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BRIEF NOTE

18S Ribosomal RNA Gene Sequence Characters Place the Human Pathogen *Sporothrix schenckii* in the Genus *Ophiostoma*

MARY L. BERBEE AND JOHN W. TAYLOR

Department of Plant Biology, 111 Genetics and Plant Biology Building, University of California, Berkeley, California 94720

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BERBEE, M. L., AND TAYLOR, J. W. 1992. 18S ribosomal RNA gene sequence characters place the human pathogen *Sporothrix schenckii* in the genus *Ophiostoma*. *Experimental Mycology* 16, 87–91. Using the sequence of the gene for the 18S subunit of ribosomal RNA, we show that the asexual human pathogen *Sporothrix schenckii* lies phylogenetically within the sexual genus *Ophiostoma*. By distance or maximum parsimony criteria, *S. schenckii*, *Ophiostoma stenoceras*, and *O. ulmi* (the Dutch elm disease fungus) are all related, but *S. schenckii* and *O. stenoceras* are more closely related to each other than either is to *O. ulmi*. The 18S RNA gene sequences of *S. schenckii* and *O. stenoceras* are identical except at three sites, and the two species group together in 99% of the trees generated using the statistical method of bootstrapping. This is the first time that DNA sequence data have been used to place an asexual fungus, phylogenetically and with statistical support, in a sexual genus.

INDEX DESCRIPTORS: *Ceratocystis; Ophiostoma ulmi; Ophiostoma stenoceras; Sporothrix schenckii;* 18S ribosomal RNA; phylogeny; nomenclature.

Sporothrix schenckii Hektoen *et* Perkins infects human skin and lymph systems when implanted into susceptible wounded tissue, causing sporotrichosis (Rippon, 1988). *Ophiostoma ulmi* (Buism.) Nannf. [=*Ceratocystis ulmi* (Buism.) C. Moreau] infects American elm trees, causing Dutch elm disease. Unlike *O. ulmi*, *S. schenckii* has never been known to produce a sexual state. Otherwise, the vegetative morphologies of the human pathogen *S. schenckii* and the tree pathogen *O. ulmi* are similar. Both produce simple, mononematous conidiophores that bear sympodulospores on denticles (Fig. 2), and both, under certain conditions, will grow as yeasts (Upadhyay, 1981). The two can be distinguished because *O. ulmi* produces synnemata, tight clusters of condiophores, while *S. schenckii* does not (Upadhyay, 1981). Asexual forms of other *Ophiostoma* species, including *O. stenoceras* (Robak) Melin et Nannf. and *O. piliferum* (Fries) H. et P., can be distinguished from *S. schenckii* biochemically or by their lack of pathogenicity, but not morphologically (Hoog, 1974; Mendonça-Hagler *et al.*, 1974; Mariat, 1977; Travassos, 1985). *S. schenckii* is morphologically distinct from the asexual form of *O. minus* (Hegc.) H. and P. Syd. (Mariat, 1977), but was closer to *O. minus* than to other *Ophiostoma* species based on DNA–DNA hybridization experiments (Mendonça-Hagler *et al.*, 1974).

Because the important taxonomic characters of perithecium and ascus morphology are missing in the asexual *S. schenckii*, the fungus could not be placed phylogenetically or nomenclatorially within the genus *Ophiostoma*. However, all fungi, sexual and asexual, have ribosomal DNA. Phylogenetic trees inferred from ribosomal DNA have the potential to resolve relationships between fungi irrespective of their sexual condition (Bruns *et al.*, 1991; Reynolds and Taylor, 1991).

We used the pattern of ribosomal DNA base substitutions to determine whether or

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not *S. schenckii* lies phylogenetically within the genus *Ophiostoma*. As outgroups, we chose *Leucostoma persoonii* Höhnel and *Neurospora crassa* Shear and Dodge. We had previously sequenced the 18S ribosomal RNA gene from *L. persoonii* and *O. ulmi* and shown that both *L. persoonii* and *N. crassa* (GenBank NEURRNAS) are related to *O. ulmi* at the class level. *L. persoonii* clustered with *O. ulmi* within the class Pyrenomycetes (Berbee and Taylor, 1992). We amplified the gene for the 18S ribosomal RNA subunit from crude (miniprep) total genomic DNA, using the polymerase chain reaction and then performed a series of overlapping, asymmetrical amplifications to generate the single-stranded DNA that we sequenced (Lee and Taylor, 1990; White *et al*, 1990; Berbee and Taylor, 1992).

