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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'.

Key words: Planthopper — Aphid — Fungus — Symbiosis —Symbiont — Uricase — Uric acid — Yeast-like — PTS

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

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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 cicticeps* (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 mM 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 mM Tris–HCl buffer (pH 7.5) containing 1 mM 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 diethyl-pyrocarbonate (DEPC)-treated H₂O and treated with DNase I (Gen-Hunter) 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).

	Specific primers for uricase genes of fungal endosymbionts
U017B	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'
U055	5'-GCGAGCAGAGCGCTGCTAAG-3'
U03R2	5'-TCGCTGTATTACTCTCGAGCTG-3'
UOR2	5'-TCGCTGTATTACTCTCGAGCTG-3'
UOR2	5'-ATCTCAGTTGGCACGGTGGCA-3'
UOS5B	5'-TGCCACCGTGCCAACTGAGAT-3'
UO55B'	5'-TGCCACCGTTCGCAGCGCG-3'
UOAP3	5'-CTGATACGGTTTGGCGGAG-3'
UOS5C	5'-CTGATACGACTAGCACGGCT-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'-GGCTGGCACCAGACTTG-3'
	Specific primers for the uricase gene of Paecilomyces tenuipes
H0	5'-CGCTACGGCAAGGACAATGTCC-3'
H0'	5'-GGACATTGTCCTTGCCGTAGCG-3'
H1'	5'-ACGCAGACGACGGTCTCGGTCGCGACGACGAC-3'
H2'	5'-GACGGTGTTCTTCATGGAGTCGGCGACGACGAC-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
TO'	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, *ApaI*, *Eco*RV, *Hind*III, and *SacI* (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 ³²P-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.

268

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

	Uric	Symbiont	
Species	acid	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).

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).

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*,



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, *ApaI*; 2, *Eco*RV; 3, *Hin*dIII; 4, *SacI*.



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).

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

1:ATGCCGTACGTCTCTGTCGCACGCTACGGCAAGGACAACGTCCGCGTCCTCAAGGTCAGCCGCGATGCCGGCATCGGTGT 80 A NI YES IS YES Rj YLS GA YIS Ts YLS Ttw YLS THE YES GE YLS CF VIS T. niveum B. bassiana' P. tenuipes NI YIS 81:CCAGACGGTCACCGAGATGACCATCCGCTGCCTCATTGAGGGCGACATCGAAGCCTCCTATACCGGTGCCGACAATAGCA 160 Ls YLS Ri YLS Gd YLS Te YIS Ttw YLS Ttk YLS GE YLS

 81:T.
 G.T.
 C.
 160

 81:T.
 CAC
 G.
 G.
 G.

 6:G.
 C.
 A.
 G.G.
 G.
 G.

 6:G.
 C.
 A.
 G.G.
 G.G.
 C.
 G.G.

 6:G.
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 G.G.
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 G.G.

 6:G.
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 G.G.
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 G.G.

 CF YLS T. niveum B. bassiana 81:G..C.GC..G.....CCGT.G..T..GTG...C.C.....GT.G....C.GC.C.....CTC.G 160 P. tenuipes 161:GCGTCGTGGCCACCGACTCCATCAAGAACACCATCTACATCCTGGCCAAGCAGCATCGCGTCAACCCGGCGGAGCTCTTC 240 N1 YES Ls YLS Rj YLS Gd YLS Ts YLS Ttw YLS T+k YIS Gb YLS CF YLS T. niveum B. bassiand P. tenuipes Ts, Ttws YLS: frameshift mutation NI YLS Ls YLS RI YLS GA YLS Ts YLS Ttw YLS T+k YIS YLS GЬ CF YLS T. niveum B. bassiana P. tenuipes 319:TGGCTCCGCATGGACGTTGACGGGAAGCCACACCCCCACAGCTTCATCAAAGACGCCGGGGAAACGCGCAACGTCCACGT 398 NI YLS Ls YLS Rj YLS Gd YLS Ttw YLS Ttk YLS Gb YLS Cf YLS T. niveum B. bassiana P. tenuipes N1 YLS 399:CAACGCAAAAACGCCAAGACGGCATCGCCATCGCAAGTTCCCATTGTCGGCCTAAGTCTTCTCAAGAGCACGGGTTCCGCCT 478 Ls YLS Rj YLS Gd YLS

 399: C.
 C.
 G.
 478

 399: C.
 C.
 G.
 478

 392: C.
 C.
 G.
 471

 392: C.
 G.
 G.
 A.
 71

 392: C.
 G.
 G.
 A.
 71

 392: C.
 G.
 G.
 A.
 71

 115: C.
 G.
 G.
 A.
 T.

