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Cryptic species in *Stachybotrys chartarum*

Michael Cruse
Robin Telerant
Thomas Gallagher
Thomas Lee
John W. Taylor[1]

Department of Plant and Microbial Biology, 321 Koshland Hall, University of California, Berkeley, California, USA 94720-3102

Abstract: *Stachybotrys chartarum* has received much attention as a possible cause of sick-building syndrome. Because morphological species recognition in fungi can hide diversity, we applied a phylogenetic approach to search for cryptic species. We examined 23 isolates from the San Francisco Bay Area, and another seven from around the US. Using markers we developed for three polymorphic protein coding loci (chitin synthase 1, beta-tubulin 2, and trichodiene synthase 5), we infer that two distinct phylogenetic species exist within the single described morphological species. We have found no correlation between genetic isolation and geographic distance.

Key Words: fungal species, molecular evolution, phylogenetic species, population genetics, sick-building syndrome

INTRODUCTION

Stachybotrys chartarum is a toxigenic fungus of worldwide distribution. It is of particular interest because it is implicated as a cause of ''sick building syndrome'' (Cooley et al 1998). This fungus can grow on the paper covering gypsum wallboard, provided that it is saturated by water, and introduce potentially toxic spores into buildings. It produces numerous toxins, including a set of highly toxic trichothecenes (Jarvis et al 1998). These toxins can cause stachybotrytoxicosis, which first was described in Russia in 1931 when thousands of horses died from consuming contaminated hay (Forgas 1972). The fungus has been shown to produce the hemolysin stachylysin (Vesper et al 2001), and has been suspected as a cause of infant pulmonary hemosiderosis (Anonymous 1994), although this association is controversial (Anonymous 2000).

Stachybotrys chartarum is one of 11 morphologically recognized *Stachybotrys* species (Jong and Davis 1976). Their phylogenetic relationships have been established by analysis of the rDNA internal transcribed spacer (ITS) (Haugland et al 2001), which suggested that two *Memnoniella* species be transferred to *Stachybotrys*, bringing to 13 the total number of morphologically recognized *Stachybotrys* species.

We wondered if more than one phylogenetic species could be recognized in the morphological species, *S. chartarum*. This possibility was suggested by variation among individuals seen in the RAPD analysis conducted by Vesper and colleagues (Vesper et al 1999, 2000), by other work showing variation in the levels of toxin production among *S. chartarum* individuals (Jarvis 1998, Vesper et al 1999), and by the discovery of one biallelic, polymorphic nucleotide position within the ITS region of *Stachybotrys chartarum* (Haugland et al 2001). To recognize phylogenetic species, one gene genealogy cannot suffice (Taylor et al 2000), therefore we used the concordance of three gene genealogies to search for phylogenetic species in *Stachybotrys chartarum*.

Phylogenetic species have been recognized in other morphological fungal species, such as, *Coccidioides immitis* (Koufopanou et al 1997), *Histoplasma capsulatum* (Kasuga et al 1999), *Aspergillus flavus* (Geiser et al 1998) and the *Letharia vulpina–Letharia columbiana* complex (Kroken and Taylor 2001). Biological species recognition was possible only in *Histoplasma capsulatum* (teleomorph *Ajellomyces capsulatus*), because the others, like *S. chartarum*, either are asexual or cannot be mated experimentally.

To determine phylogenetic species boundaries in *S. chartarum*, gene genealogies were constructed by sequencing regions from three independent genes in 30 individuals of *S. chartarum*, 23 of which were from the San Francisco Bay Area of California and seven from other regions of the United States. Maximum parsimony analysis of the variation present in the genes, separately and together, partitioned the isolates into two strongly supported phylogenetic species, which lack any obvious geographic correlation.