We sequenced over 1700 nucleotides from each fungus, the entire 18S ribosomal RNA gene except for about 50 nucleotides at the 5′ end and about 20 nucleotides at the 3′ end which we could not reach using our primers. The sequence is based on both strands of DNA except for about 30 nucleotides 5′ to the primer NS 8 and in an approximately 100 nucleotide long region near NS 5. The sequences are deposited in GenBank, and fungal strains are: *Ophiostoma stenoceras*, University of California, Berkeley 57.013 (also in T. Harrington's, Iowa State University collection as C447), *Sporothrix schenckii* American Type Culture Collection (ATCC) 14284. We also sequenced the gene for the 18S ribosomal RNA subunit from *S. schenckii* ATCC 10213. We found no differences between the two strains and used the completed, corrected sequence of one strain, ATCC 14284, for our analysis. The sequences from the five fungi were easily aligned across their whole lengths, by first using the Genalign option in the Intelligenetics Inc. package and then visually changing the positions of single-nucleotide gaps to maximize aligned sites. Aligned sequences are available on request.

We analyzed sequence data using maximum parsimony algorithms and PAUP 3.01 (Kluge and Farris, 1969; Swofford, 1989) in order to distinguish between the three possible phylogenetic relationships for *O. ulmi*, *S. schenckii*, and *O. stenoceras* in Fig. 1. From over 1700 nucleotides of sequence data, 149 sites were variable and 32 were informative. The branch and bound option in PAUP produced a single most parsimonious tree requiring 164 steps (Fig. 2). Bootstrapping is a statistical method for assessing the strength with which data support the branches in the most parsimonious tree or trees. If a branch appears in 95% of the bootstrap replicates, this support can be roughly translated into a 95% confidence

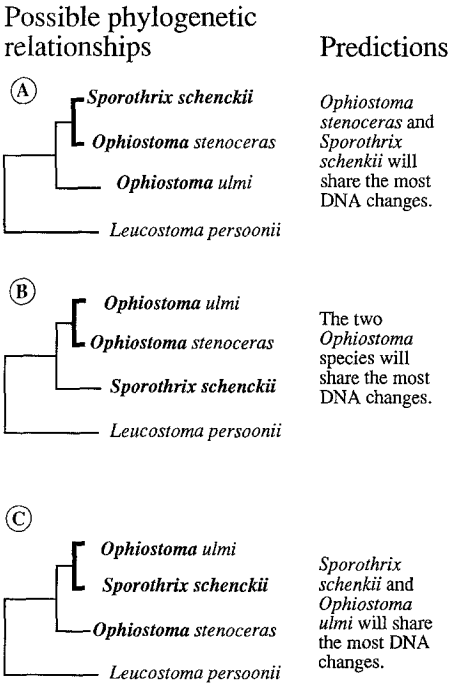

FIG. 1. The possible phylogenetic relationships between *S. schenckii*, *O. stenoceras*, and *O. ulmi* are diagrammed in these three trees rooted on the branch to *L. persoonii*. Trees A, B, and C each predict a different distribution of DNA characters. Tree A predicts the distribution of DNA changes that we actually found in analysis of our sequence data.

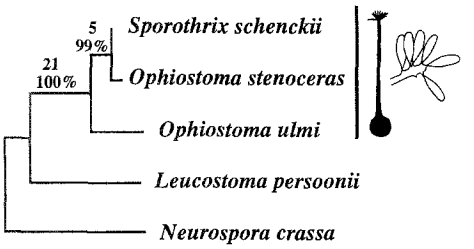

FIG. 2. This is the single most parsimonious phylogenetic tree generated from our ribosomal DNA sequence data by the branch and bound option in PAUP 3.0l. The percentages are the frequencies with which branches appeared in 1000 bootstrap replications. Above the percentages are the numbers of characters that changed unambiguously on the branches. In the tree, the clustering of the asexual species *S. schenckii* with the sexual species *O. stenoceras* inside the genus *Ophiostoma* receives strong statistical support, appearing in well over 95% of the bootstrap replicates. Diagrammed to the right is the perithecial sexual state found in the *Ophiostoma* species but missing from *S. schenckii*. Grouping of *O. ulmi* with *S. schenckii* and *O. stenoceras* is also strongly supported by bootstrapping. Sympodulospores, asexual conidia borne on denticles, characterize both of the *Ophiostoma* species as well as *S. schenckii* and are diagrammed to the far right.

