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Evolutionary Studies on Uricases of Fungal Endosymbionts of Aphids and Planthoppers

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Abstract. Aphids belonging to the three genera *Tuberaphis, Glyphinaphis,* and *Cerataphis* contain extracellular fungal symbionts that resemble endocellular yeast-like symbionts of planthoppers. Whereas the symbiont of planthoppers has a uricase (urate oxidase; EC 1.7.3.3) and recycles uric acid that the host stores, no uric acid was found in *Tuberaphis styraci,* and its fungal symbiont did not exhibit the uricase activity. However, the fungal symbionts of these aphids, including that of *T. styraci,* were shown to have putative uricase genes, or pseudogenes, for the uricase. Sequence analysis of these genes revealed that deleterious mutations occurred independently on each lineage of *Glyphinaphis* and *Tuberaphis,* while no such mutation was found in the lineage of *Cerataphis.* These genes were almost identical to those cloned from the symbionts of planthoppers, though the host aphids and planthoppers are phylogenetically distant. To estimate the phylogenetic relationship in detail between the fungal symbionts of aphids and those of planthoppers, a gene tree was constructed based on the sequences of the uricase genes including their flanking regions. As a result, the symbionts of planthoppers and *Tuberaphis* aphids formed a sister group against those of *Glyphinaphis* and *Cerataphis* aphids with high bootstrap confidence levels, which strongly suggests that symbionts have been horizontally transferred from the aphids' lineage to the planthoppers'.

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Introduction

Almost all homopterans harbor either eukaryotic or prokaryotic endosymbionts (Buchner 1965). These symbionts are obligatory and thought to play an important role in nitrogen metabolism of their hosts (Houk and Griffiths 1980). Nitrogen metabolism in the symbiotic system has been studied extensively, especially in aphids. Whereas the great majority of aphid species contain the bacterial symbiont, *Buchnera,* endocellularly (Buchner 1965; Munson et al. 1991), a few groups of the tribe Cerataphidini are exceptional in that they have extracellular yeast-like symbionts (YLSs), instead of *Buchnera* (Buchner 1958, 1965; Fukatsu and Ishikawa 1992; Fukatsu et al. 1994). These aphids comprise monophyletic three genera, *Tuberaphis, Glyphinaphis,* and *Cerataphis* (Fukatsu et al. 1994). While the roles played by *Buchnera* in the host's nitrogen metabolism are well understood (Sasaki and Ishikawa 1995; Douglas 1998; Shigenobu et al. 2000), the metabolic roles of these YLSs are totally unknown. In this regard, it is noteworthy that planthoppers bear YLSs that resemble those of aphids (Buchner 1965; Noda 1977; Chen et al. 1981). Molecular phylogenetic analyses have shown that the YLSs of planthoppers and that of the aphid, *Tuberaphis styraci,* belong equally to the class Pyrenomycetes in the subphylum Ascomycotina (Noda et al. 1995; Fukatsu and Ishikawa 1996). Evidence suggests that the YLS of

the brown planthopper, *Nilaparvata lugens*, is involved in the host's nitrogen metabolism (Sasaki et al. 1996; Hongoh and Ishikawa 1997). The planthopper produces and stores uric acid in its tissue when fed on diets rich in nitrogen and recycles the uric acid under adverse conditions (Hongoh and Ishikawa 1997). In this process, it is the uricase of the YLS that mobilizes uric acid (Sasaki et al. 1996; Hongoh et al. 2000).

In this study, in an effort to learn the origin and evolution of association between homopteran insects and their fungal symbionts, we examined whether the YLSs of Cerataphidini aphids play a similar role in the host's metabolism to that of the planthopper. Also, we compared, from an evolutionary point of view, several uricase genes of YLSs of homopteran insects and nonsymbiotic fungi that are thought to be phylogenetically related to YLSs.

Materials and Methods

Insects and Fungal Symbionts

A gall of *Tuberaphis* (or *Hamiltonaphis*) *styraci* (family Aphididae) on a twig of *Styrax obassia* was collected at Chichibu, Saitama, Japan. Aphids in the gall were sorted out, and their YLSs were isolated as described before (Hongoh et al. 2000). Other Cerataphidini aphids, *Tuberaphis taiwana*, *T. takenouchii*, *Cerataphis fransseni*, and *Glyphinaphis bambusae*, were collected in Taiwan (Fukatsu et al. 1994) and their DNA samples were provided by Dr. Fukatsu.

Nilaparvata lugens and *Laodelphax striatellus* (family Delphacidae; planthoppers) were maintained on rice seedlings at 25°C under a 16-h light:8-h dark photoperiodic regime. Isolation of their YLSs was also performed as above.

Acyrthosiphon pisum (family Aphididae) were maintained on young broad bean plants, *Vicia faba*, at 20°C under a 16-h light:8-h dark regime. Eggs and adults of *Lachnus tropicalis* (family Aphididae) were collected on the campus of Tamagawa University, Tokyo.

Other homopterans, *Ricania japonica* (family Ricanidae; a planthopper), *Geisha distinctissima* (family Flatidae; a planthopper), *Tettigella spectra* (family Tettigellidae; a leafhopper), and *Nephotettix cincticeps* (family Deltocephalidae; a leafhopper), were collected at Aizu, Fukushima, Japan, and stored at −80°C until processed.

Nonsymbiotic Fungi

Three deuteromycetous fungi, which were phylogenetically shown to belong to the class Pyrenomycetes (Nikoh and Fukatsu 2000), were used in this study. *Paecilomyces tenuipes* were collected from a lepidopteran worm at Amami-Ooshima, Kagoshima, Japan, and provided by Dr. Nikoh as a culture on a yeast extract peptone dextrose (YEPD) agar plate. *Beauveria bassiana* and *Tolypocladium niveum* were purchased from the Institute of Fermentation, Osaka (IFO), culture collection (B4848 and T31669, respectively). These fungi were maintained on Czapec yeast autolysate agar (CYA) plates containing 5 m*M* sodium urate at 20°C in the dark.

Detection of Uric Acid and Uricase Activity

Uric acid in whole tissues of planthoppers and leafhoppers was detected using uricase as described before (Sasaki et al. 1996; Hongoh and Ishikawa 1997). For detection of uric acid in eggs and whole tissues of aphids, reverse-phase high-performance liquid chromatography was employed (Hongoh and Ishikawa 1997).

Uricase assay was performed basically as described before (Sasaki et al. 1996; Hongoh et al. 2000). Insects, isolated YLSs, and mycelia of nonsymbiotic fungi were frozen in liquid nitrogen and crushed into a fine powder. The powder was suspended in 0.1 *M* Tris–HCl buffer (pH 9.25) containing 5 m*M* dithiothreitol (DTT), 2 m*M* EDTA, 10% glycerol, and a protease inhibitor cocktail (Boehringer Mannheim). The mixture was subjected to the purification steps including treatment with protamine sulfate and ammonium sulfate as described previously (Sasaki et al. 1996). The resulting mixture was dialyzed against 0.1 *M* Tris–HCl buffer (pH 9.25) containing 5 m*M* DTT overnight. The uricase activity of a sample, thus prepared, was determined by the decrease in absorbance at 292 nm (Hongoh et al. 2000). One unit was defined as the amount of enzyme necessary to convert 1 μmol of uric acid into allantoin in 1 min at 25°C and pH 9.25.

