; Chen *et al.*, 1981). The YLS of *N. lugens* (*Nl*YLS) and *L. striatellus* (*Ls*YLS) were cultured and two kinds of YLS were isolated from each planthopper species (Kusumi *et al.*, 1979; Nasu *et al.*, 1981), but the culture of the YLS was not confirmed. We therefore purified YLS from planthoppers by Percoll buoyant density gradient centrifugation (Noda and Omura, 1992).

Information on the phylogenetic position of the YLS in the kingdom of microorganisms is useful for studies of YLS origin. Recent advancements in DNA technology and accumulated information on the genome, enable us to take a molecular approach to the study of microorganisms without culturing or knowing the genetic background. Sequencing of nucleic acids or proteins with the same function provides a powerful approach to classifying and identifying the microorganisms, and to measuring the phylogenetic relationship among organisms. Genotypic information, i.e. sequence information of nucleic acids or proteins is also very useful for obligatory symbiotes, because phenotypic information is often quite limited. Ribosomal RNA (rRNA) or ribosomal RNA genes (rDNA) are studied in both prokaryotic and eukaryotic microorganisms (Woese, 1987; Bruns *et al.*, 1991). Phylogenetic positions based on the nucleotide sequences of rDNA are reported in the symbiotic bacteria of aphids (Unterman *et al.*, 1989; Munson *et al.*, 1991a, b), of weevils (Campbell *et al.*, 1992), of whiteflies (Clark *et al.*, 1992; Campbell, 1993), and the symbiotic rickettsia of the causative agent of cytoplasmic incompatibility (Breeuwer *et al.*, 1992; O'Neill *et al.*, 1992; Rousset *et al.*, 1992a, b; Stouthamer *et al.*, 1993). However, the molecular phylogeny of YLS has not been examined.

In this article, we present the results of 18S rDNA analysis to determine the fungal phylogenetic affiliation of the YLS of the three rice planthoppers.

*The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank databases under the accession numbers D38474–D38479.

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MATERIALS AND METHODS

Insects and YLS

Three rice planthoppers, the brown planthopper (*N. lugens*), the whitebacked planthopper (*S. furcifera*), and the small brown planthopper (*L. striatellus*) (Homoptera, Delphacidae), were collected in paddy fields of Izumo, a western region of Japan. Colonies of the planthoppers were maintained on rice seedlings at 26 + 2°C with a photoperiod of 16L:8D. Mature nymphs or adults, from which YLS were isolated, were collected and stored at −70°C. YLS of the planthoppers, *Nl*YLS, *Sf*YLS and *Ls*YLS, were purified by Percoll (Pharmacia LKB, Sweden) buoyant density gradient centrifugation according to the method of Noda and Omura (1992).

DNA preparation

The purified YLS were suspended in 50 mM EDTA, pH 8.0. One-third volume of Lyticase (Sigma) solution (0.2% in 0.01 M sodium phosphate buffer, pH 7.5, containing 50% glycerol) was added to the YLS suspension and incubated at 37°C for 30 min to make YLS spheroplasts (Noda and Kawahara, 1995). The procedure for further isolation followed that for the yeast DNA miniprep (Rose *et al.*, 1990). The isolated nucleic acids were treated with RNase (20 μg/ml) at 37°C for 15 min and DNA was stored at −20°C.

Template DNA of planthoppers for polymerase chain reaction (PCR) amplification was obtained by boiling part of the planthopper body (Jackson *et al.*, 1991). Simple boiling of fresh tissues in distilled water was sufficient for amplifying rDNA that is a high-copy-number sequence. The legs or head of an insect, which do not harbour YLS, were put in a microfuge tube with 200–300 μl water, boiled for 10 min, and about 30 μl was directly used for the following PCR amplification as template DNA solution.