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[1] Corresponding author: Email: jtaylor@socrates.berkeley.edu

TABLE I. Sample sheet

Sample number	Location	Sample room
1	Home in Albany, CA	
2	Environmental Sample, Berkeley, CA	
5	Environmental Sample, Berkeley, CA	
6	Environmental Sample, Berkeley, CA	
7	Home in San Rafael, CA	
8	Home in San Rafael, CA	
11	Apartment in Oakland, CA	Unit 1, Room A
12	Apartment in Oakland, CA	Unit 1, Room A
13	Apartment in Oakland, CA	Unit 2, Room A
14	Apartment in Oakland, CA	Unit 2, Room A
15	Apartment in Oakland, CA	Unit 2, Room B
16	Apartment in Oakland, CA	Unit 2, Room B
17	Apartment in Oakland, CA	Unit 2, Room B
18	Apartment in Oakland, CA	Unit 2, Room B
101	Pathcon Ohio	
102	Pathcon Texas	
103	Pathcon New Mexico	
104	Pathcon South Carolina	
105	Pathcon Florida	
108	Pathcon Massachusetts	
110	Pathcon Kansas	
201	Apartment in Oakland, CA	Unit 3, Room A
203	Apartment in Oakland, CA	Unit 3, Room A
204	Apartment in Oakland, CA	Unit 3, Room A
205	Apartment in Oakland, CA	Unit 3, Room A
206	Apartment in Oakland, CA	Unit 3, Room A
207	Apartment in Oakland, CA	Unit 4, Room B
209	Home in Elk Grove, CA	
210	Home in Elk Grove, CA	
211	Apartment in Oakland, CA	Unit 3, Room A

MATERIAL AND METHODS

Fungal isolates.—Putative isolates of *S. chartarum* were obtained from structures and leaf litter in Northern California. Isolates from other parts of the United States were purchased from a commercial lab specializing in the identification of fungi growing in structures. Putative samples of *Stachybotrys chartarum* were collected from structures by using clear adhesive tape to remove fungi from the contaminated region and affixing the tape to a glass slide in the field. Pieces of tape were cut from the slide and placed on wet sterile Whatman #1 filter paper sitting atop sterile 1% H_2O agar plates (Difco, Detroit, Michigan). Putative environmental samples of *S. chartarum* were obtained by macerating potentially contaminated leaf litter with sterile water in a Waring blender. The resulting homogenate was poured on sterile Whatman #1 filter paper sitting atop 1% H_2O agar plates. Isolates from around the United States were purchased from Pathcon Laboratories (Norcross, Georgia). Sample location and number are shown in TABLE I.

Putative *S. chartarum* colonies grown on filter paper from tape or leaf samples were transferred to sterile Whatman #1 filter paper sitting atop 1% H_2O water agar plates and maintained on the filter papers or subsequently transferred to cornmeal agar plates (Difco, Detroit, Michigan). The identities of the samples were initially checked by observing morphology as described by Jong and Davis (Jong and Davis 1976). For long-term storage, mycelium was grown in 5 mL of yeast extract broth (20 g glucose, 10 g yeast extract, 2 g peptone per 1 liter water) for 2–3 d and lyophilized. Mycelium for DNA extraction was obtained in a similar manner. Mycelium was grown in 100 mL of yeast broth at room temperature for 3–4 d with agitation. Mycelium was collected by filtration, frozen in liquid nitrogen, and lyophilized.

DNA extraction, amplification, and sequencing.—DNA was extracted using a modification of a CTAB extraction protocol (Platt 1999). Approximately 200 mg of lyophilized sample was placed in a 2 mL bead beater tube along with 2–3, 5 mm glass beads and 1 mL of 2× CTAB extraction buffer (Doyle and Doyle 1987). The tube was agitated for ca 30 s on a Beadbeater (Bartlesville, Oklahoma) at maximum speed. Following lysis, the samples were extracted with 750 μL of chloroform : isoamyl alcohol (24:1). The DNA was purified further using Qiagen's DNeasy kit (Cat. No. 69506, Qiagen, Valencia, California) following manufacturer's instructions.

As a second check of identity, amplicons for the ribosomal internal transcribed spacer (ITS) were sequenced as described below using the ITS primer pair ITS1/4 (White et al 1990) or ITS1A/4F (Gardes and Bruns 1993) from a subset of the samples (1, 2, 4, 17, 101, 102, 103, 105, 108, 109). The sequences of these PCR-amplified fragments were compared to GenBank sequences (AF081468 and AF206273) using Sequence Navigator 1.01 (Applied Biosystems, Foster City, California).

