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Molecular identification and phylogenic analysis by sequencing the rDNA of copper-tolerant soft-rot *Phialophora* spp.

Hasanthi Karunasekera, Geoffrey Daniel*

Department Forest Products/Wood Science, Box 7008, Swedish University of Agricultural Sciences, SE-750-07 Uppsala, Sweden

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ABSTRACT

The genus *Phialophora* includes some of the most copper-tolerant and frequently isolated fungi causing soft rot of copper-treated wood in service. The aim of this work was to conduct a phylogenic analysis of different *Phialophora* species and strains isolated from treated wood, evaluate strain compatibility, and examine their copper-tolerance variability under liquid- and solid-culture conditions. Phylogenic analysis of *Phialophora malorum* (four strains), *Phialophora mutabilis* (two strains), *Lecythophora mutabilis* (one strain), and *Phialophora* sp. A. (three strains) originating from different parts of the world showed clustering into three major clades. *Phialophora* sp. A. refers to a number of fungal isolates identified as *Phialophora* spp. previously isolated by our laboratory from preservative-treated stakes and poles in Sweden. *P. mutabilis* strains fell into a single cluster together with *Phialophora lignicola* and *Phialophora hoffmanii*; *P. malorum* strains clustered in their own group (93% bootstrap value) and *Phialophora* sp. A. clustered together with *Phialophora botulispora*. Compatibility studies with the different *Phialophora* species/strains showed discriminative behavior. All *Phialophora* species showed mutual inhibition (incompatibility), as did strains of *P. malorum* and *P. mutabilis*. *Phialophora* sp. A. strains showed mutual intermingling but incompatibility with *P. botulispora*. Species/strain variability regarding copper tolerance was evaluated using in-vitro liquid and solid media containing different $CuSO_4$ concentrations. *P. malorum* 211-C-15-1 showed the highest copper-tolerance growing on 6.4% $CuSO_4$ supplemented agar and 3.2% $CuSO_4$ in liquid cultures, followed by *P. malorum* ATCC 66716 (3.2% w/v $CuSO_4$-agar). *P. mutabilis* strains and *L. mutabilis* showed growth on 0.64% $CuSO_4$ incorporated in liquid and agar cultures and *Phialophora* sp. A. (25M3) at 0.32% w/v $CuSO_4$ on agar. The results emphasize that copper tolerance varies greatly within and between species of *Phialophora*.

1. Introduction

The imperfect fungal anamorph genus *Phialophora* (family Herpotrichiellaceae) and the closely related genera *Cadophora* and *Lecythophora* include many well-known fungal species isolated from both treated (i.e., with copper) and untreated wood exposed in ground contact situations. These fungi have been well documented as having a cosmopolitan distribution and causing soft-rot decay (i.e., cavities [Type 1] and/or 2 erosion [Type 2] attack of wood cells) of treated (e.g., with waterborne preservatives) wood in service (primarily utility poles) in many countries, including Sweden (Henningsson and Nilsson, 1976; Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988), Germany (Gersonde and Kerner Gang, 1976), France (Fougerousse, 1976), the U.S. (Zabel et al., 1985; Zhong et al., 1995), and Australia (Leightley, 1979, 1980). *Phialophora* species resist toxicity from heavy metals (e.g., copper, chromium, arsenic, zinc, and tin) incorporated into conventional wood preservatives (Henningsson and Nilsson, 1976; Sutter and Carey, 1986; Daniel and Nilsson, 1988). After basidiomycete decay by brown-rot fungi, soft rot is the most economically important type of wood rot in terrestrial situations and is also the most serious decay in aquatic environments – both in sea- and lake-water situations. Despite changes in wood protection formulations and a general move toward chemical modification systems consistent with environmental regulations, the various forms of copper treatment of today are likely to remain the dominating form of wood protection in the near future worldwide. Thus members of the *Phialophora* genus causing soft rot will remain important degraders of copper-treated wood in service in the future.

An important and very interesting feature of many *Phialophora* species is their considerable tolerance to copper in in-vitro conditions (Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988) at levels far exceeding that of basidiomycete fungi and that is may

* Corresponding author. Tel.: +46 18 67 24 89;fax: +46 18 67 34 89.
E-mail addresses: hasanthi.karunasekera@slu.se (H. Karunasekera), geoffrey.daniel@slu.se (G. Daniel).

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be possible and economically viable to use them as protective agents in wood. Earlier ultrastructural studies (Daniel and Nilsson, 1989) have provided knowledge of the interaction of certain *Phialophora* spp. with metals (Cu, Cr, As) in wood, but the true mechanism(s) of copper tolerance involved—whether biochemical or structural or both—and the manner of Cu detoxification in wood during attack is not understood.

The aims of the present work were: (1) to determine molecular identity and phylogeny of economically important *Phialophora* spp. previously isolated from copper-treated and untreated wood from different parts of the world using polymerase chain reaction (PCR) of the ITS region of ribosomal DNA coupled with sequencing; (2) to evaluate strain compatibility: (3) to relate strain variability with an ability to tolerate copper under solid- and liquid-culture conditions; and (4) to allow selection of important copper-tolerant species/strains for subsequent studies on the role of copper detoxification during decay of copper-treated wood. Inter-compatibility tests and microscopic features of morphological characters were further used as additional tools to complement molecular identity. In the present study we consider copper tolerance as the upper level found in which growth of the fungi was inhibited but was not necessarily lethal.