PCR amplification

Six primers for amplification of small nuclear rDNA were used according to the primer design of White *et al.* (1990). Their sequences and position on *Saccharomyces cerevisiae* 18S rRNA (Mankin *et al.*, 1986) are:

NS1, 5′-GTA GTC ATA TGC TTG TCT C-3′ (20–38)

NS2, 5′-GGC TGC TGG CAC CAG ACT TGC-3′ (573–553)

NS5, 5′-AAC TTA AAG GAA TTG ACG GAA G-3′ (1129–1150)

NS6, 5′-GCA TCA CAG ACC TGT TAT TGC CTC-3′ (1436–1413)

NS7, 5′-GAG GCA ATA ACA GGT CTG TGA TGC-3′ (1413–1436)

NS8, 5′-TCC GCA GGT TCA CCT ACG GA-3′ (1788–1769)

NS represents small nuclear rDNA. NS1, NS5, and NS7 are forward primers and the others are reverse ones. These primer DNAs were synthesized by a DNA synthesizer (Applied Biosystems Model 392). The synthesized DNA was partly purified by phenol extraction and ethanol precipitation.

Either most of the entire length, or a part of 18S rDNA, was selectively amplified by the PCR from the genomic DNA preparations. Amplifications were performed in 50 μl of buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ 0.001% gelatin), with 0.12 mM each dNTP, 20 pmol primers, template DNA (10–100 ng) and 2.5 U *Taq* DNA polymerase (Perkin Elmer Cetus). The PCR thermal program was 96°C for 2 min; followed by 30 cycles of 96°C for 30 s, 55°C for 30 s, 72°C for 2–2.5 min, and 72°C for 5 min as a final extension after the last cycle. A part of the amplified DNA was electrophoresed in an 0.8–2.0 agarose gel and stained with ethidium bromide to examine the DNA size. The remaining amplified DNA was precipitated with the same volume of 4 M ammonium acetate and two-fold isopropanol.

Nucleotide sequencing

Sequences of amplified 18S rDNA were determined by the *Taq* dye deoxy terminator cycle sequencing kit (Applied Biosystems) using a DNA Sequence System (Applied Biosystems Model 373A). According to the manufacturer's recommendation, 1 μg of PCR product and 3–4 pmol primers were mixed with premix buffer solution containing dNTP, dye deoxy terminators (A, T, G, C), and *Taq* DNA polymerase. The primers used for sequencing were the same as those used for the PCR. Temperature regimen was 96°C for 1 min, followed by 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The reaction mixture was passed through a handmade spin column of Bio-gel P30 (Bio-Rad) to remove the remaining dye deoxy terminators.

Database search and phylogenetic analysis

Fungi and yeasts which have taxonomic affinities to YLS were selected after a search for sequence similarities between 18S rDNAs of YLS and those registered in the DNA databases, EMBL and GenBank. This search was performed using the FASTA family programs of Pearson and Lipman (1988), installed in the program package of FLAT at the DNA Data Bank of Japan (DDBJ), Mishima. The 18S rDNA sequences of the selected fungi and yeasts were obtained from the DNA databases, EMBL and GenBank. They were then analyzed by software packages installed in a desktop computer. The sequences were aligned by a software for multiple sequence alignment, CLUSTAL V distributed by D. G. Higgins, European Molecular Biology Laboratory, and the aligned sequences were analyzed by neighbor-joining method in PHYLIP (Phylogeny Inference Package) version 3.5C distributed by J. Felsenstein, University of Washington.

RESULTS

Comparison of nucleotide sequences of planthoppers and YLS

The primers successfully amplified the 18S rDNAs of the YLS and planthoppers. Yamada and Kawasaki (1989) noted that comparison of the partial sequences in the positions around 1490 of 18S rRNA in *S. cerevisiae* gave the most decisive phylogenetic information in their study of partial sequences of rRNA in basidiomycetous yeasts. Therefore, we first determined the sequence of the 18S rDNA toward downstream from the position of NS7 primer. To avoid the possibility of amplifying rDNA of host planthoppers owing to impurities in the purified YLS, the sequences of the planthoppers at the same position as that of the YLS were also determined.

Nucleotide sequences of about 210 bp, which correspond to positions 1451–1660 in the sequence of 18S rRNA of *S. cerevisiae* (Mankin *et al.*, 1986), are shown in Fig. 1. Nucleotide sequences were highly conserved among the three species of YLS and among the planthoppers. However, the sequences in positions of 1488–1491 and 1501–1509 were variable between *S. cerevisiae* and the YLS as well as in the basidiomycetous yeasts (Yamada and Kawasaki, 1989). The sequences of the planthoppers differed more from *S. cerevisiae* than did those of the YLS. Among the three species of planthoppers, only one base was not common at position 1557 (Fig. 1).