Three loci were sequenced, as described below, in order to recognize phylogenetic species by the concordance of gene genealogies. The trichodiene synthase 5 fragment (*tri5*) was obtained by designing primers for the published sequence of trichodiene synthase 5 (GenBank AF053926) using Oligo 4.0 (National Biosciences Inc., Plymouth, Minnesota). The 5′ primer used was CATCAATCCAACAGTTTCAC and the 3′ primer GCAACCTTCAAAGACTATTG. The beta-tubulin 2 (*tub2*) primers were designed by aligning sequences of *tub2* for closely related ascomycetes (*Gibberella fujikuroi* Genbank U27303, *Aspergillus flavus* Genbank M38265, *Neurospora crassa* Genbank M13630, *Colletotricum gloeosporioides* Genbank U14138, and *Acremonium chrysogenum* Genbank X72789). A consensus sequence was made and a degenerate primer pair was designed by hand and then checked in Oligo. The *tub2* used was CTGTCCAACCCCTCTTACGGCGACCTGAAC for the 5′ primer and ACCCTCACCAGTATACCAATGCAAGAAAGC for the 3′ primer. The chitin synthase 1 fragment (*chs1*) was obtained using the degenerate chitin synthase primer set MYK1/2 (Bowen et al 1992). Sequence from this PCR amplification was used as the basis for designing taxon specific primers. The taxon specific primers were ATCTCACCACAAGCACCGCCACACA

for the 5′ primer and GGAAGAAGATCGTTGTGTGCGTGGT for the 3′ primer.

Tri5 and *tub2* of the *Stachybotrys chartarum* genome were amplified from DNA samples using 50-μL polymerase chain reactions (PCR) containing 0.5 units of AmpliTaq DNA polymerase, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/mL gelatin, and 200 μM of each of four deoxyribonucleotide triphospates. Primers were added in 0.5 μM concentrations in *tub2*, *tri5*, and ITS. *Chs1* was amplified in 20 μL reactions with a primer concentration of 1 μM.

PCR products were prepared for sequencing using Qiagen's QIAquick PCR purification kit or using an isopropanol precipitation (Platt and Spatafora 1999). All *chs1* products were cleaned using an isopropanol precipitation. Purified PCR product was sequenced using an ABI model 377 Sequencer and ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California). We analyzed and aligned the obtained sequences using Sequencing Analysis 3.0 and Sequence Navigator 1.01 (Applied Biosystems, Foster City, California). The initial sequences obtained from the loci were tested for identity by using a nucleotide-nucleotide BLAST search (Altschul et al 1997) with a low complexity filter and word size set to 11. A translated BLAST search (Altschul et al 1997) was then performed with a low complexity filter and a word size set to 3 on the sequences obtained for *tub2* and *chs1* to approximate intron and exon sites.

Phylogenetic analysis.—Sequences obtained from *tri5, tub2,* and *chs1* were aligned and then checked by eye using Sequence Navigator 1.01 (Applied Biosystems, Foster City, California). There were no gaps in the alignments. The aligned sequences were exported to a NEXUS file and analyzed using PAUP 4.0b8 (Swofford 2001). ITS sequence data was used only for identification and it was not included in the phylogenetic analysis.

All sequences were placed in a single NEXUS file and partitioned by each locus to create the complete data set. These data were then analyzed using maximum parsimony. Due to low amounts of variation and virtually no homoplasy in the data set, unless otherwise stated all parameters were set to the default suggested by PAUP, in which gaps were treated as missing data, multiple states were treated as uncertainties, character states were optimized using the accelerated transformation algorithm, and zero length branches were collapsed. Analysis was done individually on each locus and on all loci combined. Heuristic searches were carried out using tree-bisection reconnection and 1000 random sequence additions. The set of the most parsimonious trees was compared using maximum likelihood by the Kishino-Hasegawa test (Kishino and Hasegawa 1989) as implemented in PAUP. Algorithm-specific biases were tested for by comparing parsimony trees to trees created using neighbor-joining and maximum likelihood. Support for internal branches was assessed using a heuristic parsimony search of 1000 bootstrapped data sets. To assess congruence among trees produced by the different loci, the partition-homogeneity test was conducted in PAUP using only the parsimony informative characters. The likelihoods of the trees shown were not significantly different from those of the other equally parsimonious trees, as determined by the Kishino-Hasegawa test. The trees shown are rooted at the midpoint.

