Insect Symbiosis: Derivation of Yeast-like Endosymbionts Within an Entomopathogenic Filamentous Lineage

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Yeast-like endosymbionts (YLSs) of insects often are restricted to specific hosts and are essential to the host’s survival. For example, in plant hoppers (Homoptera: Delphacidae), endosymbionts function in sterol utilization and nitrogen recycling for the hosts. Our study, designed to investigate evolutionary changes in the YLS lineage involved in the plant hopper association, strongly suggests an origin of the YLSs from within the filamentous ascomycetes (Euscomycetes), not the true yeasts (Saccharomycetes), as their morphology might indicate. During divergence of the plant hopper YLSs, dramatic changes would have occurred in the insect-fungus interaction and the fungal morphology that have previously been undescribed in filamentous ascomycetes. Phylogenetic trees were based on individual and combined data sets of 2.6 kb of the nuclear small- and large-subunit ribosomal RNA genes for YLSs from three rice plant hoppers (Laodelphax striatellus, Nilaparvata lugens, and Sogatella furcifera) compared with 56 other fungi. Parsimony analysis placed the plant hopper YLSs within Cordyceps (Euscomycetes: Hypocreales; Clavicipitaceae), a genus of filamentous insects and a few fungal pathogenic ascomycetes. Another YLS species restricted to the aphid Hamiltonaphis styraci (Homoptera: Aphididae) was a sister taxon to the plant hopper YLSs. Filamentous insect pathogens (Matarhizium and Beauveria) specific to the same species of insect hosts as the YLSs also formed lineages within the Clavicipitaceae, but these were distinct from the clade comprising YLS species. Trees constrained to include the YLSs in families of the Hypocreales other than the Clavicipitaceae were rejected by the Kishino-Hasegawa test. In addition, the results of this study support a hypothesis of two independent origins of insect-associated YLSs from among filamentous ascomycetes: the plant hopper YLSs in the Clavicipitaceae and the YLSs associated with anobiid beetles (Symbiotaphrina species). Several lineages of true yeasts (Saccharomycetes) also formed endosymbiotic associations with beetles, but they were not closely related to either group derived from the filamentous ascomycetes.

Introduction

Over the last century, the discovery of microbial endosymbionts in a wide variety of arthropods has been a significant finding in arthropod biology. For example, the recognition that prokaryotic rickettsial endosymbionts were widespread among arthropods and may induce sterility in their hosts brought a new perspective to studies of arthropod speciation (Shoemaker, Katju, and Janek 1999). In contrast, although a number of fungal endosymbionts of insects were previously reported, relatively few were substantiated (e.g., Buchner 1965, pp. 24–25, 236). Obligate fungal gut endosymbionts are known, however, in plant hoppers and aphids (Homoptera) and three families of beetles (Coleoptera: Anobiidae, Cerambycidae, and Scolytidae; Nardon and Grenier 1989). The fungal endosymbionts all appear to play important roles in insect nutrition, broadening the range of available resources by supplying enzymes for degradation or detoxification of plant material. For example, Symbiotaphrina kochii occurs in the gut of the anobiid tobacco beetle Lasioderma serricorne, a pest in stored tobacco. The single-celled fungus detoxifies plant materials ingested by the beetles (Dowd 1989, 1991). Most known fungal endosymbionts of insects are true yeasts (Saccharomycetes), but phylogenetic analyses based on partial sequences of the nuclear small-subunit ribosomal RNA gene (rDNA) have suggested that two groups of endosymbionts were derived from within the filamentous ascomycetes (Euscomycetes): the anobiid beetle yeast-like endosymbionts (YLSs) (S. kochii and Symbiotaphrina buchneri) and several unnamed YLSs of plant hoppers (Homoptera, Delphacidae: Laodelphax striatellus, Nilaparvata lugens, and Sogatella furcifera) and an aphid (Homoptera, Aphididae: Hamiltonaphis styraci). Studies of the anobiid YLSs indicated that they were derived from a filamentous ascomycete lineage outside the pyrenomycetes. Because of problems in acquiring and sampling vast numbers of taxa, the close filamentous relatives of the Symbiotaphrina species could not be determined to provide more information on changes occurring during evolution of endosymbiosis (Jones and Blackwell 1996; Noda and Kodama 1996; Jones, Dowd, and Blackwell 1999).