Protein concentration was estimated using the Bio-Rad protein assay (Bradford 1976) with bovine serum albumin as a standard.

DNA Preparation

Whole insect tissues were ground to powder in liquid nitrogen, and DNA was extracted according to the method described by Fukatsu and Ishikawa (1996).

DNE extraction from isolated YLSs of *T. styraci* and *N. lugens* was performed using Zymolyase-100T (Seikagaku) as described before (Hongoh et al. 2000).

DNA extraction from nonsymbiotic fungi was performed using DNAZOL (GIBCO). Mycelia peeled off the agar plate were frozen in liquid nitrogen and ground until powdered. The powder was suspended in DNAZOL solution and processed according to the manufacturer's instruction. The purified DNA was resuspended in 10 m*M* Tris–HCl buffer (pH 7.5) containing 1 m*M* EDTA (TE buffer) and stored at −30°C.

RNA Preparation

Isolated YLSs and mycelia of the nonsymbiotic fungi were frozen in liquid nitrogen and ground vigorously to a fine powder. The powder was suspended in Trizol solution (GIBCO) and processed according to the Trizol manual. The resulting precipitate was dissolved in diethylpyrocarbonate (DEPC)-treated H_2O and treated with DNase I (GenHunter) at 37°C for 30 min. Total RNA was extracted from the solution with phenol/chloroform and precipitated in ethanol. The purified total RNA was stored in 75% ethanol at −30°C.

Primers

Primers used in this study for polymerase chain reactions (PCRs) and sequencing are listed in Table 1. The degenerate primers, UO1 and UO3′, were designed based on the consensus amino acid sequences of fungal uricases (Hongoh et al. 2000). The primers, UO17B, UOAP4, UOR1, UOAP1, 5R3A, 5R2A, 5R2B, 5R3B, 5R4B, UO55, UO3R2, UOR2, UO55B, UO55B′, UOAP3, and UO55C were designed based on the sequence of the uricase gene of *N. lugens* YLS (Hongoh et al. 2000). Primers of the P0-7′, T0′-4, and B1′-4 series were designed based on the sequences of the uricase genes of *P. tenuipes*, *T. niveum*, and *B. bassiana*, respectively. The locations of these primers on the uricase genes are shown in Fig. 1.

Detection of a Uricase Gene from T. styraci *YLS*

A uricase gene on the genome of *T. styraci* YLS was detected basically according to the method we described previously (Hongoh et al. 2000).

Table 1. Primers used in this study for PCR, reverse transcriptase reaction, and sequencing

Specific primers for uricase genes of fungal endosymbionts	
UO17B	5′-ACGACTATCTCTGCACGTACCT-3′
UOAP4	5′-AAGGACACCCACGCACTACCC-3′
UOR1	5′-AGATGACCATAAGCTGCCTCAT-3′
UOAP1	5′-ATGGTGTTCTTGTAGGTGTC-3′
5R3A	5′-TGATGATGGAAGCGAAGAGCTC-3′
5R2A	5′-GGCGACGTGAATATGGCTGTA-3′
5R2B	5′-CGCATGGACGTTGACGGGAA-3′
5R3B	5′-CCCACAGCTTCATCAAAGACG-3′
5R4B	5′-GCGAAACGCGCAACGTCCAC-3′
UO55	5′-GTCGAGCAGAGCGCTGCTAAG-3′
UO3R2	5′-TCGCTGTATTACTCTCGAGCTG-3′
UOR2	5′-GCTCAAAATTGTGCTTGTTAGGCA-3′
UO55B	5′-ATCTCAGTTGGCACGGTGGCA-3′
UO55B′	5′-TGCCACCGTGCCAACTGAGAT-3′
UOAP3	5′-CTGATACGGTTTGGCGGAG-3′
UO55C	5′-GGATAACGACTAGCACGGCT-3′
Consensus primer sets for fungal uricase genes	
UO1	5′-TACGGCAAGGACAAYGT-3′
UO3′	5′-CAYTTDATNAGNCCRTTNGG-3′
Consensus primer sets[a] for fungal 18S rDNA	
NS1	5′-GTAGTCATATGCTTGTCTC-3′
NS2	5′-GGCTGCTGGCACCAGACTTG-3′
Specific primers for the uricase gene of *Paecilomyces tenuipes*	
H0	5′-CGCTACGGCAAGGACAATGTCC-3′
H0′	5′-GGACATTGTCCTTGCCGTAGCG-3′
H1′	5′-ACGCAGACGACGGTCTCGGTCGTCACGCTGTGC-3′
H2′	5′-GACGGTGTTCTTCATGGAGTCGGTGGCGACGAC-3′
H3	5′-CATCAACAGCGGCATCGCCGACCTCACCGTCC-3′
H4	5′-TCACGGCTTTATCCGCGACGAGTTCACCAC-3′
H6	5′-ACTTGAGCTGGCACAAGGGCATCAAGAGCACCG-3′
H7′	5′-CTCGACCTTGATGAGGCCCGTTGG-3′
Specific primers for the uricase gene of *Tolypocladium niveum*	
T0′	5′-TCACGGGTGACCTTGAGGA-3′
T2′	5′-GTGTTCTTSATGGAGTCGGTGG-3′
T3	5′-CTGGCCTGACGGTCCTTAAGAGC-3′
T4	5′-TGGCTTCGTGCGCGACGAGTTC-3′
Specific primers for the uricase gene of *Beauveria bassiana*	
B1′	5′-CTCGATGGCGCCCTCGAGGAGC-3′
B3	5′-CGGACCTCACGGTGCTCAAGAGC-3′
B4	5′-ACGGCTTCGTCCGCGACGACTTC-3′
Primers for reverse transcriptase reactions	
5′-PRT	5′P-AGGGTGGTGAAC-3′
3′-Adaptor	5′-GGCCACGCGTCGACTAGTAC-3′

[a] These primers were designed by White et al. (1990).

Purified DNA from whole tissues of *T. styraci* was completely digested with one of the restriction enzymes, *Apa*I, *Eco*RV, *Hin*dIII, and *Sac*I (Takara). The digested DNA was electrophoresed on 0.7% GTG–Agarose (FMC) and transferred onto Hybond N^+ (Amersham). The transferred DNA was hybridized with a PCR product from the uricase gene of *N. lugens* YLS as a probe. PCR for producing this probe was performed using the primers UOR1 and UOR2 (Table 1), and the products were labeled with ^{32}P-deoxycytidine 5′-triphosphate (dCTP), using a Bca-BEST DNA Labeling Kit (Takara). Hybridization was performed as described before (Hongoh et al. 2000). Signals were detected and analyzed by a MacBAS-2500 image analyzer (Fuji Film).

PCRs in this study were performed using EX-Taq polymerase (Takara).