RESULTS

The full data matrix and the trees used in the analysis are available from TreeBASE, study accession number S724, matrix accession number M1155. The ITS sequences of the putative *S. chartarum* individuals all matched one of the two expected sequences of *S. chartarum* (Haugland and Heckman 1998), which differ at only one polymorphic nucleotide position.

A translated BLAST search indicated that *chs1* sequences obtained were all protein coding. A sample from each clade was chosen for submission to Genbank (sample 5 AF468158 and sample 6 AF468159). The alignments are available on TreeBASE. Maximum parsimony analysis of *chs1* produced one tree shown in FIG. 1. This tree has a consistency index (CI) of 1, retention index (RI) of 1, and a homoplasy index (HI) of 0. There are 11 informative, and two variable, uninformative characters in this data set.

The sequences obtained for *tub2* have a 63 bp putative intron, according to data obtained from a translated BLAST search, and character changes are not present in the intron at a higher frequency than in the exons. A sample from each clade was chosen for submission to Genbank (sample 5 AF468156 and sample 6 AF468157). Maximum parsimony analysis of *tub2* produced eight trees. The occurrence of multiple trees is due to unsequenced characters in the sequence of isolate 14. All eight trees have a CI of 1, and RI of 1 and a HI of 0. The tree shown in FIG. 2 has the highest log-likelihood of the eight most parsimonious trees as determined by the Kishino-Hasegawa test, though there is no significant difference among the eight likelihood scores. There are 27 informative and two variable, uninformative characters in this set.

Maximum parsimony analysis of *tri5* produced a single tree shown in FIG. 3. A sample from each clade was chosen for submission to Genbank (sample 5 AF468154 and sample 6 AF468155). This tree has a CI of 1, RI of 1, and HI of 0. There are 30 parsimony-informative characters and one variable, uninformative character.

Phylogenetic analysis of the complete data set with the three genes made use of 1350 characters for 30 taxa. Of these characters, 68 were potentially parsimony-informative, and five were potentially uninformative. The initial heuristic search produced three equally parsimonious trees each having a consistency index of 1, a retention index of 1, and a homoplasy index of 0. All three trees were part of one island.