The plant hopper YLSs, also suggested to be more closely related to euscomycetes than to true yeasts, were tentatively placed among taxa in the Hypocreales on the basis of partial sequences of the nuclear small-subunit rDNA (Noda, Nakashima, and Koizumi 1995). More recently, Fukatsu and Ishikawa (1996) found that the aphid YLS was closely related to those of the plant hoppers. Although these YLSs were placed phylogenetically among taxa in Hypocreales, a more exact phylogenetic position was not determined because of incomplete sequence data and taxon sampling. A number of workers have suggested a need for additional sequences such as large-subunit rDNA to obtain better resolution and statistical support within the Hypocreales (Spatafora and Blackwell 1993; Noda, Nakashima, and Koizumi 1995; Rehner and Samuels 1995; Fukatsu and Ishikawa 1996).
Ishikawa 1996; Suh et al. 1998; Suh and Blackwell 1999). In contrast to the situation encountered in the study of Symbiotaphrina, taxon sampling was much less problematic among the better-understood pyrenomycetes, including the Hypocreales. The dramatic changes required for the transition in morphology (filamentous growth to budding cells) and host relations (pathogenesis to obligate intracellular symbiosis) in the Hypocreales drew us to the planthopper YLSs in an attempt to pinpoint the lineage from which the endosymbionts were derived.

The planthopper YLSs have never been found to be free-living in nature; they occur in the host fat body and are transmitted to the offspring through the ovary (Nasu 1963; Noda 1977). The YLSs apparently are involved in an obligate association in which they utilize sterol and help to recycle nitrogen within the fat bodies of the insect hosts (Wetzel et al. 1992; Sasaki, Kawai-mura, and Ishikawa 1996; Hongoh and Ishikawa 1997). Planthopper YLSs have not been cultured; their cells, however, can be separated by Percoll buoyant density gradient centrifugation to allow for DNA extraction (Noda and Omura 1992). In this study, we determined about 2.6 kb of partial sequences of nuclear small-subunit (SSU) and large-subunit (LSU) rDNA from each planthopper YLS and compared these with other major groups of ascomycetes. We also report sequences of SSU and LSU rDNA, respectively, by the polymerase chain reaction (PCR). PCR products were purified using a DNA purification kit (Bio-Rad Laboratories, Hercules, Calif.), and the purified double-stranded PCR products were used directly as templates for sequencing with an ABI PRISM BigDye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, Calif.). Primers used in sequencing were NS1, NS2, NS3, 18H, and NS8 for SSU rDNA and LS1, LR3, and LR5 for LSU rDNA (White et al. 1990; Hausner, Reid, and Klassen 1993; Rehner and Samuels 1995). DNA sequences were determined by an ABI PRISM 377 automated DNA sequencer (PE Applied Biosystems).

Data Analyses

Sequences were aligned with other data obtained from nucleotide sequence libraries by using the multiple-alignment program Clustal X (Thompson et al. 1997). Species included in this study and their GenBank accession numbers of SSU and LSU rDNA sequences, respectively, were as follows: Aphyphysostroma stercorarium (U32398; U47820), Aspergillus fumigatus (M60300; —), Atkinsonella hypoxylon (U44034; U57087), Balansia sclerotica (U32399; U47821), Beauveria bassiana IFO 4848 (AB027336; AB027382), Beauveria brongniartii (AB027335; AB027381), Blastomyces dermatitidis (M55624; —), Candida albicans (M60302; —), Candida tropicalis (M55527; —), Ceratocystis fimbriata (U32418; —), Cerocphora septentrionalis (U32400; —), Chromocleista malachitea (D88323; —), Claviceps pampali (U32401; U47826), Claviceps purpurea (U44040; U57085), Cordyceps bisporus (AH006986; AF096543), Cordyceps capitata (U44041; U57086), Cordyceps ophioglossoides (U46881; U47827), Cryptothecia parasitica (L42441; —), Daldinia concentrica (U32402; —), Diaporthe phaseolorum (L36985; —), Diatrypella disciformis (U32403; U47829), Echinostachys tuberiformis (U44042; —), Fusarium solani (U44043; U47828), Fusicystis solani (U44044; U57089), Humicola grisea (AF009958), Humicola insolens (U44045; U57090), Hyalohyphae sp. (U32404; —), Laetisaria fuciformis (U44046; U47829), Lecanicillium lecanii (U32405; U47830), Metarhizium flavoviride var. minus (U57091; U57092), Nectria haematococca (U32406; U47831), Nipaecoccus vicinus (U32407; U47832), Paecilomyces blakesleeanus (U32408; U47833), Paecilomyces variotii (U32409; U47834), Phoma medicaginis (U32410; U47835), Pristidium roseum (U32411; U47836), Rhizopus stolonifer (U32412; U47837), Verticillium dahliae (U32413; U47838), and Xodolectryon sp. (U32414; U47839). DNA Extraction, PCR, and Sequencing