 399: C.
 T.
 T.
 471
 471

 115: C.
 G.
 G.
 A.
 T.

 399: C.
 T.
 T.
 471
 471

 399: C.
 T.
 T.
 471
 471

 399: C.
 T.
 T.
 471
 471

 399: C.
 T.
 T.
 472
 524

 399: C.
 G.
 T.
 G.
 472

 399: C.
 T.
 T.
 C.
 C.
 G.

 399: C.
 T.
 G.
 G.
 G.
 G.
 478

 399: C.
 G.
 T.
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 478

 399: G.
 G. Ts YLS Ttw YLS Ttk YLS Gb YLS CF YLS T. niveum B. bassiana P. tenuipes N1 YES 479;TCGGCGGTTTCGTCCGCGACGAGTTCACCACCCTGCCCGAGTCGTGGGATCGCATCTTGGCCACTGACGTCGATGCCAGC 558 Is YIS Rj YLS 64 115 TS YLS Ttw YLS

 472: . A.
 T.
 T.
 T.
 S51

 195: . A.
 C.
 A.
 274

 473: . A.
 C.
 T.
 S52

 479: . A.
 C.
 T.
 S52

 479: . A.
 C.
 T.
 S58

 479: . TCAT.
 C.
 G.
 T.

 473: . C.
 C.
 T.
 S52

 479: . A.
 C.
 T.
 S58

 479: . TCAT.
 C.
 A.
 C.
 C.

 479: . TCA.
 C.
 A.
 C.
 A.