Detection of Uricase mRNAs from T. styraci *YLS*

Purified total RNA from the isolated YLSs of *T. styraci* was subjected to a reverse transcriptase reaction, performed with random hexamers and Superscript II (GIBCO), according to the manufacturer's instruction. An aliquot of the reaction mixture was subjected to PCR using the degenerate primer set UO1 and UO3′ and the specific primer set UOR1 and UO55B′ for amplification of uricase cDNA and using the primers

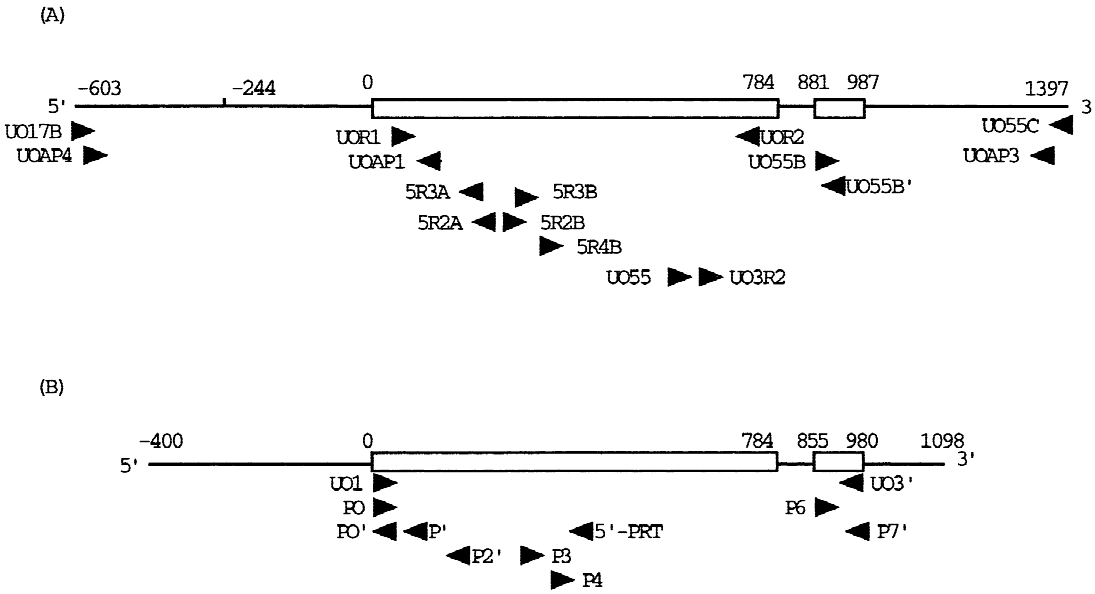


Fig. 1. Location of primers used in this study. **A** Primers for the uricase genes of the symbiotic fungi, represented by that of *N. lugens* YLS. **B** Primers for the uricase genes of the nonsymbiotic fungi, represented by that of *P. tenuipes. Open boxes* represent the two exons of the uricase genes. For the sequences of these primers, see Table 1.

NS1 and NS2 for that of cDNA of 18S rRNA (Table 1). Products from the former PCR were purified with agarose gel electrophoresis and Geneclean III (BIO 101) and cloned into pCR TOPO of a TOPO TA cloning kit (Invitrogen). The clones were sequenced.

Cloning of Uricase Genes from Fungal Symbionts of Homopterans

The entire uricase genes and their flanking regions from YLSs of the aphids and planthoppers were amplified by PCR using the primers designed based on the sequence of the uricase gene of *N. lugens* YLS (Table 1). The PCR products were purified, cloned as above, and sequenced.

Cloning of Uricase Genes and Their cDNAs from Nonsymbiotic Fungi

A reverse transcriptase reaction was performed with an oligo(dT)-polylinked adapter primer and Superscript II (GIBCO). An aliquot of the reaction mixture was subjected to PCR using the degenerate primers, UO1′ and UO3′. The products were purified, cloned as above, and sequenced.

For analysis of the 3′ end region of cDNA nested PCR was performed using the adapter primer and the specific primers (Table 1). The products were purified, cloned and sequenced.

A 5′-Full RACE Core Set (Takara) was used for analysis of the 5′ end region of cDNA, according to its protocol. A reverse transcriptase reaction was performed using the 5′-phosphorylated primer, 5′-PRT, and the products were circularized or concatemerized with T4 RNA ligase. These products were used as templates for nested PCR with the two specific primer sets (Table 1 and Fig. 1). The products were purified, cloned, and sequenced.

Uricase genes were amplified by PCR using the specific primers (Table 1). The products were directly sequenced.

DNA Sequencing

Clones were sequenced using an ABI PRISM 310 autosequencer. Samples were prepared using the DNA Cycle Sequencing Kit with Big-Dye Terminator (Perkin Elmer). The subcloned plasmids as templates were prepared in an Automatic DNA Isolation System (Kurabo). For direct sequencing, PCR products were purified with the Spin Column S-300HR (Pharmacia) for templates.

Sequence Analysis

GENETYX-MAC V8 (Software Development) was used for basic analysis of sequences except a multiple alignment of gene sequences, which was performed by DNASIS-MAC V3.7 (HITACHI), using Higgins's (1991) method. Distances in synonymous and nonsynonymous sites were calculated according to the method described by Li (1993). Phylogenetic analysis was performed by MEGA V1.01 (Kumar et al. 1993) with TreeExplore V2.12 (Tamura 1999) and PHYLIP V3.573 (Felsenstein 1993).

Results

Absence of Uric Acid in T. styraci

The presence of uric acid in the whole tissue was examined for *T. styraci* and other homopteran species. No uric acid was found in *T. styraci* or two other aphids containing *Buchnera,* while appreciable amounts were detected in planthoppers and leafhoppers (Table 2). Since homopterans, in general, do not excrete uric acid (Bursell 1967; Houk and Griffiths 1980; Sasaki et al. 1996; Hongoh and Ishikawa 1997), the presence of uric acid in the latter two groups is taken to suggest that the compound is utilized in these insects.

Absence of Uricase Activity in T. styraci

Uricase activity was not detected in either the whole tissue of *T. styraci* or its isolated YLSs, while activity of 1.2 and 19.8 mU/mg protein was detected in the whole tissue of *N. lugens* and its isolated YLSs, respectively.

Table 2. Presence of uric acid in whole tissues of homopteran insects

Species	Uric acid	Symbiont type
Auchenorrhyncha		
Fulgoroidea		
Nilaparvata lugens	+	Yeast-like[a]
Laodelphax striatellus	+	Yeast-like[a]
Ricania japonica	+	Yeast-like[b]
Geisha distinctissima	+	Yeast-like[b]
Cicadelloidea		
Tettigella spectra	+	Bacterial[a]
Nephotettix cicticeps	+	Bacterial[a]
Sternorrhyncha		
Aphidoidea		
Tuberaphis styraci	−	Yeast-like[c]
Acyrthosiphon pisum	−	Bacterial[a]
Lachnus tropicalis		
Adult	−	Bacterial[b]
Egg	−	

[a] Houk and Griffiths (1980).
[b] T. Fukatsu, personal communication.
[c] Fukatsu and Ishikawa (1992).