2. Materials and methods

2.1. Origin and culturing of Phialophora species

The three most common copper-tolerant *Phialophora* species (Henningsson and Nilsson, 1976; Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988) and respective strains were selected for the study (Table 1). Apart from *Phialophora malorum* ATCC 66716 and *Lecythophora mutabilis* ATCC 44034 (=*P. mutabilis* [J.F.H. Beyma] Schol-Schwarz [1970]), all other strains were obtained from the Department of Forest Products, SLU culture collection. *Phialophora* sp. A. refers to a number of fungal isolates identified as *Phialophora* spp. previously isolated by our laboratory from preservative-treated stakes and transmission poles, primarily in Sweden. They have frequently been shown to be one of the most important soft-rot fungi colonizing preservative-treated poles and stakes in ground contact (Henningsson and Nilsson, 1971, 1976; Nilsson and Henningsson, 1978) impregnated with a variety of different preservatives (i.e., Zn/Cr/As; Cu/Zn/Cr/As; Cu/Cr/As; F/Cr/As; Cr/F; F; Cy/PCP; CCA; creosote). In one study, *Phialophora* sp. A. represented approximately 65% of all the *Phialophora* species isolated from CCA-treated pine stakes exposed at two different test sites in Sweden (Nilsson and Henningsson, 1978) and also about 30% of all isolates colonizing wood treated with different types of wood preservatives (Henningsson and Nilsson, 1976). *Phialophora* sp. A. was formerly named *Phialocephala* sp. A. (Henningsson and Nilsson, 1971) and was often isolated from treated wood in Sweden and Denmark during the 1970s, when great interest was given to the failure of treated transmission poles. As far as the authors know, *Phialophora* sp. A. has so far not been taxonomically described despite its earlier isolation from preservative treated wood in service.

All fungi were cultured on 2.5% w/v malt extract agar (MEA) at 20 °C for morphological and molecular identification.

2.2. Identification of fungal strains

Three approaches were used to identify the *Phialophora* species/strains: (1) molecular identification by PCR amplification of the ITS region of rDNA sequence; (2) microscopic identification of morphological characters according to Schol-Schwarz (1970); and (3) in-vitro compatibility screening of mixed cultures for confirmation of strain discrimination. It is quite difficult to distinguish between different species/strains of the *Phialophora* genus using only morphological characters since many of these differences are based on complex properties of colony morphology, and characteristic features of sexual spores and fungal hyphae. Emphasis was given to molecular identification using PCR to establish a reliable identification tool. Inter-compatibility tests and microscopic features of fungal hyphae were used to confirm the PCR results.

2.2.1. Molecular identification

Genomic DNA was isolated from related *Phialophora* morphotypes from Sweden and ATCC and CBS culture collections (Table 1; Henningsson and Nilsson, 1976; Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988, 1989). DNA extraction was performed using a modified phenol/chloroform-extraction method (Larena et al., 1999). Agar plugs with mycelia were inoculated (five plugs/flask) in 250-ml Erlenmeyer flasks containing 2% w/v corn steep in 100 ml Abrams medium (pH 6.5) and incubated for 4–5 days as stationary cultures at 20 °C. Resulting mycelia were harvested by filtration, followed by blotting and freeze drying at 45 °C overnight in an Edwards Freeze dryer before grinding to a fine powder under LN_2. Mycelia powder suspended in extraction buffer (50 mM EDTA [pH 8.5] containing 0.2% SDS [dodecyl sulphate sodium salt]) and incubated at 65 °C for 30 min was cooled to room temperature and centrifuged at 12,000 rpm for 15 min. The supernatant was transferred to a fresh tube and RNAse added to a final concentration of 50 μg ml^{-1} and incubated for 1 h at 30 °C. After centrifuging for 15 min at 10,000 rpm, the supernatant was collected and added to a cold fresh tube. A one-tenth volume of 5 M potassium acetate (pH 5.2) was added and incubated on ice for at least 1 h. The solution was then centrifuged at 13,000 rpm for 15 min before extracting twice with an equal volume of phenol/chloroform/isoamyl alcohol (49/49/2). DNA was precipitated by 2 vol. of 95% ethanol and incubated at −20 °C for 30 min. After final centrifugation at 12,000 rpm for 15 min, the recovered pellet was washed with 80% cold ethanol, vacuum-dried, resuspended in 100 ml sterile water, and stored at −20 °C until further use.

Table 1
Details of the fungi used in the study.

Fungal species	Strain	Culture collection/Depositor	Origin/Isolated from
1. *Phialophora malorum*	211-C-15-1	Dept. Forest Products	2% K33 impregnated poles, Sweden
	ATCC 66716	Wang	CCA treated Douglas fir poles, New York
	CBS 245.60	Kidd & Beaumont	Eucalyptus poles, Denmark
	ATCC 42795	Nilsson	Beech posts, Sweden
2. *Phialophora mutabilis*	24-E-1-1	Dept. Forest Products	CCA treated transmission poles, Sweden
	ATCC 42792	Nilsson	CCA treated transmission poles, Sweden
Lecythophora mutabilis	ATCC 44034	Leightley	Treated Eucalyptus poles, Australia
3. *Phialophora* sp. A.	35-1	Dept. Forest Products	Telephone poles, Sweden
	25M3	Dept. Forest Products	Telephone poles, Sweden
	204-1	Dept. Forest Products	Telephone poles, Sweden