 479: . TCA.
 C.
 T.
 S58
 S6

 479: . TCA.
 C.
 T.
 S58
 S6

 479: . TCA.
 C.
 T.
 C.
 S58

 Gb YLS: nonsense mutation
 Gb YLS: nonsense mutation
 Gb YLS: nonsense mutation

 Ttk YLS

270

Gb YLS Cf YLS T. niveum B. bassiand P. tenuipes

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B N	I YLS	559:TGGAAGTGGTCCAAGTTTGCCAACCTGGAGGCCGTCGAGCAGAGCGCTGCTAAGTTTGACGCIGCC	IGGGAGICGGCICG	638
	s YIS	559:CC		638
D	G VIS	559:CC		638
		559. 6 6 6		638
6	0 115		• • • • • • • • • • • • • • • • • • • •	621
	s YLS	552	· · · A · · · · · · · · · · · · ·	0.51
т	tw YLS	552:CC		631
т	tk YLS	275:CCC		354
G	5 YIS	553:AC	C	632
č	C VIC	550 6 6 6 6		638
<u> </u>	1 163		· · · · · · · · · · · · · · · · · · ·	639
T	. niveum	559:	· · · · · · · · · · · · · · · · · · ·	636
В	. bassiana	484;G.CAI.GCLCC.GGCCCG.G.CGCGC.CGLGL	ACAG.CC	565
P	. tenuipes	559:G.CGGTCC.GGTCCGGCGCGCCGCGGA.CCGCCC	CACGC	638
		······································	** * ** **	
		GD ILS: NONSENSE MUTATION		
				74.0
N	I YES	639:CTGTATIACICICGAGCIGIIIGCCCAAGACGACICCCCAGCGIGCAAAACACCAIGIACAAGAIG	GIGCCAGCAGATIC	/18
L	s YLS	639:		718
R	1 115	639:		718
	A VIC	630+		718
	- VIC	632.		711
1	S YLS	632:		711
Ŧ	'tw YLS	632:		111
т	tk YLS	355:T.	A	434
G	ib YLS	633:A	c.	710
ſ	FYIS	639:CC		718
			6 C	718
	. neveum			643
8	. bassiana	564: AAC. (C. CI. A. G. C. C. G. G. A. AG. G. C. C. G. G. A. A. A. A. A. G. G. C. C. G. G. A.		74.0
P	'. tenuipes	639: GACCCI.GCGC.GCCAA.LAAG.GGGC		/18
			• • • • • •	
		▲ Ch YIS: frameshift mutation	5 . E. S. S.	
		ab its. indimession mutaction	intro	on
N	I VIC	719-TCAATGTCCTTCCCGACACCCAAAGCGTGACGTACGTTTTGCCTAACAAGCACAATTTTGAGCTCG	GTICAGTCTGATT	796
	- 115		7	706
Ľ	S TLS	713:		750
R	IJ YLS	719:	••••••••••••••	796
G	id YLS	719:		796
Т	s YLS	712:C	GA	791
T	+	712		791
, T	THE VIE	435 · C G		512
	LK ILS			700
G	ib YLS	711:	• • • • • • • • • • • • • • • • • • • •	/88
c	.f YLS	719:C.A	T	796
т	. niveum	719:.GGC.G.CTG.GTGTGTCGCC	AG	792
8	. bassiana	644:G.CGG.GA.T.T.A.C.AGCTACTCGCC	A T	717
p	, tenuines	719: TGCC.G.CGC.GGT.AGC.AGTACTCGCCTT.CT.	ACT.CT	796
			** ••	
N	1 1 1 5	797 · ACTTTTTTCGTTACTCCGCTGCCCCCCCACTCCAACAAGCATCATCGTT	GGCTTCAGTCGGG	858
			r	855
	S FLS	/3/		859
н	ig ves	/9/:	•••••••••••••••••••••••••••••••••••••••	0.00
G	id YLS	797:		858
T	's YLS	792:TT		855
т	tw YES	792:TT		859
Ť	+1 YI C	513	 .	573
			TG CT	847
9				
ι,	T TLS	/9/:=1	r	877
. т			c	872
8	. niveum	793:T.C.G.TAACA.TC.T.A		872 830
-	. niveum . bassiana	793:T.C.G.TAACA.TC.T.A		872 830 750
P	'. niveum 1. bassiana 1. tenuipes	793:T.C.G.TAACA.TC.T.A	C AAAAC.A AGTAT.A.A A.GCC.TG	872 830 750 839
P	'. niveum . bassiana . tenuipes -	793:T.C.G.TAACA.TC.T.A		872 830 750 839
P	'. niveum I. bassiana I. tenuipes sequence fo	793:T.C.G.TAACA.TC.T.A, 718:G.AA.TGA.AGTA, 797:TT.ACTTTC.C.CGATTTT, pr splicing intron		872 830 750 839
P	". niveum 3. bassiana 9. tenuipes sequence fo	793:T.C.G.TAACA.TC.T.A		872 830 750 839
P	 niveum bassiana tenuipes sequence for 1 YLS 	793:T.C.G.TAACA.TC.T.A		872 830 750 839 937
P	 niveum bassiana tenuipes sequence for 1 YLS s YLS 	793:T.C.G.TAACA.TC.T.A		872 830 750 839 937 934
P	 niveum bassiana tenuipes sequence for 'l YLS s YLS i YLS 	793:T.C.G.TAACA.TC.T.A		872 830 750 839 937 934 937
P	 niveum bassiana tenuipes sequence for NLS S YLS J YLS 	793:T.C.G.TAACA.TC.T.A		872 830 750 839 937 934 937 937
P	 niveum bassiana tenuipes sequence fi YLS YLS YLS YLS YLS YLS 	793:T.C.G.TAACA.TC.T.A- 718:G.AA.T.GA.AGTA- 797:TI.ACTTIC.C.CGA.TTTT. or splicing intron 859: CTAAC-TTGGGTTCTATTCCCAGATCTCAGTTGGCACGGTGGCATCCAAAACACCGGAAAGCAGGCG 856:		872 830 750 839 937 934 937 937 934
P N L R G	 niveum bassiana tenuipes sequence f(YLS YLS YLS YLS YLS YLS YLS YLS 	793:T.C.G.TAACA.TC.T.A- 718:G. AA.T.GA.AGTA- 797:TT.ACTTTC.C.CGA.TTTT or splicing intron 859: CTAAC-TTGGGTTCTATTCCCAGATCTCAGTTGGCACGGTGGCATCCAAAACACCGGAAAGCAGGCC 856:		872 830 750 839 937 934 937 937 934