Nucleic acids were extracted and purified following the procedure of Lee and Taylor (1990). The primer sets NS1–NS8 (White et al. 1990) and LS1–LSD (Hausner, Reid, and Klassen 1993) were used for amplifying SSU and LSU rDNA, respectively, by the polymerase chain reaction (PCR). PCR products were purified using a DNA purification kit (Bio-Rad Laboratories, Hercules, Calif.), and the purified double-stranded PCR products were used directly as templates for sequencing with an ABI PRISM BigDye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, Calif.). Primers used in sequencing were NS1, NS2, NS3, 18H, and NS8 for SSU rDNA and LS1, LR3, and LR5 for LSU rDNA (White et al. 1990; Hausner, Reid, and Klassen 1993; Rehner and Samuels 1995). DNA sequences were determined by an ABI PRISM 377 automated DNA sequencer (PE Applied Biosystems).

Materials and Methods

Strains Used in the Study

YLSs from L. striatellus (Ls YLS), N. lugens (Nl YLS), and S. furcifera (Sf YLS) were isolated and purified by the methods of Noda and Omura (1992), and the freeze-dried cells were used directly for extracting DNA. Isolates of Metarhizium and Beauveria were grown for 1 week in 2% malt extract broth in 1.5-ml tubes. Information on the sources and GenBank accession numbers for the sequences determined in this study is given in Table 1.

Table 1

<table>
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<tr>
<th>Fungus</th>
<th>Sourcea</th>
<th>Host</th>
<th>SSU rDNA Accession No.</th>
<th>LSU rDNA Accession No.</th>
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<td>AF280631</td>
<td>AF280634</td>
</tr>
</tbody>
</table>

a H. Noda is with the National Institute of Sericultural and Entomological Sciences, Japan; ARSEF = United States Department of Agriculture, Agricultural Research Service (USDA-ARS) Collection of Entomopathogenic Fungal Cultures.
Leucostoma persoonii (M83259; Ð), Hypoxylon atroroseum (Z75578; Ð), Saccharomyces cerevisiae (U00971; Ð), Taphrina deformans (U44117; U47836), Neothyphodium coenophialum (U59422; U57681), Neurospora crassa (X04971; Ð), Ophiostoma stenoceras (M85054; Ð), Ophiostoma ulmi (M83261; Ð), Paezilomyces tenue (D85136; U47838), Penicillium chrysogenum (M55628; Ð), Phialophora verrucosa (L36999; Ð), Protomyces inoueyi (D11377; Ð), Saccharomycetes cerevisiae (Z55757; Ð), Sordaria fimicola (X69851; Ð), Sporothrix schenckii (M85053; Ð), Taphrina deformans (U00971; Ð), Trichoderma koningii (AF218790; Ð), Trichoderma viride (AF218788; Ð), Xylaria hypoxylon (U20378; U47841), yeast-like symbiont of Hamiltoniphilus styrae (D55719; Ð).

