Yeasts Associated with Bark Beetles of the Genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae): Molecular Identification and Biochemical Characterization

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Introduction

The bark beetles (Scolytinae) increase their potential to colonize their hosts by associating symbiotically with microorganisms, particularly fungi, which are carried on specialized structures called mycangium (Harrington 1993; Klepzig 2001; Paine and others 1997; Six 2003; Whitney 1982). Additionally, there is experimental evidence that some yeasts and bacteria from the alimentary canal of these insects might be involved in digestion and detoxification processes and pheromone production. The latter are compounds fundamental to the chemical communication of these insects (Borden 1982; Harrington 1993; Paine and others 1997). The role of bark beetles-associated yeast cannot be studied if we do not know their diversity and location. Yeasts taxonomic identification have been commonly performed on morphological characters of vegetative and sexual stages as well as by physiological tests; however, the variable responses of yeasts to the physiological tests and their phenotypic plasticity have hindered their taxonomic identification. Recent investigations have demonstrated that the 18S rDNA and D1/D2 domain of the large subunit 26S rDNA are reliable attributes for yeasts identification. Thus, the purpose of this study is to elucidate yeasts diversity associated to different species of *Dendroctonus*. In order to asses how many biological entities (putative species) are present in our samples, we used species concept based on phylogenetic analysis of D1/D2 26S, 18S, and ITS1 rDNA data. In this context, we assume that the categories "species" or "groups" are not ranges, but different biological entities; the reference species or taxa were used to specify the name to which the group applies.

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Methods

Four hundred and fifty beetles of nine *Dendroctonus* species from 36 geographical sites were dissected under sterile conditions. The yeasts were isolated from gut and ovaries using RPMI 1640 medium for a 48 h period. Inocula were taken from the RPMI culture, placed in Sabouraud dextrose broth, and incubated for 24 to 48 h at 28 °C. The yeasts were isolated in Sabouraud dextrose agar and restreaked until pure cultures were obtained. Bark beetle frass and eggs were directly inoculated in Sabouraud broth and incubated under the same conditions above mentioned. Total genomic DNA was isolated from each yeast strain according to the protocol described in Lehmann and others (1992). The gene regions of D1/D2 domain 26S, 18S, and ITS1 of rDNA were amplified and sequenced. The sequences obtained were compared with yeast sequences from GenBank. The phylogenetic relations among the strains were established by parsimony methods using PAUP 4.0b10. The number of nucleotide differences per site among D1/D2 domain 26S sequences was estimated according to Nei and Li (1979). *Schizosaccharomyces pombe* was used as outgroup in all analyses. Finally, yeast groups were characterized by 29 assimilations and seven fermentation tests of carbohydrates, four additional assimilation tests, and morphological features following Yarrow (1998).

Results and Discussion

A total of 430 yeast isolates were obtained: 331 (gut), 33 (ovaries), 50 (frass), and 16 (eggs). Thirteen consistent groups were found throughout the phylogenetic trees of D1/D2 26S and ITS1 rDNA, however, some of them were not consistent on 18S topology. Eleven groups were associated to *Candida arabinofermentans, C. ernobii, C. oregonensis, C. picea, C. terebra, Pichia americana, P. canadensis, P. capsulata, P. glucozyma, P. guilliermondii, P. scolyti*, and two with no equivalent within the GenBank database. The great majority of yeast isolates, within groups, showed less that 1 percent nucleotide substitution respect to reference species, and among groups, more that 1 percent.

Phylogenetic analysis show, independent of the analyzed gene, the yeast community associated with *Dendroctonus* species only belong to *Candida* and *Pichia* genera. This observation agrees with previous studies carried out on bark beetles using conventional tests (Brand and others 1977; Bridges and others 1984; Leufvén and others 1984; Moore 1972; Shifrine and Phaff 1956), however, the species or groups are different. Also, this analysis shows that bark beetle-associated yeast groups do not present correlation with the isolate sites, geographic location, or *Dendroctonus* species. The only exception was *Pichia scolyti* group, which showed an association with its host, *Dendroctonus pseudotsugae*. Additionally, this analysis suggests that *C. arabinofermentans, C. ernobii*, and *P. capsulata* groups from *D. pseudotsugae* could have a vertical transmission from parents to progeny since they were isolated from eggs, ovaries, larvae, and adults in the same geographic site. However, it is necessary to carry out specific investigations to test if the transmission of the yeasts is really vertical.

Bark beetle-associated yeasts exhibit a physiological response highly variable within and among groups (data not shown, but obtainable upon request). Therefore, there is not correlation between groups and the fermentation or

assimilation of some carbohydrates. Nevertheless, a greater part of the isolates assimilated cellobiose, salicin, and D-xylose. Some studies reported that these carbohydrates are indicator substrates of enzymes involved in metabolic routes associated with digestive and detoxification processes (Vega and Dowd 2005). Unfortunately, we know little or nothing about the symbiotic association between *Dendroctonus species* and its associated yeast. Our findings of no association of yeasts with *Dendroctonus* species or geographic localities suggested no significant commensal or symbiotic relationship. However, we assume that this relationship can be very important in a nutritional context, as has been shown in other insect groups.

Future Research Plans

The following studies are planned for the future:

- Determine the role of yeast in the digestion process of bark beetles.
- Perform molecular analysis of multigenic family P450, esterases, and glutathione S-transferase of yeasts to learn about their role in the detoxification process in bark beetles.
- Determine if the transmission of the yeasts is vertical or horizontal.

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