P N L R G T T	 niveum bassiana tenuipes sequence foil YLS 	793:T.C.G.TAACA.TC.T.A.		872 830 750 839 937 934 937 937 934 937
P N L R G T T T T	 niveum bassiana tenuipes sequence for NLS YLS 	793:T.C.G.TAACA.TC.T.A-		872 830 750 839 937 934 937 934 937 934 938 652
P L R G T T T G	 niveum bassiana tenuipes sequence fi YLS 	793: - T. C. G. TAACA. TC. T. A T TG		872 830 750 839 937 934 937 934 938 652 926
P L R G T T T G	 niveum bassiana tenuipes sequence fill YLS YLS YLS YLS YLS YLS YLS tw YLS tk YLS tk YLS tk YLS tk YLS tk YLS 	793:T.C.G.TAACA.TC.T.A.		872 830 750 839 937 934 937 934 937 934 938 652 926 951
P N L R G T T T T G C	. niveum . bassiana . tenuipes sequence fi Il YLS .s YLS .j YLS .j YLS .j YLS .j YLS .tw YLS .tw YLS .tw YLS .th YLS .f YLS . riveum	793:T.C.G.TAACA.TC.T.A.		872 830 750 839 937 934 937 937 934 937 938 652 926 9251 903
P N L R G T T T T G C T	 niveum bassiana tenuipes sequence fi IL S SY LS JY LS YLS YLS YLS tw YLS tw YLS tw YLS tk YLS TLS TLS TS 	793:T.C.G.TAACA.TC.T.A.		872 830 750 839 937 934 937 934 937 934 938 652 926 926 926
P NLRG TT G C T B	. niveum J. bassiana . tenuipes sequence fi ls YLS is YLS id YLS is YLS itw YLS itw YLS itw YLS itw YLS itw YLS itw YLS . niveum bassiana	793:T.C.G.TAACA.TC.T.A.		872 830 750 839 937 937 937 937 937 938 652 926 951 903 823
P NLRGTTT GOTBP	 niveum bassiana tenuipes sequence f ILLS s YLS YLS YLS<	793: - T. C. G. TAACA. TC. T. A.		872 830 750 839 937 934 937 937 937 937 937 937 938 652 926 951 903 823 912
P N L R G T T T G G T T B P	 niveum tenuipes sequence f ll YLS is YLS ij YLS id YLS id YLS itw YLS <l< td=""><td>793:T.C.G.TAACA.TC.T.A. </td><td></td><td>872 830 750 839 937 934 937 937 934 937 934 938 652 926 951 903 823 912</td></l<>	793:T.C.G.TAACA.TC.T.A.		872 830 750 839 937 934 937 937 934 937 934 938 652 926 951 903 823 912
P N L R G T T T T G G T T B P	 niveum bassiana tenuipes sequence f YLS SYLS YLS YLS<td>793: - T. C. G. TAACA. TC. T. A. </td><td></td><td>872 830 750 839 937 934 937 937 934 937 938 652 926 951 903 823 912</td>	793: - T. C. G. TAACA. TC. T. A.		872 830 750 839 937 934 937 937 934 937 938 652 926 951 903 823 912
P NLRGTTTTGC TBP N	 niveum bassiana tenuipes sequence f IL YLS sy YLS YLS AYLS inveum bassiana tenuipes IL YLS 	793: - T. C. G. TAACA. TC. T. A. T TG		872 830 750 839 937 934 937 937 934 937 934 937 934 923 926 926 9251 903 912 9087
P N L L R G G T T T T G G C T T B B N L L	 niveum bassiana tenuipes sequence f YLS HUS HUS HUS ib yLS YLS is analytic tenuipes Il YLS SYLS 	793:T.C.G.TAACA.TC.T.A-		872 830 750 839 937 934 937 937 937 937 937 937 937 937 933 937 933 937 937
P N N L L R G G T T T T G G C T T B P N N R G G T T T R G G R R G R R R G R R R R	 niveum bassiana tenuipes sequence f IL YLS s YLS YLS Niveum bassiana tenuipes IL YLS S YLS S YLS YLS S YLS YLS S YLS YLS 	793: - T. C. G. TAACA. TC. T. A. T TG		872 830 750 .839 937 934 937 937 937 937 937 937 937 937 937 937
P N L R GG T T T G G C T T B P N L R G	 niveum bassiana tenuipes sequence f IL YLS s YLS j YLS j YLS itk YLS 	793:T.C.G.TAACA.TC.T.A.		872 830 750 937 934 937 937 934 937 937 937 937 934 938 926 951 963 823 912 987 987 987
P NLL RG TT TG CC T B B P NLL RG T	 niveum bassiana tenuipes sequence f IL YLS sy LS YLS YLS YLS YLS tw YLS tw YLS TYLS niveum bassiana tenuipes IL YLS Sy LS YLS is YLS 	793: - T. C. G. TAACA. TC. T. A. T TG		872 830 750 937 937 937 937 937 937 937 937 937 937