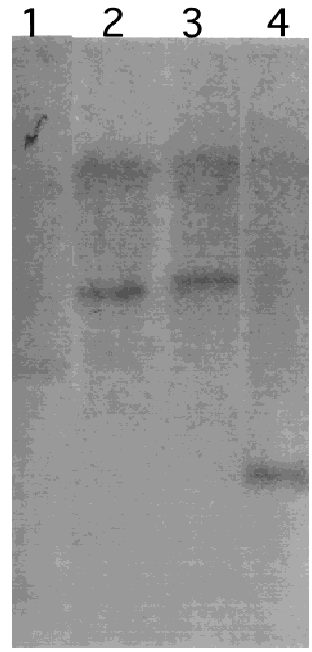


Fig. 2. Southern hybridization analysis of a genomic mixture of *T. styraci* and its symbiont. The genomic mixture was completely digested with one of the four restriction enzymes. A PCR product from the uricase gene of *N. lugens* YLS was used as a probe. 1, *Apa*I; 2, *Eco*RV; 3, *Hin*dIII; 4, *Sac*I.

Detection of a Uricase Gene and Its mRNAs from T. styraci *YLS*

The result that neither uric acid nor uricase activity was detected in *T. styraci* prompted us to detect a uricase gene and its transcripts in *T. styraci* YLS. As a result, a single copy of putative uricase gene was detected by Southern hybridization on a genomic mixture of *T. styraci* and its YLS (Fig. 2). Whereas a probe that was prepared based on the uricase gene of *N. lugens* YLS successfully detected the gene, probes prepared based on that of *Drosophila virilis* (X57114) did not (data not shown), suggesting that the uricase gene detected here was not of the insect, but of the YLS.

RT-PCR detected two transcripts of the uricase gene of *T. styraci* YLS (Fig. 3). Sequencing of these two transcripts revealed that they were completely identical to each other except for an insert of 100 bp and that their sequence was 96% identical to that of the uricase gene of *N. lugens* YLS. In spite of this high identity, an insert of dinucleotide was found in the cDNA of *T. styraci* YLS, which probably caused a frameshift mutation (Fig. 4).

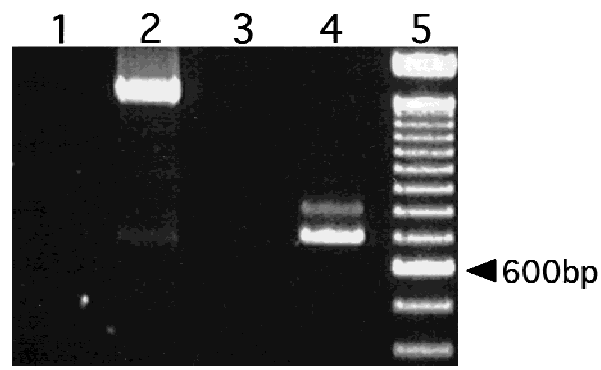
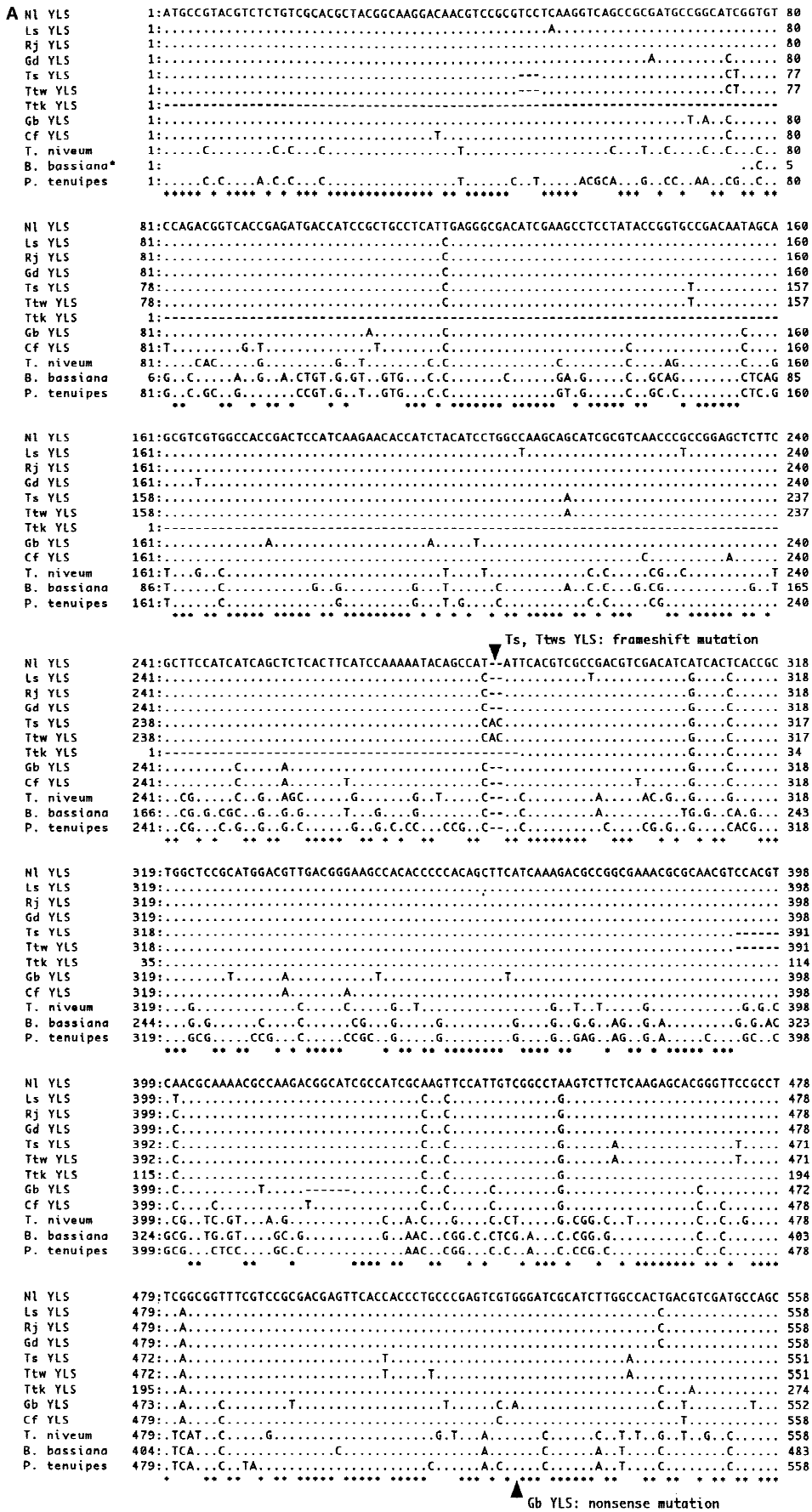


Fig. 3. Detection of transcripts of the uricase gene from *T. styraci* YLS. RT-PCR was performed with total RNA extracted from the isolated YLSs. The primers NS1 and NS2 (Table 1) were used for the amplification of cDNA of 18S rRNA (lanes 1 and 2), and UOR1 and UO55B′ (Table 1 and Fig. 1) were used for the uricase cDNA (lanes 3 and 4). 1, −RT; 2, +RT; 3, −RT; 4, +RT; 5, 100-bp ladder (GIBCO).