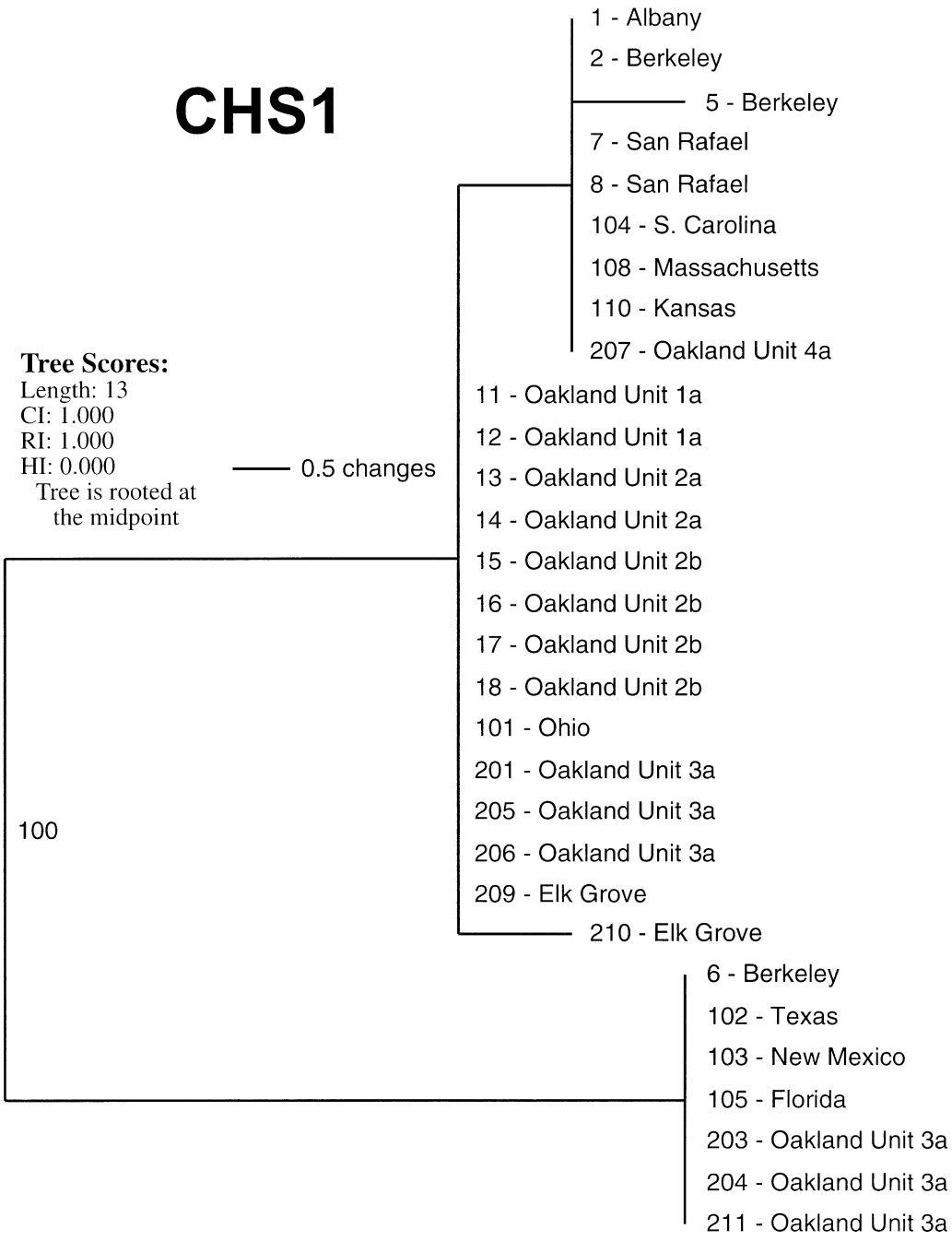

FIG. 1. Chitin synthase gene genealogy. The single most parsimonious tree based on *chs1* gene sequence using a heuristic search with 1000 random sequence additions as described in the text. Support for internal branches represents the percentage of 1000 bootstrap resampled data sets containing the branch. A scale for the number of nucleotide substitutions is given.

All three trees showed two distinct clades separated from one another by one branch supported by 65 characters, which was found in of 100% of bootstrapped data sets. The tree shown in FIG. 4 has the highest log-likelihood of the three most parsimonious trees. There is no significant difference in the likelihood scores among the trees. Trees created using neighbor-joining algorithms had the same topology as those created with maximum parsimony.

DISCUSSION

The trees produced from our data set show that the single morphological species *S. chartarum* actually is