P N L R G C T T T G C C T T B P N L R G C T T	 niveum bassiana tenuipes sequence f ILLS sYLS jYLS dYLS is YLS itw YLS truy YLS truy YLS truy YLS truy YLS tenuipes tenuipes ILYLS sYLS jYLS dYLS sYLS jYLS dYLS sYLS jYLS dYLS itw YLS sYLS itw YLS 	793:T.C.G.TAACA.TC.T.A.		872 830 750 937 934 937 934 937 934 938 938 926 926 926 9251 903 823 922 926 938 938 938 938 938 938 938 938 937
P N L R G G T T T T G G C T T T G G C T T T G G C T T T G G C T T T G G T T T G G T T T T	 niveum bassiana tenuipes sequence f YLS SYLS YLS 	793: - T. C. G. TAACA. TC. T. A. T TG		872 830 750 839 937 934 937 934 937 934 937 934 938 652 951 983 926 951 983 912 987 984 987 987 987 987
P N L L R G G T T T S G C T T B P N L L R G T T T T T T	 niveum bassiana tenuipes sequence f ILLS sYLS JYLS JYLS JYLS HLS YLS TVLS 	793: - T. C. G. TAACA. TC. T. A.		872 830 750 839 937 934 937 934 938 652 926 938 938 926 938 938 938 938 938 938 938 937 934 938 938 937 934 938 937 934 938 937 937 937 937 937 937 937 937 937 937
P Nilrggttttgcctb P Nilrggttt G Ctb R G Ctt G Ctt G	 niveum bassiana tenuipes sequence f YLS SYLS YLS Y	793: - T. C. G. TAACA. TC. T. A. T TG. 718: - G AA. T GA. AGTA C A. 797: - TT ACTTTC. C. CGA. T TTT. TC. sor splicing intron 859: CTAAC TTGGGTTCTATTCCCAGATCTCAGTTGGCACGGTGGCATCCAAAACACCGGAAAGCAGGCC 856: T. T. 859: T. T. 859: T. T. 856: T. T. 860: T. T. 874:		872 830 750 839 937 934 937 934 938 652 926 652 9251 903 823 912 987 987 987 987 987 987 987 987 987
P N LL R GG T T T T G G C T T B P N LL R G T T T G C T T T T T G C T T T T G C T T T T T T G C T T T T T T G C T T T T T T T T T T T T T T T T T T T	. niveum . bassiana . tenuipes sequence f II YLS .s YLS .s YLS .iy YLS .iy YLS .t YLS .t YLS .niveum .bassiana .tenuipes II YLS .s YLS	793: - T. C. G. TAACA. TC. T. A. T TG		872 8390 750 8399 937 934 937 937 934 937 937 934 937 937 934 937 937 934 938 912 987 984 982 984 988 702 984 984 984 937
P Nijrgg Tttgg Ttgg Nijrgg Ttgg Ttgg T	. niveum . bassiana . tenuipes sequence f II YLS .s YLS .s YLS .s YLS .s YLS .tk YLS .tk YLS .tk YLS .niveum .bassiana .tenuipes II YLS .s YLS	793:T.C.G.TAACA.TC.T.A.		872 830 339 937 934 937 934 937 934 937 934 937 924 925 926 920 987 988 903 823 912 987 9887 9887 9887 9887 9887 9887 9987
P N LLRRGTTTTGCTTB P N LLRRGTTTTGCCT B	. niveum . bassiana . tenuipes sequence f II YLS .s YLS .j YLS .j YLS .j YLS .itw YLS . triveum . bassiana . tenuipes II YLS .s YLS .j YLS .j YLS .s YLS .itw YLS .s YLS .itw YLS .itw YLS .itw YLS .itw YLS .j YLS .niveum .bassiana	793: - T. C. G. TAACA. TC. T. A. T TG		872 839 750 839 937 934 937 937 937 937 937 937 937 937 937 937
P NLLRGTTTGCTBP NLLRGTTTGCTBB	. niveum b. bassiana . tenuipes sequence f II YLS is YLS is YLS is YLS is YLS it YLS is YLS it YLS . niveum bassiana . tenuipes II YLS is YLS	793:T.C.G.TAACA.TC.T.A.	A	872 830 339 937 934 937 937 934 937 938 852 926 928 926 928 928 912 987 987 987 987 988 702 978 988 702 978 988 702 976 988 702 978 988 702 978 988 702 978 988 702 978 978 978 978 978 978 978 978 978 978
P N LLRGTTTTGCTTBP N LLRGTTTTGCTTBP BP	 niveum bassiana tenuipes sequence f YLS SYLS YLS Y	793: - T. C. G. TAACA. TC. T. A. T TG. 718: - G AA. T GA. AGTA C A. 797: - TT. ACTTTC. C. CGA. T TTT. C A. or splicing intron TC. 859: CTAAC TTGGGTTCTATTCCC AGATCTCAGTTGGCACGGTGGCATCCAAAACACCGGAAAGCAGGCC 859: CTAAC TTGGGTTCTATTCCC AGATCTCAGTTGGCACGGTGGCATCCAAAACACCGGAAAGCAGGCC 859: T.		872 830 333 937 934 937 934 937 937 938 652 926 951 903 823 912 987 987 988 912 987 984 987 984 987 984 9987 9984 9987 9984 9989 9989
P N LLRRGTTTGCCTB BP N LLRRGCTTGCCTB P	. niveum . bassiana . tenuipes sequence f Il YLS .s YLS .s YLS .i YLS .i YLS .i YLS .i YLS . niveum . bassiana . tenuipes Il YLS .s YLS	793: - T. C. G. TAACA. TC. T. A. T TG. 718: - G AA. T GA. AGTA C		872 830 839 937 934 937 937 937 938 652 926 928 926 938 912 987 984 987 984 984 984 984 984 984 984 984 984 984