Cloning and Sequencing of Uricase Genes from Symbionts of Aphids and Planthoppers

Since the uricase cDNA of *T. styraci* YLS was almost identical to that of *N. lugens* YLS, we attempted to amplify entire lengths of uricase genes and their flanking regions from several YLSs by PCR, using the specific primers designed based on the sequence of the gene of *N. lugens* YLS (Table 1). Entire lengths of uricase genes were successfully amplified for the YLSs of *T. styraci*, *T. taiwana*, *T. takenouchii*, *G. bambusae*, and *C. fransseni*, as well as for those of the planthoppers, *L. striatellus*, *R. japonica*, and *G. distinctissima*. Their coding sequences that were split by single introns and the deduced amino acid sequences are aligned in Figs. 4 and 5, respectively. The complete sequence data including those on flanking regions will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers listed in the legend to Fig. 4.

It should be noted that the uricase genes cloned from the YLSs of *T. styraci* and *T. taiwana* shared the frameshift mutation described above and, in addition, a


A
Nl YLS
Ls YLS
Rj YLS
Gd YLS
Ts YLS
Ttw YLS
Ttk YLS
Gb YLS
Cf YLS
T. niveum
B. bassiana*
P. tenuipes
Ts, Ttws YLS: frameshift mutation
Gb YLS: nonsense mutation

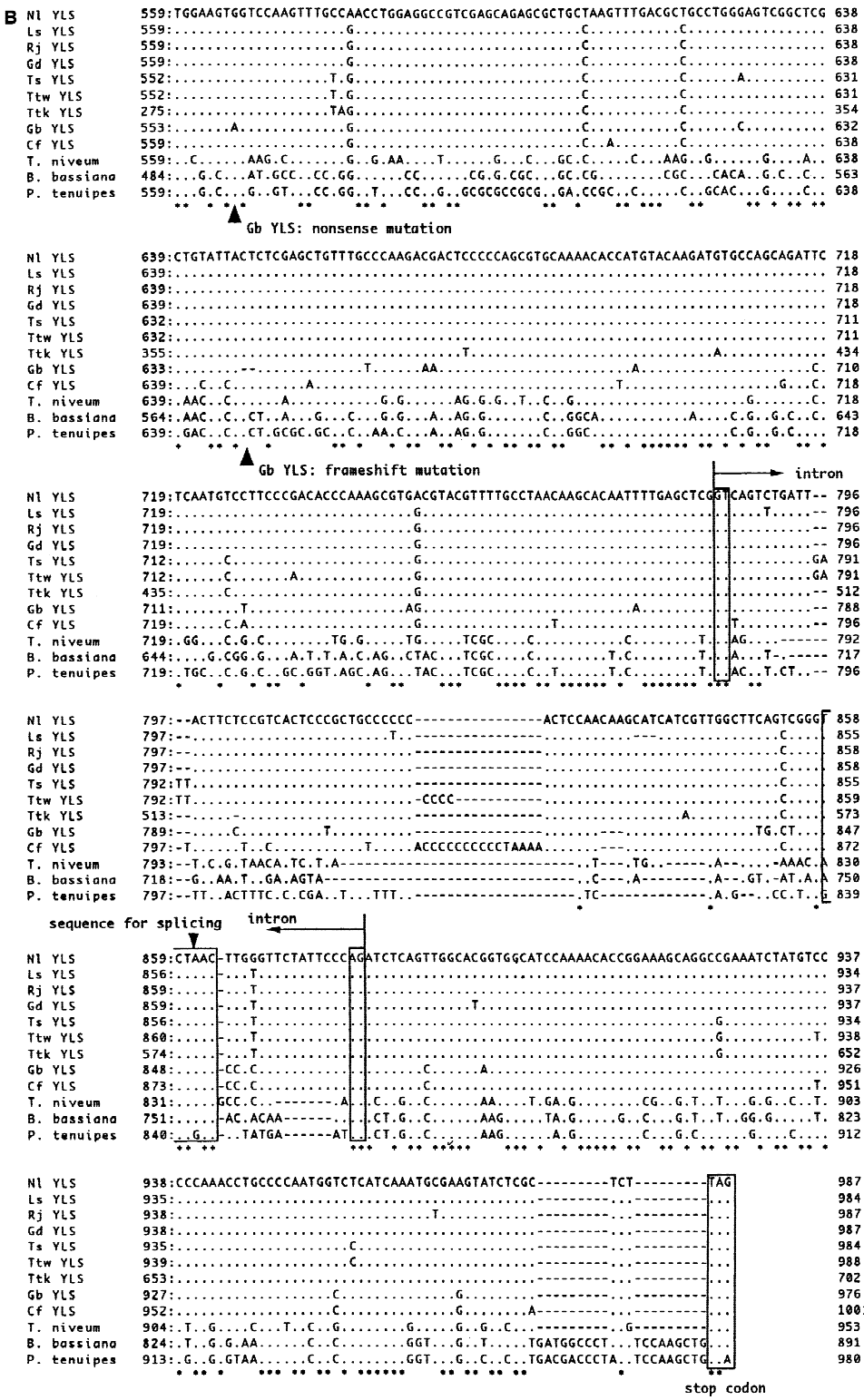


Fig. 4. Comparison of the sequences of the coding region and intron of the uricase genes of the symbiotic and nonsymbiotic fungi. The deleterious mutations found in the uricase genes of the YLSs of *T. styraci*, *T. taiwana*, and *G. bambusae* are indicated by *arrowheads.* A conserved region for splicing in the intron (Legoux et al. 1992) is *boxed.* Names of the organisms used are abbreviated as below, with accession numbers for complete sequences including flanking regions in parentheses. For planthoppers: Nl, *Nilaparvata lugens* (AB027293); Ls, *Laodelphax striatellus* (AB038699); Rj, *Ricania japonica* (AB038700); Gd, *Geisha distinctissima* (AB038701). For aphids: Ts, *Tuberaphis styraci* (AB038702); Ttw, *Tuberaphis taiwana* (AB038703); Ttk, *Tuberaphis takenouchii* (AB038704); Gb, *Glyphinaphis bambusae* (AB038705); Cf, *Ceratophis fransseni* (AB038706). For nonsymbiotic fungi: T. niveum, *Tolypocladium niveum* (AB038708); B. bassiana, *Beauveria bassiana* (AB038707); and P. tenuipes, *Paecilomyces tenuipes* (AB038709).