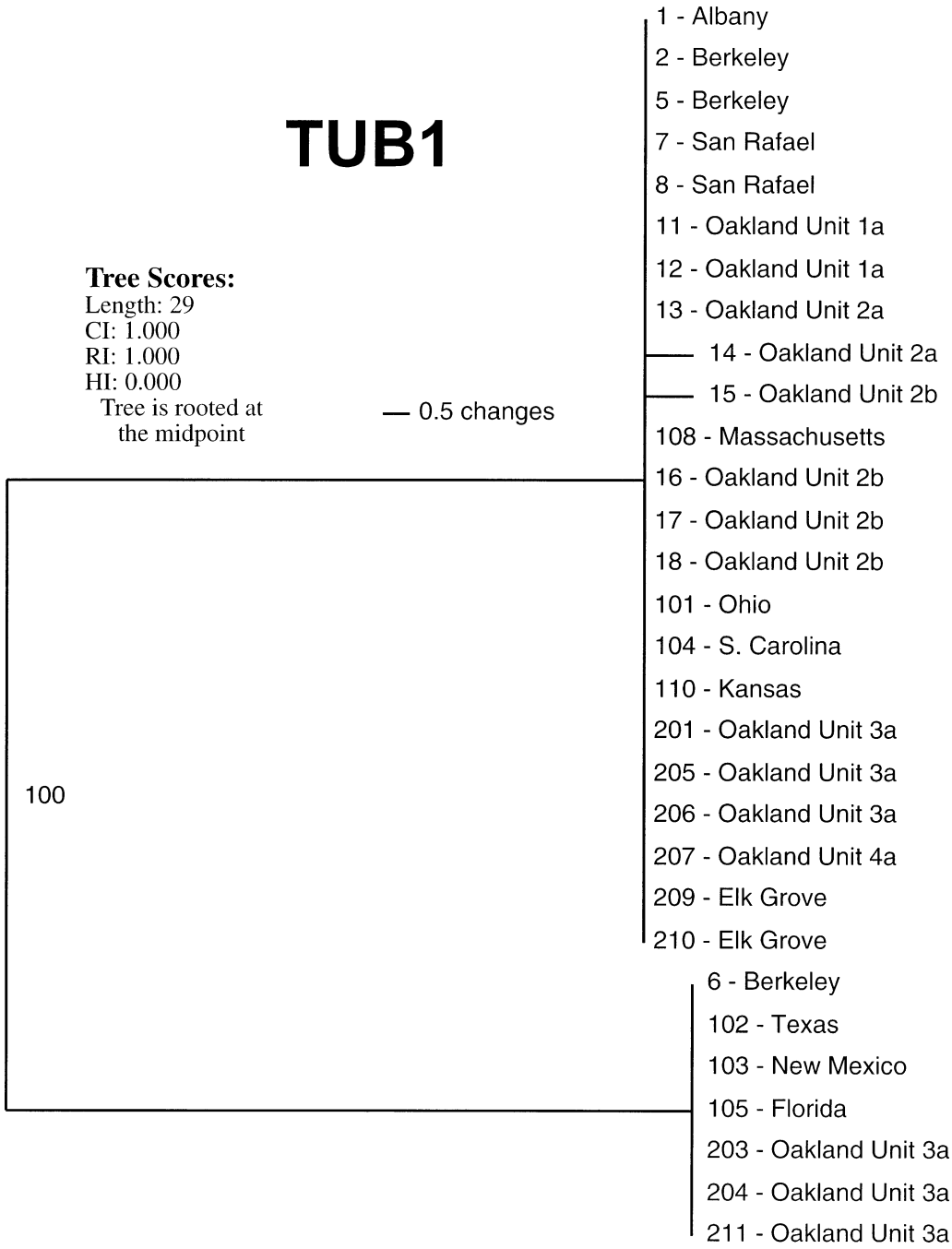

FIG. 2. Beta-tubulin gene genealogy. Shown is the one of eight most parsimonious trees based on *tub2* gene sequence using a heuristic search with 1000 random sequence additions as described in the text, for which the data set is the most likely based on maximum likelihood analysis. Support for internal branches represents the percentage of 1000 bootstrap resampled data sets containing the branch. A scale for the number of nucleotide substitutions is given.

composed of two phylogenetic species. The large percentage (approximately 5%) of nucleotide positions in the three protein-coding loci that are fixed for alternate nucleotides between the two species is consistent with a long period of genetic isolation. Of 73 variable characters, 63 were fixed between the species while three were confirmed variable within one species but not the other. Unsequenced characters could potentially make this number as high as ten. For comparison, in the two phylogenetic species of *Coccidioides immitis* only half of the variable nucleotide positions are fixed in both species; the other half are fixed in one and variable in the other (Koufopanou et al 1997). This result might seem contradictory to pre-