Alignments were optimized visually, and ambiguously were excluded from the analyses. Maximum-parsimony analyses were performed using PAUP*, version 4.0b4a (Swofford 2000). Heuristic tree searches were executed using the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. The bootstrap values in most-parsimonious trees were obtained from 1,000 replications. Maximum-likelihood analyses (Kishino and Hasegawa 1991) for tree scoring were performed using PAUP*, version 4.0b4a, or the DNAML program in the PHYLIP package, version 3.572c (Felsenstein 1995), with empirical frequencies and a transition/transversion ratio of 2.0. The partition homogeneity test option in PAUP* with 1,000 replicates was used to determine whether the SSU and LSU rDNA data sets were in conflict. MacClade software was used to trace host preferences of the clavicipitaceous fungi (Maddison and Maddison 1999).

Results and Discussion

Approximately 1,730 bp of SSU rDNA representing most of the gene were obtained from PCR products of the YLSs of the planthoppers and Metarhizium and Beauveria isolates. The SSU rDNA of B. bassiana (ARSEF 2427) and M. flavoviridae var. minus (ARSEF 1764) had insertions of several hundred bases. The insertions occurred at positions 943 and 1199 of the SSU rDNA of Escherichia coli and were found to be major groups of group I introns (IC or IE) based on secondary structure analysis (Michel and Westhof 1990; Suh, Jones, and Blackwell 1999). Introns sequences were excluded from the analyses, and details of the introns will not be discussed here. For the LSU rDNA, about 900 bp of the gene including the variable D1/D2 region were sequenced from the PCR products. Two isolates of M. barbatus were sequenced from the same amplicon with 100% bootstrap support.

Partial sequences of SSU rDNA from 56 taxa, which were selected from most major groups of ascomycetes, were compared with those of the planthopper YLSs using parsimony criteria; two archiascomycetes were used as outgroup taxa (fig. 1). Of the 771 characters remaining after the ambiguous regions were excluded, 522 were constant, 56 were parsimony-uninformative, and 193 were parsimony-informative. The species of ascomycetes compared were well defined at the level of order, with comparatively high bootstrap values in the consensus of 272 most-parsimonious trees obtained from 1,000 bootstrap samplings derived from 1,000 samples supporting the internal branches by ≥50%.

flavoviridae var. minus had identical sequences in LSU rDNA.

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**Fig. 1.**—Consensus of 272 most-parsimonious trees obtained from SSU rDNA sequence data. The planthopper yeast-like endosymbiont clade appears in boldface type, and orders of ascomycetes and families of Hypocreales are indicated. Tree length = 561; consistency index = 0.5455; homoplasy index = 0.4545; retention index = 0.8175; rescaled consistency index = 0.4459. Numbers on tree branches indicate the percentages of bootstrap samplings derived from 1,000 samples supporting the internal branches by ≥50%.
The best of five most-parsimonious trees obtained by analysis of combined SSU and LSU rDNA data sets places the yeast-like endosymbiont (YLS) clade in Clavicipitaceae with somewhat better resolution and support than with analyses of the individual gene sequences (see also fig. 1). Tree length = 684; consistency index = 0.5760; homoplasy index = 0.4240; retention index = 0.6827; rescaled consistency index = 0.3933; 2 ln likelihood = 6,366.76796. Trees constrained to place the YLS lineage within Hypocreaceae or Nectriaceae had log likelihoods significantly worse than the most-parsimonious tree shown here.

In the partition homogeneity test for the SSU and LSU rDNA sequence data, the observed summed tree lengths of 675 steps fell within the distribution of randomized data sets (P = 0.501), indicating that there was not significant conflict between them, and the data were combined. The combined data set from 28 taxa using two species of the Xylariales as outgroup taxa was analyzed using parsimony criteria (fig. 2). After excluding ambiguous regions of the remaining 1,577 characters in the combined data set, 1,255 were constant and 228 were parsimony-informative. Of five most-parsimonious trees, the tree with the best score in the Kishino-Hasegawa test provided somewhat better resolution and support for the branches within the Clavicipitaceae (fig. 2).