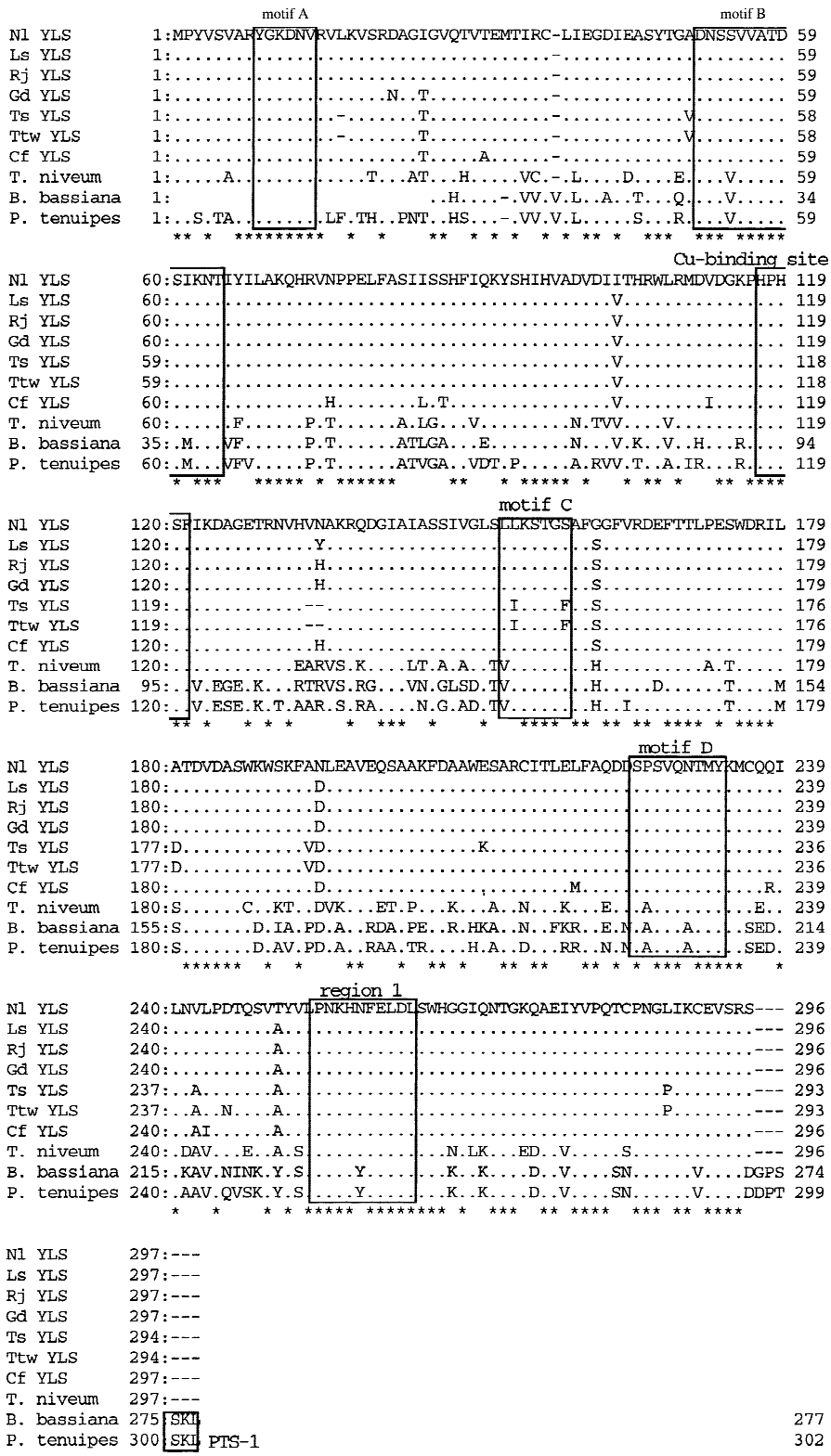


Fig. 5. Comparison of the deduced amino acid sequences of uricases of symbiotic and nonsymbiotic fungi. The consensus motifs [motifs A–D and region 1 (Hongoh et al. 2000; Koyama et al. 1996)] and the putative copper-binding site (Chu et al. 1996) are *boxed.* It is noted that the uricases of the symbionts and *T. niveum* lack the standard PTS-1 at the C-terminal extremity. The abbreviated names of species are the same as in Fig. 4.

deletion of 350 bp in the 5′ flanking region. In *T. takenouchii,* the deletion was larger, up to about 800 bp, reaching 300 bp downstream from the initiation codon. These uricase genes shared 96% nucleotides with that of *N. lugens* YLS, even in the 3′ flanking region. The large deletion and the frameshift were not found in the uricase genes of the YLSs of *G. bambusae* and *C. fransseni.* In the former, however, two nonsense mutations and a deletion of dinucleotide that probably caused a frameshift were found. Positions of these mutations are summarized in Fig. 6. In the latter, no loss-of-function mutation was found.

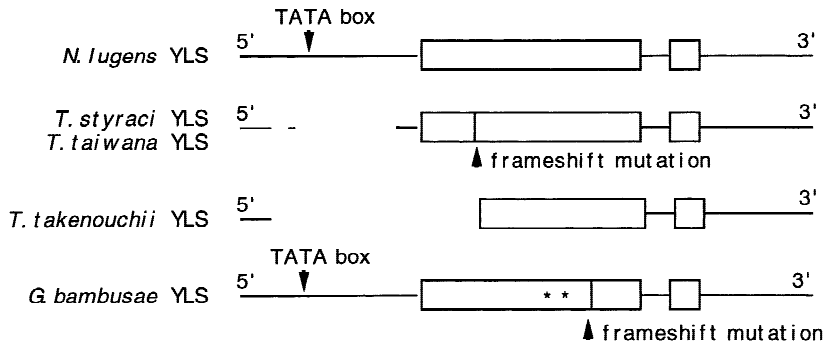


Fig. 6. Deleterious mutations on the uricase genes of fungal symbionts of Cerataphidini aphids. *Open boxes* represent the two exons of the uricase genes. *Arrowheads* indicate the location of putative TATA boxes. In *Tuberaphis* species, the 5′ flanking region, including the putative TATA box, was largely deleted. In addition, an insert of dinucleotide in the coding region causes frameshifts in *T. styraci* YLS and *T. taiwana* YLS. In *G. bambusae* YLS, two nonsense mutations (∗) were found in addition to the deletion of a dinucleotide.

Detection of Uricase Activity in Nonsymbiotic Fungi

To examine the effects of symbiosis on the evolution of uricases of the symbionts, uricase activity and sequences of uricase genes from nonsymbiotic fungi were determined for the sake of comparison.

Uricase activity was detected in all three deuteromycetes used in this study. The activities were 25, 15, and 30 mU/mg protein for *P. tenuipes, B. bassiana,* and *T. niveum,* respectively.

Cloning and Sequencing of Uricase Genes and Their cDNAs from Nonsymbiotic Fungi

Entire or partial segments of uricase genes and their cDNAs were cloned and sequenced from the nonsymbiotic fungi. Sequences of coding regions and the amino acid sequences deduced therefrom were aligned together with those of the YLSs in Figs. 4 and 5, respectively. All of the deuteromycetous uricase genes were split by single introns at the positions corresponding to those of the symbionts. Deduced molecular weights, isoelectric points, lengths, identities, and carboxyl extremities are compared in Table 3. The higher p*I* predicted for the uricases of *P. tenuipes* and *B. bassiana* were due mostly to an increase in arginine residues in variable regions.