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: NI, *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, Cerataphis fransseni (AB038706). For nonsymbiotic fungi: T. niveum, Tolypocladium niveum (AB038708); B. bassiana, Beauveria bassiana (AB038707); and P. tenuipes, Paecilomyces tenuipes (AB038709).

NI YLS 1. 1. MYVSCALFÜRENVERVLÄVSERAGLOV/JUTYEPTTRLIEDDIEASUTAJENSEVATE 59 RJ YLS 1			motif A motif B	
Le YLS 11	Nl	YLS	1:MPYVSVAFYGKDNVRVLKVSRDAGIGVQTVTEMTIRC-LIEGDIEASYTGADNSSVVATD	59
Rj TLS 1. 1. 59 TW YLS 1. 7. 7. 58 TW YLS 1. 7. 7. 58 TW YLS 1. 7. 7. 58 Tr VLS 1. 7. 7. 7. 58 Cf YLS 1. 7. 7. 7. 7. 59 T. niveum 1. 7. 7. 7. 7. 7. 7. 7. B. bassiana 1. 7. 7. 7. 7. 7. 7. 7. B. VIS 60: 60: 7. <td< td=""><td>Ls</td><td>YLS</td><td>1:</td><td>59</td></td<>	Ls	YLS	1:	59
Gd YLG 1:	Rj	YLS	1:	59
TW YLS 1:	Gđ	YLS	1:	59
TW YLS 1:	Τs	YLS	1:VV	58
Cr VIS 1 1	Tt	W YLS	1:VV	58
T. Diveum 1:A.	Cf	YLS	1:	59
B. Daesiana 1:	т.	niveum	1:A	59
P. Cenuipes 1::::::::::::::::::::::::::::::::::::	в.	bassiana		34
Cu-binding site N1 YLS 60: SIRNTYILAKQHKVNPPELASIISSHFJQXYSHHVADVDJTTHWULMVUVUKIHH 119 PK 1LS 60:	Ρ.	tenuipes	1:S.TA	59
NI YLS 60: SIXMI YILAKQHRVNPPELFASIISSHFIQXYSHIHVADVDI ITHEWURDVDGILHEH IJ910 La YLS 60:			On hinding.	
Int 1:00 00: 21:NII 1:12:QUIVE FILE FILES FILES FILES FILES FILENCE VICE FILENCE VICE FILES RY LIS 60:	דא	VIC		110
R TLS 60:	Te	TDS VIC	60. U	110
Gd YLS 60:	Ri	VLS	60:	119
TS ULS 59:	Gđ	YLS	60:V	119
Ttw YLS 59:	Ts	YLS	59:V	118
Cf YLS 60:	Tt	w YLS	59 :	118
T. niveum 60: F P. T A. I.G V M. TVV V 119 B. bassiana 35: M F P. T ATUGA E N V.K. V. H R 94 P. tenuipes 60: M F P. T ATUGA VDT. P A. RVV. T. A. IR R 119 T This 120: STIKDACETRNVHVNAKRQDCIALASSIVCLSTLKSTCSPROEPVROEFTILESWDRIL 179 RI VLS 120: Y S 179 RJ VLS 120: H S	Cf	YLS	60:V	119
B. bassiana 35: M. VF P. T ATUGA E N. V.K. V. H. R 94 P. tenuipes 60: M. VFV. P. T ATUGA .VDP.P. A.RVV.T. A.IR 119 motif C NI YLS 120: FIKDAGETERNVHVNARKOGCIALASSIVULSATISTCSPEGEPVEPETTLPESWDELL 179 Ls YLS 120: Y S 179 Gd YLS 120: H S 179 Gd YLS 120: H S 179 Ts YLS 119: I. F. S 176 Cf YLS 120: H S 176 Cf YLS 120: EARVS.K. LT A. T S 179 B. bassiana 95: V. EGE K. RTRVS.KG. VN.GLSD.TV H A. T M 154 P. tenuipes 120: EARVS.K. LT A. A. TV. H M. T M 154 P. tenuipes 120: EARVS.K. LT A. A. TV. H A. T M 154 P. tenuipes 120: EARVS.K. LT A. A. TV. H M. T M 154 P. tenuipes 120: D CARVS.K. LT A. A. TV. H A. T M 154 P. tenuipes 120: D CARVS.K. LT A. A. TV. H A. T M 154 P. tenuipes 120: D CARVS.K. LT A. A. TV. H D T M 154 P. tenuipes 120: D CARVS.K. LT A. A. TV. H D T M 154 P. tenuipes 120: D CARVS.K H C. AD. TV H D T M 154 P. tenuipes 120: D 239 FX YLS 180: D 239 FX YLS 180: D 236 TT YLS 180: D 236 TT YLS 180: D 236 TT YLS 180: D M A 236 TT YLS 177: D VD K A. N K. E. A E. 239 B. bassiana 155: S D. IA. PD. A. RAA. R A A E. 239 B. bassiana 155: S D. IA. PD. A. RAA. R A 236 TT YLS 240: A	т.	niveum	60:	119