As in the case of the tree derived from the independent SSU data set, the YLSs and the insect parasites, C. bisporus and H. thompsonii, formed a clade, but it was supported by a higher (90%) bootstrap value. Once again, the YLS lineage was separated from C. bisporus and H. thompsonii by a comparatively long branch length. Placement of the planthopper YLSs within the Clavicipitaceae was supported by the Kishino-Hasegawa test. Two constraint trees placing the YLSs in other families of the Hypocreales (Hypocreaceae and Nectriaceae) were significantly worse than the best tree (fig. 2). The differences of ln L values (−ln L), the standard deviation of the difference (SD), and the P value for significance between the best and other constraint trees were as follows: (1) for YLSs in the Hypocreaceae, −ln L = −119.39787, SD = 28.55769, P < 0.0001; (2) for YLSs in the Nectriaceae, −ln L = −116.92290, SD = 27.85217, P < 0.0001 (fig. 3).

The insect pathogen C. bisporus is characterized by perithecia produced in a Cordyceps-like stroma, dark ascospores, and a Hirsutella anamorph. It has been placed among species of Cordyceps based on its morphological and molecular characteristics (Suh et al. 1998). Since culturing of the YLSs from planthoppers has not been successful, the morphological characteristics could not be fully compared with related taxa in the Clavicipitaceae, but they appeared very dissimilar. Under the compound microscope, the purified cells were typical yeast-like cells with budding; they lacked pseudohyphae or true hyphae. The YLSs appear to have adapted to the intracellular habit in planthoppers, perhaps even having lost some genes essential for hyphal growth. Because of their unique habit as endosymbionts and their restricted yeast-like growth form, the YLSs not only stand out among the Clavicipitaceae, but they are also distinct among all of the Hypocreales.

The host ranges of a set of taxa of Clavicipitaceae are shown mapped on the tree (fig. 3, based on the combined data of fig. 2). Although some lineages were not strongly supported by bootstrap analysis and the number of taxa was limited, host ranges of the taxa were related to their phylogenetic positions within the family. Members of the Clavicipitaceae were divided into several groups. Plant parasites, endophytes, fungal parasites, and some insect pathogens were separated from a Cordyceps clade of insect pathogens (C. bisporus and H.

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**Fig. 2.**—The best of five most-parsimonious trees obtained by analysis of combined SSU and LSU rDNA data sets places the yeast-like endosymbiont (YLS) clade in Clavicipitaceae with somewhat better resolution and support than with analyses of the individual gene sequences (see also fig. 1). Tree length = 684; consistency index = 0.5760; homoplasy index = 0.4240; retention index = 0.6827; rescaled consistency index = 0.3933; 2 ln likelihood = 6,366.76796. Trees constrained to place the YLS lineage within Hypocreaceae or Nectriaceae had log likelihoods significantly worse than the most-parsimonious tree shown here.

**Fig. 3.**—Tree diagram based on the combined data of figure 2 (branches with <80% bootstrap values collapsed) indicates that host preferences of clavicipitaceous taxa, including the insect endosymbiotic habit, have a phylogenetic distribution. The yeast-like endosymbionts are derived within an insect-pathogenic Cordyceps clade (Cordycepioides bispous and Hirsutella thompsonii).
thompsonii) and the planthopper YLSs. In addition, a few species of Cordyceps are parasites of false truffles (Basidiomycetes), and Nikoh and Fukatsu (2000) estimated that a member of the genus Cordyceps jumped from an arthropod to a fungal host about 43 MYA. If their estimate is correct, the YLS lineage would be younger than 43 Myr old because it diverged after the fungal (C. ophioglossioides and C. capitata)—insect pathogen split in Cordyceps (fig. 3).

Species of Hypocreales are known for their wide variety of associations with other organisms; in particular, host shifts appear to have occurred repeatedly in the Clavicipitaceae, a family that contains many species that are leaf endophytes and parasites of arthropods, fungii, and plants (Alexopoulos, Mims, and Blackwell 1996, p. 330). The work reported here is unique because it describes yet another host association, obligate endosymbiosis with Homoptera, derived from within a Cordyceps lineage of obligate insect pathogens with filamentous growth. The planthopper and aphid YLS lineage represents a relatively recent independent divergence of endosymbionts within Euascomycetes, and these fungi are related only distantly to the Symbiotaphrina YLSs of anobiid beetles. The planthopper YLSs are even more distantly related to lineages of the true yeasts that are associates of several groups of beetles.

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LITERATURE CITED


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