The carboxyl extremity of the uricase of *T. niveum* was Ser–Arg–Ser, the same as that of the YLSs, while those of the other two nonsymbiotic fungi were Ser–Lys–Leu, which represents the standard peroxisomal targeting signal, PTS-1 (Table 3, Fig. 5).

GC Content and Transition/Transversion Substitution Rate

GC contents and rates of transition/transversion substitutions (Ts/Tv ratio) in the uricase genes were estimated. High GC contents were observed through all the uricase coding regions, which were 57.2–58.5% in the YLSs and 61.8–66.9% in the nonsymbiotic fungi. In the YLSs, the GC content was also high in the noncoding regions, especially in the 5′ flanking region (62.2–66.7%). The Ts/Tv ratio was determined by MEGA V1.01 and found to be biased in these fungal uricase genes. High Ts/Tv ratios were estimated among the uricase genes of the symbionts, which were 2.8–3.8 in aphid YLSs and 3.5–8.1 in planthopper YLSs. In contrast, the Ts/Tv ratios in nonsymbiotic fungi were as low as 0.58–0.68.

Synonymous and Nonsynonymous Substitutions in Uricase Genes

Since no deleterious mutation was found in the uricase gene of *C. fransseni* YLS, it was conceivable that the product is active and plays a certain role unlike in YLSs of *Tuberaphis* and *Glyphinaphis* aphids. To assess this possibility, d_s/d_N (distance at synonymous sites/distance at nonsynonymous sites) ratios were estimated among the fungal uricase genes and are shown in Table 4. If two genes have been inactive and free from selection pressure since they bifurcated, the theoretical d_s/d_N ratio between them should be no more than 1.0. Actually, the ratio was 1.1 in the comparison between *T. styraci* YLS and *T. taiwana* YLS, while the ratios were 3.0–7.5 among the YLSs of planthoppers and among the nonsymbiotic fungi. However, when compared between *T. styraci* YLS and *C. fransseni* YLS or *G. bambusae* YLS, the d_s/d_N ratio was as high as 8.6 or 3.5, respectively, both of which substantially exceeded 1.0 (Table 4).

Phylogenetic Analysis of Uricase Genes

The phylogenetic relationship of the YLSs was estimated by a gene tree of uricase constructed using the neighbor-joining method (Fig. 7). The YLSs of planthoppers and *Tuberaphis* aphids formed a sister group against the YLSs of the *Glyphinaphis* and *Cerataphis* aphids, with bootstrap confidence levels as high as 93–100. The maximum-likelihood tree constructed by PHYLIP V3.573 was completely compatible with the neighbor-joining tree (data not shown). The topology of the maximum-likelihood tree was not significantly affected by changing the Ts/Tv ratio setting from 2.0 to 10.0.

Table 3. Comparison of the length, molecular weight (MW), isoelectric point (p*I*), C-teminal tripeptide and identity (%) against *N. lugens* YLS among the uricases from symbiotic and nonsymbiotic fungi (MW and p*I* were deduced from the amino acid sequences)

Organism	Length (aa)	MW	p*I*	Carboxyl terminus	Identity[a] aa	Identity[a] nt
N. lugens YLS	296	32,882	6.06	–SRS	100.0	100.0
L. striatellus YLS	296	32,918	5.91	–SRS	98.3	98.0
R. japonica YLS	296	32,892	5.99	–SRS	98.3	98.3
G. distinctissima YLS	296	32,879	6.13	–SRS	97.6	98.0
C. fransseni YLS	296	32,919	6.18	–SRS	94.9	94.2
T. niveum	296	32,714	5.62	–SRS	77.0	78.4
B. bassiana[b]	—	—	7.03	–SKL	64.0	68.5
P. tenuipes	302	33,432	7.67	–SKL	61.9	67.7

[a] aa, amino acids; nt, nucleotides.
[b] The sequence was not completed.

Table 4. Distances in synonymous (d_S) and nonsynonymous (d_N) sites for the uricase genes[a]

Comparison	d_S	d_N	d_S/d_N
N. lugens YLS–*L. striatellus* YLS	0.0595 ± 0.0003	0.00789 ± 0.00001	7.5
L. striatellus YLS–*G. distincti* YLS	0.0253 ± 0.0001	0.00498 ± 0.00001	5.1
N. lugens YLS–*G. distincti* YLS	0.0397 ± 0.0001	0.0133 ± 0.0000	3.0
T. styraci YLS–*T. taiwana* YLS	0.0217 ± 0.0001	0.0195 ± 0.0003	1.1
T. styraci YLS–*G. bambusae* YLS	0.0921 ± 0.0004	0.0267 ± 0.0001	3.5
T. styraci YLS–*C. fransseni* YLS	0.133 ± 0.001	0.0154 ± 0.0000	8.6
P. tenuipes–B. bassiana	0.474 ± 0.020	0.0966 ± 0.0002	4.9

[a] The distances and standard errors were calculated according to the method described by Li (1993). The alignment gaps and the nonsense mutations in *G. bambusae* YLS were excluded.

Discussion

Neither uric acid nor uricase activity was detected in *T. styraci* (Table 2). In addition, the uricase gene of *T. styraci* YLS was shown to have a frameshift mutation (Fig. 6). The large deletion in the 5′ flanking region is also important, because the putative TATA box was lost due to this deletion (Fig. 6). These data indicate that, unlike planthoppers, *T. styraci* does not have a uric acid-mediated nitrogen recycling system. Since loss-of-function mutations in the uricase genes were also found in the YLSs of other *Tuberaphis* species and *G. bambusae* (Fig. 6), it is likely that a uric acid-mediated nitrogen recycling system does not exist in aphids that harbor fungal endosymbionts.

That uric acid was detected in the four species of planthoppers suggests that a uric acid-mediated nitrogen recycling system generally exists in planthoppers. Interestingly, uric acid was also detected in leafhoppers that bear bacterial endosymbionts (Buchner 1965; Houk and Griffiths 1980) (Table 2). It is likely that the presence of uric acid is related not to the type of symbiont, but to the phylogenetic lineage of host insects. This suggests that loss of the uricotelic ability of aphids occurred in their very early stage of evolution.

Although the uricase activity is not necessary for the aphid–fungus symbiosis as discussed above, it was suggested that an active uricase had been playing a certain role in fungal symbionts of aphids, at least, in the past. Otherwise, the d_s/d_N ratios in the comparison between the Cerataphidini aphid species should have been nearly 1.0. In reality, this was true only when compared between the *Tuberaphis* species (Table 4). When *T. styraci* YLS was compared with *G. bambusae* YLS, though their uricases are no longer active (Fig. 6), the d_s/d_N ratio was as high as 3.5 (Table 4). Comparison between *T. styraci* YLS and *C. fransseni* YLS showed an even higher ratio, 8.6 (Table 4). Taking these high d_s/d_N values together with the result that no loss-of-function mutation was found in the uricase gene of *C. fransseni* YLS (Fig. 4), it is possible that the uricase of this YLS still retains its activity and plays a certain role in the YLS's own nitrogen metabolism. It is conceivable that the uricase used to be necessary for a nonsymbiotic ancestor or ancestors of YLSs and that endosymbiosis with aphids, which were devoid of uricotelic ability, somehow lowered their dependence on the uricase activity. Probably, this led to the fixation of deleterious mutations such as deletion and frameshift on the uricase genes of many YLSs, while *C. fransseni* YLS alone happened to escape such mutations.