P. tenuipes 60: M. JFVP.T. ATVGA. UDT. PA.RVV. T. A.I.R. R. 119 motif C motif C NI YLS 120: FIXDAGETRNVHVNAKRQDGIALASSIVELSLEXSTCSPRGEFVRDEFTLPESWDRIL 179 Ri YLS 120:	в.	bassiana	35:.MVFP.TATLGAENV.KVHR.	94
The second sec	₽.	tenuipes	60:.MVFVP.TATVGAVDT.PA.RVV.TA.IRR.	119
Instit C Instit C N1 YLS 120:STIKDAGETENVHVNAKKQCJAIASSIVGLSIJKSTGSPCGFVRDEPTTLPESWDRLL 179 Rj YLS 120:			* *** ***** * ****** ** * ***** * * * *	
NI YLS 120: STIKDACETENNYHVNAKROCIALASSIVGLSTEKSTYGLSTEKSTYGLEVENCEVRDETTLPESWORL 179 Rj YLS 120:			motif C	
Ls YLS 120:	Nl	YLS	$120: {\tt SFIKDAGETRNVHVNAKRQDGIAIASSIVGLS} {\tt LKSTGSAFGGFVRDeFTTLPESWDRIL}$	179
Rj YLS 120:	LS	YLS	120:Y	179
Gd YLS 120:	Rj	YLS	120:H	179
TS YLS 119:	Gđ	YLS	120:	179
Twy NS 119: H.	Ts	YLS	119:	176
CL 1LS 120: 1	TU	WIC VIC	119:	170
11. Hovedin 100.1.1	UL T	niucum	120. FARVER IT A TRY H A T	170
D. DESSIMIN 30.1 P. tenuipes 120:: V.ESE.R.T.AAR.S.RAN.G.AD.TV	1. B	haceiana	95 VECE K RURUS RG VN GLSD TW H D T M	154
motif D motif D NI YLS 180: ATDVDASWKWSKPANLEAVEQSAAKFDAAWESARCITLELFAQDISPSVQNTMYMCQ01 239 Ri YLS 180: D. D. 239 Gd YLS 180: D. 239 Gd YLS 180: D. 239 To YLS 177: D. 200 To YLS 100: S C. KT. DVKET P. K A. N K. K D RA	р. Р	tenuipes	1207. V. ESE, K. T. AAR, S. RA N. G. AD. TV	179
motif D N1 YLS 180:ATDVDASWKWSKFANLEAVEQSAAKFDAAWESARCITLELFAQDISPSVQNTMYLMCQQI 239 Ls YLS 180:		conar <u>p</u> es	*** * * * * * * * * * * * * * * ****	
N1 YLS 180:ATDVDASWKWSKFANLEAVEQSAAKFDAAWESARCITLELFAQDISPSVQNTMY (MCQQI 239 Ls YLS 180:			motif D	
Ls YLS 180:	Nl	YLS	180: ATDVDASWKWSKFANLEAVEQSAAKFDAAWESARCITLELFAQDDSPSVQNTMYKMCQQI	239
Rj YLS 180:DDD	Ls	YLS	180:	239
Gd YLS 180:	Rj	YLS	180:D	239
Ts YLS 177:DVDK	Gđ	YLS	180:DD	239
Ttw YLS 177: D.	Ts	YLS	177:DVDK	236
Cf YLS 180:	Ttr	V YLS	177:DVD	236
T. niveum 180:SCRT. DVAET.PKA.NK.L.E.A	CI	YLS	180:M	239
B. Dassiana 155:5D.A.PD.A. RAA.PD.A. RAA.TRH.A.D. RR.I.M.A.A.D. SED. 214 P. tenuipes 180:SD.AV.PD.A. RAA.TRH.A.D. RR.N.N.A.A.D. SED. 239 ***** ******************************	т.	niveum	180: S C KT DVK ET. P K A N K E A	239
P. tellinges 180:35	в.	bassiana	100.C	214
region 1 N1 YLS 240:LNVLPDTQSVTYV/PNKHNFELDISWHGGIQNTGKQAEIYVPQTCPNGLIKCEVSRS296 Rj YLS 240:A. Gd YLS 240:A. Ts YLS 240:P. S YLS 240:P. S YLS 240:P. S YLS 240:P. S YLS 237:A. A. P. P. 293 Ttw YLS 237:A. A.	₽.	centripes	100:5D.AV.PD.AAA.IKII.ADKW.I <u>.A</u> .SED.	239
N1 YLS 240: LNVLPDTQSVTYVLPDKHNFELDISWHGGIQNTGKQAEIYVPQTCPNGLIKCEVSRS 296 Ls YLS 240:A.			region 1	
Ls YLS 240:A.	Nl	YLS	240: LNVLPDTOSVTYVIPNKHNFELDISWHGGIONTGKOAEIYVPOTCPNGLIKCEVSRS	296