Partial neucleotide sequences of YLS

The PCR products amplified by the primers NS1 and NS2, and those amplified by the primers NS5 and NS8, were sequenced. The primers NS1, NS5, and NS7 were used for the sequencing reaction toward downstream of 18S rDNA and the primers NS2, NS6, and NS8 toward upstream. The sequences of the regions NS1-2 (position 46 531 in *S. cerevisiae*) and NS5-8 (position 1152–1761) of the 18S rDNA from *Nl*YLS are presented in Fig. 2 with the corresponding regions of *S. cerevisiae*. Variable positions in the two regions among the rDNAs of YLS are shown in Table 1. The nucleotide sequences of the three YLS were similar and those of *Sf*YLS and *Ls*YLS were the same in region NS1-2. Seven positions were variable among the three YLS species in region NS1-2 and six in region NS5-8.

Phylogenetic relationships

Yeasts and fungi, which have 18S rDNA sequences similar to those of the *Nl*YLS, were searched for in DNA database libraries. The homology search in regions NS1-2 and NS5-8 was individually performed in EMBL and GenBank using FASTA family programs. In both regions many higher fungi were selected; ascomycetous yeasts and fungi showed particularly high scores in similarity.

Twenty species of yeasts and fungi, most of which are ascomycetes, were selected and their rDNA sequences were taken from the EMBL and GenBank. The selected species in the subphylum Ascomycotina were: *Chaetomiun elatum*, *Hypomyces chrysospermus*, *Ophiostoma ulmi* (the class Pyrenomycetes), *Ascosphaera apis*, *Aspergillus fumigatus*, *Talaromyces flavus* (the class Plectomycetes), *Aureobasidium pullulans*, *Leptosphaeria doliolum*, *Septoria nodorum* (the class Loculoascomycetes), *Cudonia confusa*, *Spathularia flavida* (the class Discomycetes), *Pichia membranaefaciens*, *Saccharomyces cerevisiae* (the class Hemiascomycetes), *Pneumocystis carinii*, *Schizosaccharomyces pombe*, *Saitoella complicata*, *Taphrina wiesneri* [basal ascomycetes designated by Berbee and Taylor (1993)]. Three species in the subphylum Basidiomycotina were also selected: *Athelia bombacina*, *Tilletia caries*, and *Ustilago maydis*. *Aureobasidium pullulans* is the name of the anamorph and is considered to belong to the class Loculoascomycetes (Wilmotte *et al.*, 1993). *Pneumocystis carinii* has a group I intron in its 18S rDNA (Sogin and Edman, 1989) and therefore the intron was removed from the sequences for the analysis. The corresponding sequences to the region NS1-2 and NS5-8 were taken out and concatenated into a sequence of about 1100 bp. A phylogenetic tree was constructed by neighbor-joining method in the PHYLIP using the edited 18S rDNA sequences (Fig. 3). In the subphylum Ascomycotina, the three YLS clearly belong to the Euascomycetes. Hemiascomycetous, basal ascomycetous, and basidiomycetous fungi formed different branches and were far from the YLS. Among euascomycetes, the YLS formed a monophyletic group with pyrenomycetes. Bootstrap analysis indicated 100% support for this lineage monophyly.

The relationship of the YLS among pyrenomycetes was then analyzed. The pyrenomycetes of which 18S rDNA sequences were deposited in the DNA databases were searched and 11 species were selected: *Microascus cirrosus*, *Pseudoallescheria boydii* [the family Microascaceae by Müller and von Arx (1973), Microascales by Hawksworth *et al.* (1983)], *Ophiostoma schenckii*, *O. stenoceras*, *O. ulmi* (the family

Table 1. Variable positions in the partial ribosomal sequences among yeast-like symbiotes of three rice planthoppers

	46–531							1152–1761					
Species	72	222	234	236	272	522	523	1447	1687	1694	1703	1728	1752
N. lugens YLS	A	A	T	G	T	T	C	T	—	—	G	G	A
S. furcifera YLS	—	—	C	—	C	C	T	T	A	—	A	A	A
L. striatellus YLS	—	—	C	—	C	C	T	C	A	G	G	A	G

Numbers refer to position on the *S. cerevisiae* sequence.