level for the grouping (Felsenstein, 1985). In 1000 bootstrap replicates, the two *Ophiostoma* species grouped with *S. schenckii* 100% of the time, indicating that the confidence level on the branch grouping the three is better than 95%. *O. stenoceras* and *S. schenckii* grouped together 99% of the time, indicating that the confidence level on the branch leading to the two exceeds 95%. To further examine the strength with which our data support the most parsimonious tree, we used the exhaustive search option in PAUP which evaluated 15 trees requiring from 164 to 191 steps. The second and third most parsimonious trees, grouping *O. ulmi* with either *O. stenoceras* or *S. schenckii*, required 5 additional steps. The fourth most parsimonious tree, including *Neurospora* within the *Ophiostoma* and *Sporothrix* group, required an additional 18 steps. Using distance criteria, *O. stenoceras* and *S. schenckii* are much more similar to each other than to *O. ulmi*. The sequences of *O. stenoceras* and *S. schenckii* are identical except at 3 sites of over 1700 sites, while *S. schenckii* and *O. ulmi* differed at 28 sites and *O. stenoceras* and *O. ulmi* differed at 30 sites.

We have demonstrated with strong statistical confidence that *S. schenckii* is more closely related to *O. stenoceras* than *O. stenoceras* is to *O. ulmi*. *Ophiostoma* is not a monophyletic genus unless it includes *S. schenckii*.

Previous studies used DNA hybridization, antigenicity, cell wall carbohydrate content, virulence studies, cultural characteristics, and morphology to indicate the close relationship between *S. schenckii* and *Ophiostoma* species (Taylor, 1970; Mendonça-Hagler *et al.*, 1974; Mariat, 1977; Travassos, 1985). None of these methods, however, resolve the relationships of the *S. schenckii* and *Ophiostoma* species relative to an outgroup or distinguish between the possibilities outlined in Fig. 1 (Mariat, 1977). The strength of DNA sequence characters is that they nest *S. schenckii* in *Ophiostoma* relative to an outgroup.

Phylogenetic taxonomy would be best served by placing *S. schenckii* formally in the sexual genus *Ophiostoma*. At present, Article 59 of the Botanical Code of Nomenclature makes no provisions for including asexual fungi in holomorphic or "whole fungus" (Hennebert and Weresub, 1977) genera (but see Reynolds and Taylor, 1992). In most cases, as with *S. schenckii*, fungi lacking sexual states lack important, morphological taxonomic characters necessary for generic placement. However, as we have shown, DNA sequence characters can compensate for missing sexual characters.

O. ulmi and *S. schenckii* are two closely related, economically important fungi. Both have been objects of significant research by people whose primary interest is not taxonomy. That they are not in the

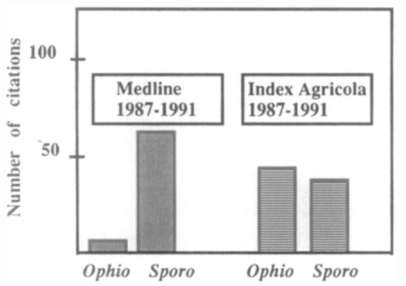

FIG. 3. Are medical researchers studying the physiology of *S. schenckii* aware of research by plant pathologists on *O. ulmi* and vice-versa? If they rely on indexing services in their fields for literature citations, they might not be. This histogram illustrates a lack of cross-referencing in the papers cited by medical and agricultural indexing services. Medline includes only five citations with the key word *Ophiostoma* but 71 with *Sporothrix* as a key word, where Index Agricola includes 44 references with *Ophiostoma* and 35 with *Sporothrix* as key words. A search for citations on the subject "*Sporothrix*" will not yield the papers indexed under the subject "*Ophiostoma*" in either data base.