Endosymbiosis itself may have facilitated a spread of deleterious mutations. It has been shown that *Buchnera* have accumulated more deleterious mutations on their genes, as a result of acceleration of the evolutionary rate,

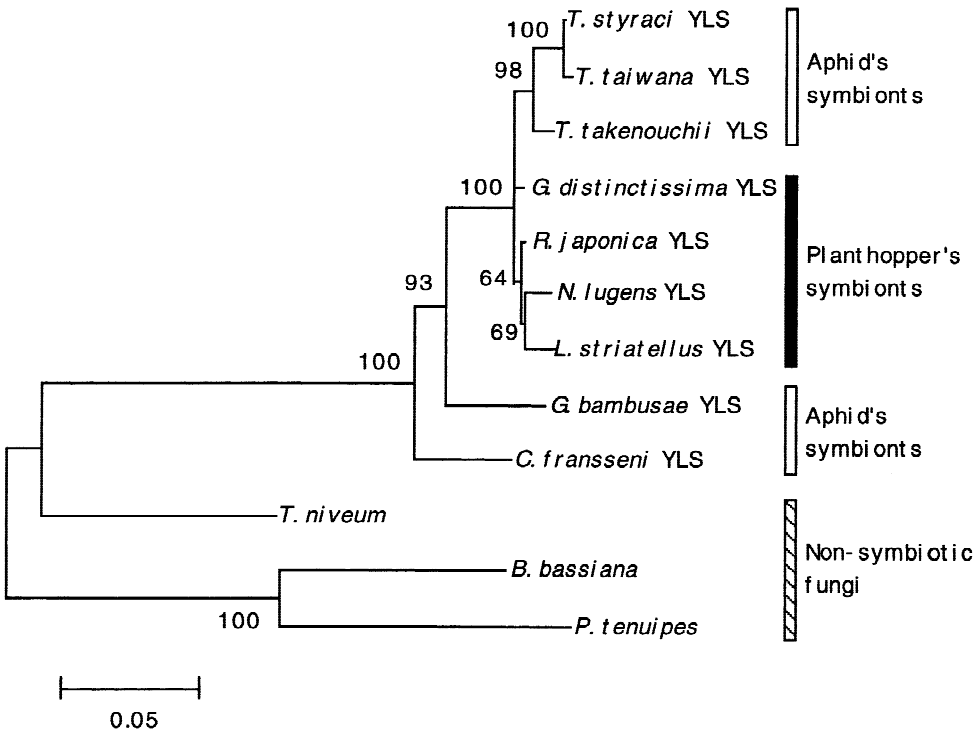


Fig. 7. Phylogenetic analysis of the uricase genes of the fungal symbionts. A bootstrap tree from the distance method (neighbor-joining) was constructed by MEGA V1.01. The entire uricase genes with their introns and flanking regions (total 2012 sites) were subjected to the analysis, with alignment gaps excluded pairwisely. The coding sequences of the uricase genes of the nonsymbiotic fungi were used as outgroups. The distances were calculated using the Tamura–Nei (1993) distance, because of the biased base contents and Ts/Tv ratios of these uricases (see text). The bootstrap test (Felsenstein 1985) was performed with 500 resamplings. The tree was condensed by a 50% cutoff level.

compared with their free-living relatives (Moran 1996; Lambert and Moran 1998; Wernegreen and Moran 1999). Like *Buchnera*, YLSs are vertically transmitted through host's generations and reproduce only in an asexual manner (Noda 1977). This implies that the effective population size of YLSs is relatively small and that the chance for interstrain recombination is limited. In addition, they experience a bottleneck effect at each host's generation. These factors may have caused a spread of deleterious mutations through the populations of YLS in a relatively short period of evolution.

Phylogenetic analysis based on 18S rDNA sequences has suggested a monophyletic relationship between YLSs of aphids and those of planthoppers (Fukatsu and Ishikawa 1996). Indeed, our analysis based on their uricase gene sequences strongly supported this view (Fig. 7). In this regard, it is necessary to discern between the two alternatives: (1) independent acquisitions of the same or closely related fungi by the two lineages and (2) horizontal transfer of a symbiont that was acquired by one lineage to the other. Our results clearly favor the second alternative. If it had not been for the horizontal transfer of a symbiont, acquisition of the symbiont by aphids and planthoppers would have had to occur, at least, four times independently to form the tree depicted in Fig. 7. Fukatsu et al. (1994) claimed that acquisition of YLSs by aphids should have been a single event because YLSs were found only in the three genera in the tribe Cerataphidini among the number of aphids surveyed to date, implying that the replacement of *Buchnera* by YLS is an extremely rare event. In addition, the molecular phylogenies of YLSs and their host aphids based on 18S rDNA sequences were concordant with each other, supporting their view (T. Fukatsu, personal communication). Taking these together, the most plausible scenario is as follows: a fungus that was once acquired by a lineage of Cerataphidini aphids became YLSs, one of which was transferred afterward to the lineage of planthoppers.

It has been suggested that the common ancestor of all extant aphid species acquired a bacterium about 200 million years ago, which eventually became *Buchnera* symbionts (Munson et al. 1991; Moran et al. 1993; Moran and Baumann 1994). It has also been suggested that only in a lineage of Cerataphidini was the *Buchnera* symbiont replaced later by a fungus, the ancestor of extant YLSs (Fukatsu et al. 1994). If this is the case, it follows that the transfer of YLSs from an ancestor of *Tuberaphis* aphids to the lineage of planthoppers, as assumed above, is a fairly recent event, suggesting that the recycling of uric acid exploiting YLS is a recent innovation in the lineage of planthoppers. In this context, it is noticeable that the uricase genes of planthopper YLSs remain active and those of *Tuberaphis* YLSs do not. This implies that the uricase gene of an ancestor of *Tuberaphis* YLSs re-

mained functional, at least, until being transferred to a planthopper and fixed deleterious mutations later in a relatively short length of time.

YLSs of aphids and planthoppers are thought to share a common ancestor belonging to the class Pyrenomycetes (Fukatsu and Ishikawa 1996). Indeed, our results suggest that the uricase of one of the pyrenomycetous fungi, *Tolypocladium niveum*, shares a common feature with those of YLSs. In these uricases, the C-terminal extremities were deleted in common, and as a result, they lack a standard PTS-1, Ser–Lys–Leu (Fig. 5). This tripeptide is known to comprise a necessary and sufficient peroxisomal targeting signal (Terlecky et al. 1996). As a peroxisomal protein, all the eukaryotic uricases sequenced to date have the consensus signal sequence, Ser/Ala–basic–Leu (Terlecky et al. 1996). The C-terminal tripeptide, Ser–Arg–Ser, shared by YLSs and *T. niveum* implies a close relationship among these fungi.

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