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                                1460*                1480*                1500*                1520*                1540*
1  Saccharomyces cerevisiae     GGGCCGCACG CGCGCTACAC TGACGGAGCC AGCGAGTCTA- ACCTTGGCCG AGAGGTCTTG GTAATCTTGT GAAACTCCGT CGTGCTGGGG
2  Nilaparvata lugens YLS       .......... .......... .......... .......AACT C......... GA...C.CG. .......... T......... ..........
3  Sogatella furcifera YLS      .......... .......... .......... .......AACT C......... GA...C.CG. .......... T......... ..........
4  Laodelphax striatellus YLS   .......... .......... .......... .......AACT C......... GA...C.CG. .......... T......... ..........
5  Nilaparvata lugens           .......... .......... ...A...AT. ....T....T- C...-.T... .A...A.CG. ....C.CGC. ...C....T. ......T...
6  Sogatella furcifera          .......... .......... ...A...AT. ....T....T- C...-.T... .A...A.CG. ....C.CGC. ...C....T. ......T...
7  Laodelphax striatellus       .......... .......... ...A...AT. ....T....T- C...-.T... .A...A.CG. ....C.CGC. ...C....T. ......T...

                          1560*                1580*                1600*                1620*                1640*                1660*
1  ATAGAGCATT GTAATTATTG CTCTTCAACG AGGAATTCCT AGTAAGCGCA AGTCATCAGC TTGCGTTGAT TACGTCCCTG CCCTTTGTAC ACACCGCCCG TCGCTAGTAC CGATTGAATG
2  .......... .C........ .......... ......C... .......... .......... .......... .......... .......... .......... ......C... ..........
3  .......... .C........ .......... ......C... .......... .......... .......... .......... .......... .......... ......C... ..........
4  .......... .C........ .......... ......C... .......... .......... .......... .......... .......... .......... ......C... ..........
5  ..T.G.GC.. .C.......C .C.A.G.... .........C .........G ......A... .C........ .......... .......... .......... ...G..C... ..........
6  ..T.G.GC.. .C....G..C .C.A.G.... .........C .........G ......A... .C........ .......... .......... .......... ...G..C... ..........
7  ..T.G.GC.. .C....G..C .C.A.G.... .........C .........G ......A... .C........ .......... .......... .......... ...G..C... ..........