same genus is an impediment to communication between the medical mycologists working on *S. schenckii* and the plant pathologists working on *O. ulmi* (Fig. 3). In the data base Medline, indexed from 1987 to October 29, 1991, no paper on molecular transformation systems was indexed under the key words "transformation" and "*Sporothrix*." However, a protocol for transforming *O. ulmi* (Royer *et al.*, 1991), which could probably be adapted to transformation of *S. schenckii* with minimal modification, was indexed under the key words "*Ophiostoma*" and "transformation."

The teleomorph or sexual state for *S. schenckii*, if one exists, may turn out to be a previously described or a new species but it will certainly be an *Ophiostoma* species. This is the first example where DNA sequence techniques have demonstrated an anamorph–teleomorph connection in a statistically supported, evolutionary sense.

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REFERENCES

BERBEE, M. L., AND TAYLOR, J. W. 1992. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. *Mol. Biol. Evol.*, 9(2), in press.

BRUNS, T. D., WHITE, T. J., AND TAYLOR, J. W. 1991. Fungal molecular systematics. *Annu. Rev. Ecol. Syst.* **22**: 525–564.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–791.

HENNEBERT, G. L., AND WERESUB, L. K. 1977. Terms for states and forms of fungi, their names and types. *Mycotaxon* **6**: 207–211.

HOOG, G. S. DE. 1974. The genera *Blastobotrys, Sporothrix, Calcarisporium* and *Calcarisporiella* gen. nov.. *Stud. Mycol.* 7: 1–84.

KLUGE, A. G., AND FARRIS, J. S. 1969. Quantitative phyletics and the evolution of anurans. *Syst. Zool.* **18**: 1–32.

LEE, S. B., AND TAYLOR, J. W. 1990. Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 282–287 Academic Press, San Diego.

MARIAT, F. 1977. Taxonomic problems related to the fungal complex *Sporothrix schenckii/Ceratocystis* spp. In *Recent Advances in Medical and Veterinary Mycology* (K. Iwata, Ed.), pp. 265–270. University Park Press, Baltimore, MD.

MENDONÇA-HAGLER, L. C., TRAVASSOS, L. R., LLOYD, K. O., AND PHAFF, H. J. 1974. Deoxyribonucleic acid base composition and hybridization studies on the human pathogen *Sporothrix schenckii* and *Ceratocystis* species. *Infect. Immun.* **9**: 934–938.

REYNOLDS, D. R., AND TAYLOR, J. W. 1991. DNA specimens and the 'International code of botanical nomenclature'. *Taxon* **40**: 311–315.

REYNOLDS, D. R., AND TAYLOR, J. W. 1992. Article 59: Reinterpretation or revision? *Taxon*, **41**, in press.

RIPPON, J. W. 1988. *Medical Mycology, The Pathogenic Fungi and the Pathogenic Actinomycetes*. pp. 325–352. Saunders, Philadelphia.

ROYER, J. C., DEWAR, K., HUBBES, M., AND HORGEN, P. A. 1991. Analysis of a high frequency transformation system for *Ophiostoma ulmi*, the causal agent of Dutch elm disease. *Mol. Gen. Genet.* **225**: 168–176.

SWOFFORD, D. L. 1989. *PAUP Phylogenetic Analysis Using Parsimony Version 3.0l.* Champaign, IL.

TAYLOR, J. J. 1970. A comparison of some *Ceratocystis* species with *Sporothrix schenckii*. *Mycopathol. Mycol. Appl.* 42: 233–240.

TRAVASSOS, L. R. 1985. *Sporothrix schenckii*. In *Fungal Dimorphism with Emphasis on Fungi Pathogenic for Humans*. (P. J. Szaniszlo and J. L. Harris Eds.), pp. 121–163. Plenum, New York.

UPADHYAY, H. P. 1981. *A Monograph of Ceratocystis and Ceratocystiopsis*. Univ. of Georgia Press, Athens, GA.

WHITE, T. J., BRUNS, T., LEE, S., AND TAYLOR, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols*, (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 315–322. Academic Press, San Diego.