RJ YLS 240:A. 296 Gd YLS 240:A. 296 Ts YLS 237:.A. A. 293 Ttw YLS 240:.AI A. 296 T. niveum 240:.DAVE. A.S 296 B. bassiana 215:.KAV.NINK.Y.S	Ls	YLS	240:A.	296
Gd YLS 240:A. P	Rj	YLS	240:A.	296
TS YLS 237:AA. P	Gđ	YLS	240:A	296
Ttw YLS 237:A.NA.	TS	YLS	237:	293
Cf YLS 240:AIA	Ttı	V YLS	237: . AN A	293
T. niveum 240:.DAVEA.S	Cf	YLS	240:AIA	296
B. bassiana 215: KAV.NINK.Y.SYK.KD.VSNVDGPS 274 P. tenuipes 240: AAV.QVSK.Y.SYK.KD.VSNVDDPT 299 * * * * * * * * * * * * * * * * * * *	т.	niveum	240: .DAVEA.S	296
P. tenuipes 240:.AAV.QVSK.Y.SY	в.	bassiana	215: .KAV.NINK.Y.SYKKDVSNVDGPS	274
* *	Ρ.	tenuipes	240: .AAV.QVSK.Y.SY	299
N1 YLS 297: Ls YLS 297: Rj YLS 297: Gd YLS 297: Ts YLS 294: Ttw YLS 294: Cf YLS 297: T. niveum 297: B. bassiana 275 SKI PTS-1 302			* * * * ***** ****** * *** ** *** *** ***	
NI YLS 297: Ls YLS 297: Rj YLS 297: Gd YLS 297: Ts YLS 294: Ttw YLS 294: Cf YLS 297: T. niveum 297: B. bassiana 275 SKI PTS-1 302				
Ls YLS 297: Rj YLS 297: Gd YLS 297: Ts YLS 294: Ttw YLS 294: Cf YLS 297: T. niveum 297: B. bassiaa 275 SKI 75 277 P. tenuipes 300 SKI PTS-1 302	Nl	YLS	297:	
KJ YLS 29/: Gd YLS 297: Ts YLS 294: Ttw YLS 294: Cf YLS 297: T. niveum 297: B. bassiana 275 SKI PTS-1 302	LS	YLS	297:	
Ga YLS 297: Ts YLS 294: Ttw YLS 294: Cf YLS 297: T. niveum 297: B. bassiana 275 SKI 277 P. tenuipes 300 SKI PTS-1 302	Кj	YLS	29/:	
15 15 294: Ttw YLS 294: 294: Cf YLS 297: 7 T. niveum 297: 8 B. bassiana 275 5KI P. tenuipes 300 5KI 975-1	Gα. m~	тьs	231:	
Cf YLS 297: T. niveum 297: B. bassiana 275 P. tenuipes 300; SKI PTS-1 302	15 m+-	VTC	204 -	
T. niveum 297: B. bassiana 275 SKI 277 P. tenuipes 300 SKI PTS-1		YLS	294:	
B. bassiana 275 SKI 277 P. tenuipes 300 SKI PTS-1 302		YLS VYLS VLS	294: 294: 297	
P. tenuipes 300 SKI PTS-1 302	Cf T	YLS V YLS YLS niveum	294: 294: 297: 297:	
	Cf T. B.	YLS v YLS YLS niveum bassiana	294: 294: 297: 297: 275[SK]	277

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 pI 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 neighborjoining 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 maximumlikelihood tree was not significantly affected by changing the Ts/Tv ratio setting from 2.0 to 10.0.

	I an ath	th MW	p <i>I</i>	Carboxyl terminus	Identity ^a	
Organism	(aa)				aa	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

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)

^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	$d_{\rm S}$	$d_{\mathbf{N}}$	$d_{ m S}/d_{ m N}$
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 sug-

gested 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 se-

quences 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 remained 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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