```

FIGURE 1. Alignment of a region of small nuclear rDNA from three species of planthoppers and their YLS. Only bases different from those of *S. cerevisiae* are shown and the same bases are indicated by dots. Hyphens show spaces. Numbers refer to position on 18S rDNA of *S. cerevisiae*.

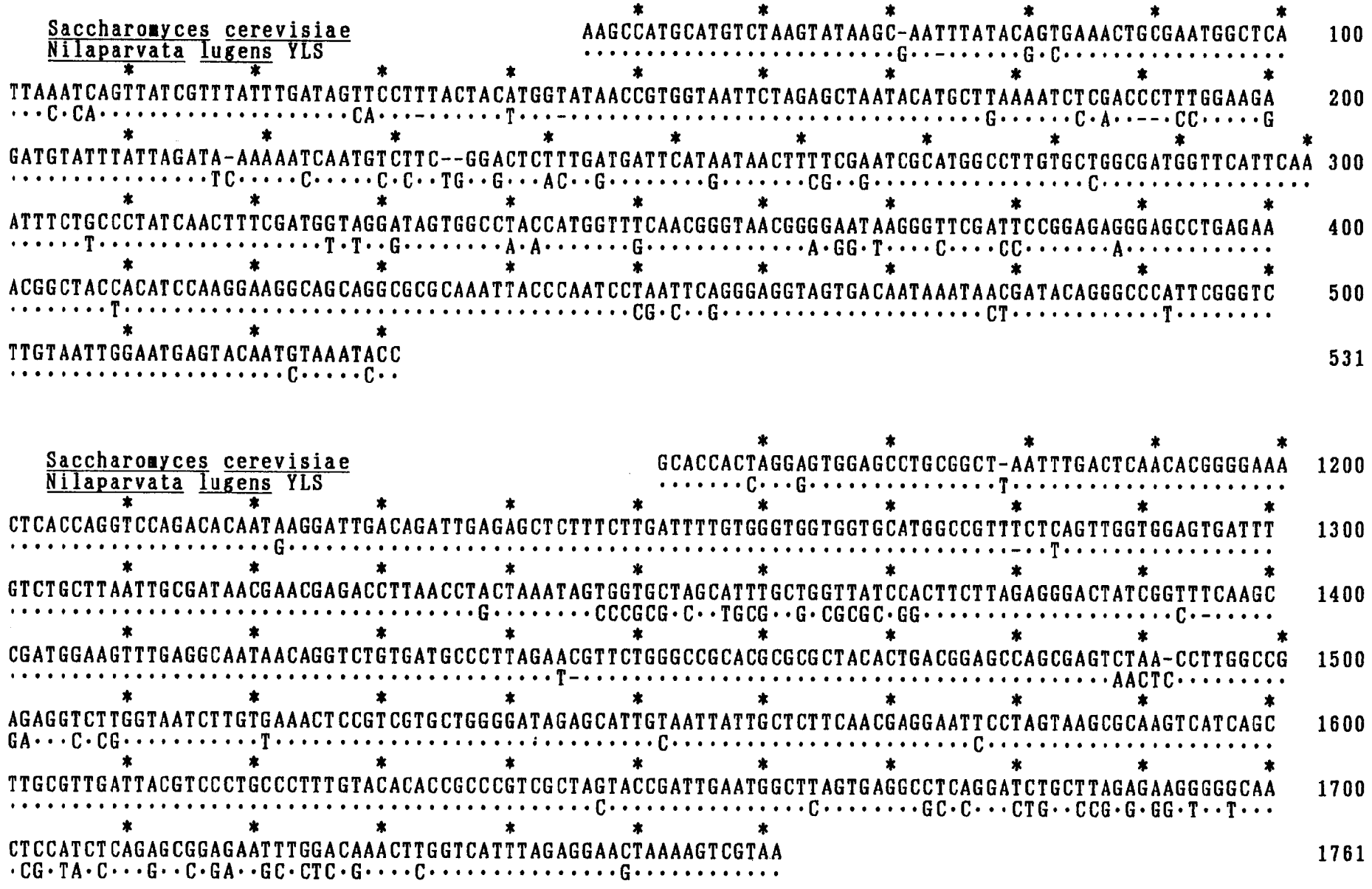

FIGURE 2. Partial sequence of small nuclear rDNA from *N. lugens* YLS. Top, the sequence amplified by NS1 and NS2 primers, 46–531 on 18S rDNA of *S. cerevisiae*; bottom, amplified by NS5 and NS8 primers, 1152–1761. Spaces (hyphens) are introduced to maintain optimal alignment.

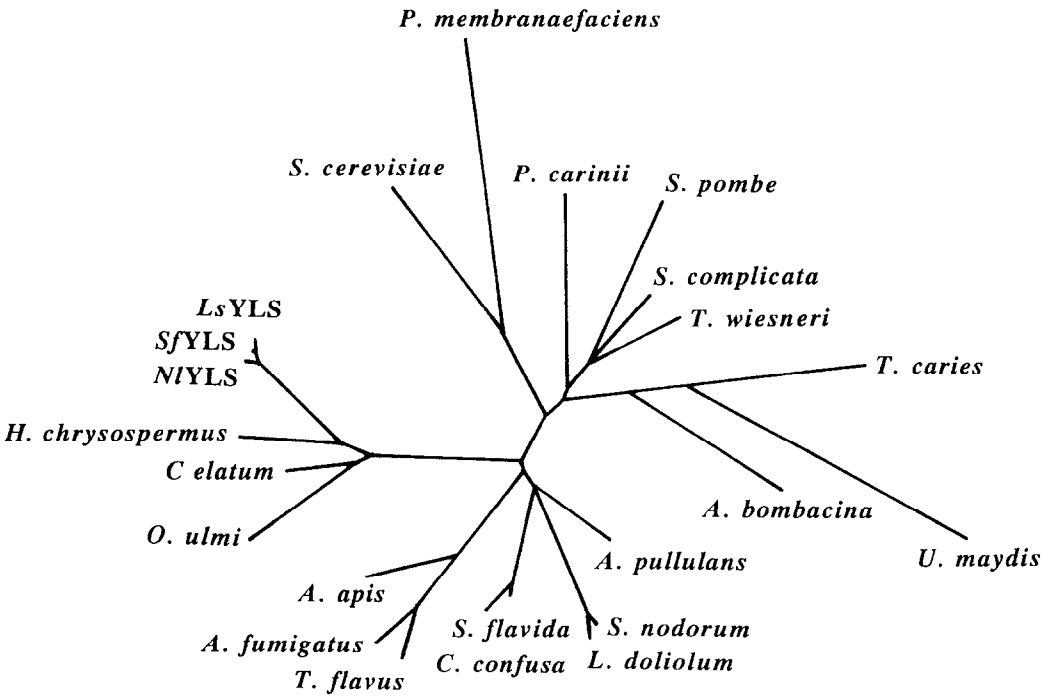

FIGURE 3. Phylogenetic relationships of three species of YLS to 20 selected species of higher yeasts and fungi. The tree was constructed by neighbor-joining procedure in the software package PHYLIP, version 3.5C, on the partial nucleotide sequences of 18S rDNA.

Ophiostomataceae, Ophiostomatales), *Chaetomium elatum*, *Sordaria fimicola*, *Podospora anserina* (the family Sordariaceae, Sordariales), *Hypomyces chrysospermus* (the family Hypomycetaceae, Clavicipitales), *Leucostoma persoonii* (the family Diaporthaceae, Diaporthales), *Glomerella cingulata* [*the family Polystigmataceae, Polystigmatales*; registered in the databases with anamorph name *Colletotrichum gloeosporioides*, cf. Wilmotte *et al.*, (1993)]. In total 15 species, including the above 11, three species of the YLS and one outgroup species, *A. apis* from the class Plectomycetes, were used. The tree indicates that YLS have a monophyletic origin and that they were close in genetic distance. The phylogenetic position of the YLS was closest to *H. chrysospermus* among the pyrenomycetes tested (Fig. 4).

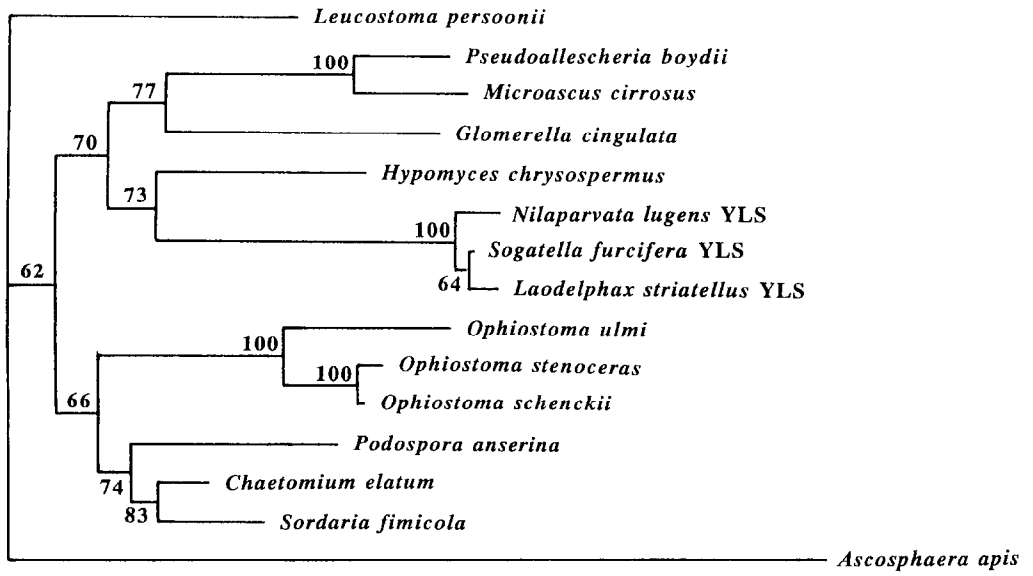

FIGURE 4. Phylogenetic relationships of three species of YLS in the class Pyrenomycetes. The tree was constructed by neighbor-joining procedure in the software package PHYLIP, version 3.5C, on the partial nucleotide sequences of 18S rDNA. The bootstrap percentages are placed alongside the node considered.

DISCUSSION

PCR is applicable to small amounts of DNA and does not require the purification of template DNA. These useful features enable the phylogenetic studies of bacterial and rickettsial symbiotes in insects (Munson *et al.*, 1991a; Campbell *et al.*, 1992; Clark *et al.*, 1992; O'Neill *et al.*, 1992; Rousset *et al.*, 1992a). In the present study, rDNA of the YLS were well amplified by taking advantage of broadly conserved regions of the rDNA. White *et al.* (1990) noted that the primers NS1 and NS2 amplify rDNA from a wide variety of fungi, protists and algae, and NS7 and NS8 also amplify some plant and vertebrate rDNAs. In the PCR, contamination of DNA of other species is sometimes a serious problem. The host insects have 18S rRNA as do the YLS, so that the YLS were purified before DNA extraction and the 18S rDNA sequences of both insect and YLS were compared first to confirm that the PCR products were from rDNA of YLS (Fig. 1).

The PCR primers could also be used for sequencing reactions. Direct sequencing avoids the time-consuming cloning processes. Moreover, we can neglect polymerase errors during PCR and sequencing reaction, which are serious problems in sequencing clones of PCR products. If different species of rDNA are mixed in template DNA, which may occur with the existence of plural species of eukaryotic microorganisms in the planthoppers, the sequencing data would become composites and hard to be identified by the direct sequencing. We were, however, able to obtain clear sequencing data. This suggests that the amplified rDNA is a single species or is composed of one dominant species. Therefore, the intracellular YLS flora in the fat body of each planthopper species must be composed of a single species.

The size and shape of YLS are not uniform among the rice planthoppers. Nasu and Suenaga (1958) noted that the *Sf*YLS is oval shaped and about 11.3 μm long, whereas *Nl*YLS is elongated and nearly 15 μm long. The size and shape of *Ls*YLS are similar to that of *Sf*YLS. The close phylogenetic relationship based on the sequence similarity of the 18S rDNAs shows that the three species of YLS have derived from a common ancestral species. Therefore, the difference in size and shape between *Nl*YLS and YLS of the other two planthopper species apparently arose in the host planthoppers after the endocytobiosis was established. Similar conclusion of the single ancient infection was also derived from the studies of symbiotic bacteria from certain aphids (Munson *et al.*, 1991a).

During the phylogenetic analyses of the members of the class Pyrenomycetes, we investigated *Taphrina deformans* because this species was shown to be a pyrenomycete (Wilmotte *et al.*, 1993). The analysis indicated that *T. deformans* and *H. chrysospermus* were monophyletic and their phylogenetic position was close to the YLS (data not shown). The genus *Taphrina*, however, together with the genus *Saitoella* is considered to have diverged prior to the separation of other ascomycetes (Nishida and Sugiyama, 1993). The present study supports this phylogenetic relationship of *Taphrina* and *Sitoella*: *T. wiesneri* and *S. complicata* are situated near *S. pombe* in Fig. 3. In this connection, other 18S rDNA sequence data of *T. deformans* is deposited in the DNA databases (Berbee and Taylor, 1993). This other sequence of *T. deformans* showed close placement to *T. wiesneri* in the phylogenetic tree (data not shown). We therefore excluded *T. deformans* from the analysis.

The phylogenetic study indicated that the YLS are the member of the euascomycetes and share ancestral species with members of the class Pyrenomycetes (Fig. 3). They have affinity with *H. chrysospermus* (Fig. 4). Müller and von Arx (1973) noted that Pyrenomycetes includes a number of different evolutionary lines which are presently placed in several orders. The order Sphaeriales, to which the YLS belong, is not well defined and the class Pyrenomycetes includes a broad range of fungi. The more rDNA sequence data from pyrenomycetes are accumulated, the more the precise phylogenetic position of the YLS will be defined. YLS are also observed in some scale insects (Buchner, 1965), aphids (Fukatsu and Ishikawa, 1992), and anobiid beetles (Jurzitza, 1979). Phylogenetic positions of the YLS of these insects are of great interest from a viewpoint of the origin of YLS in insects.

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