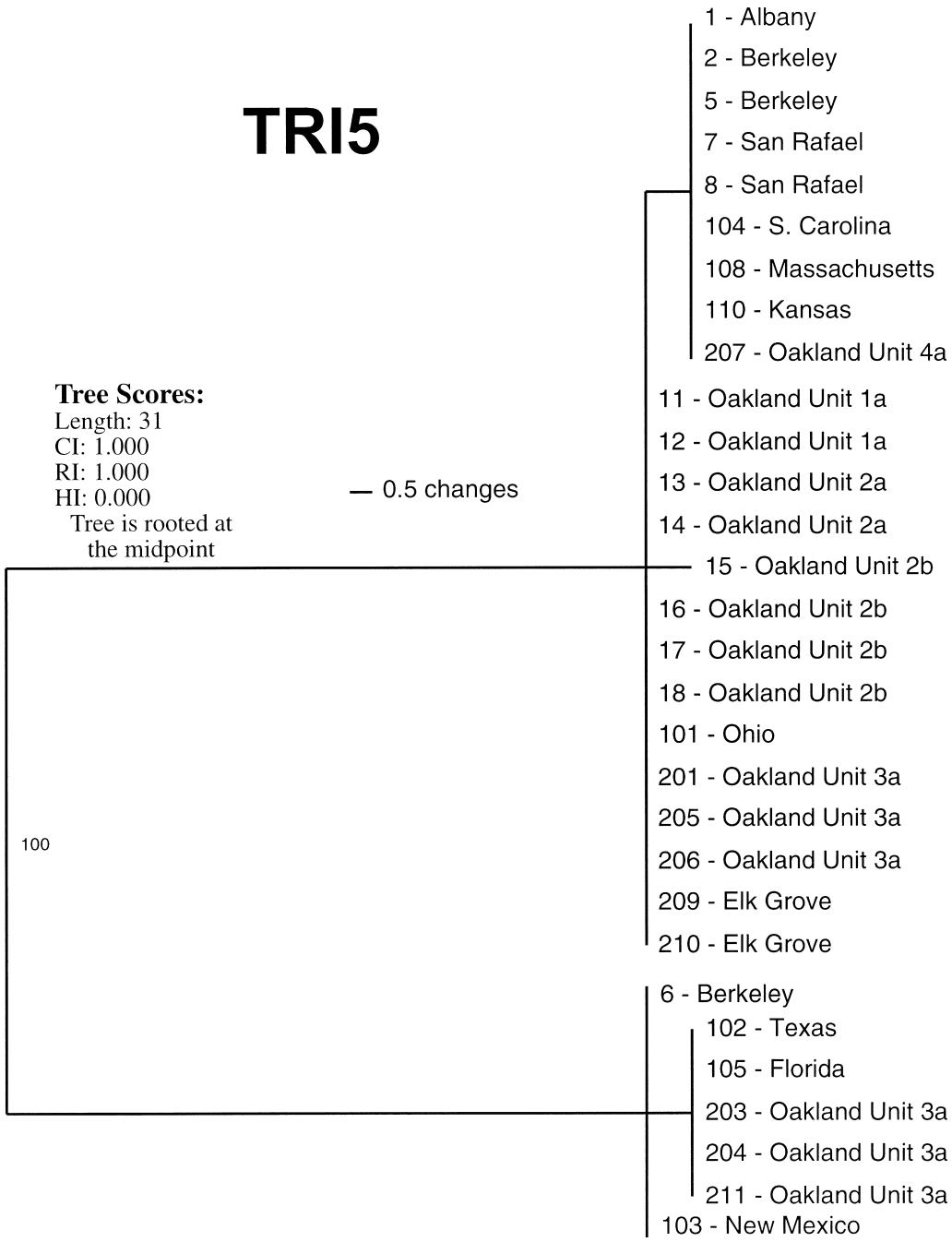

FIG. 3. Trichodiene synthase 5 gene genealogy. The single most parsimonious tree based on *tri 5* gene sequence using a heuristic search with 1000 random sequence additions as described in the text. Support for internal branches represents the percentage of 1000 bootstrap resampled data sets containing the branch. A scale for the number of nucleotide substitutions is given.

vious RAPD analysis by Vesper and colleagues (Vesper et al 1999, 2000), which did not show strongly supported branches in analyses of all isolates. This apparent conflict may be explained in two ways: the sampling of Vesper and colleagues did not include members of the one of the clades found here, or RAPD analysis was not be able to elucidate the two species (Taylor et al 1999).

Though the period since the divergence of the two phylogenetic species may have been long, the genetic isolation does not correlate with the geographic isolation. In this study the diversity seen in a single room was similar to the diversity seen throughout the US. This pattern of variation is similar to that seen in *Aspergillus flavus* (Geiser et al 1998, Tran-Dinh et al 1999) and *Neurospora species* (Spieth 1975), but is un-

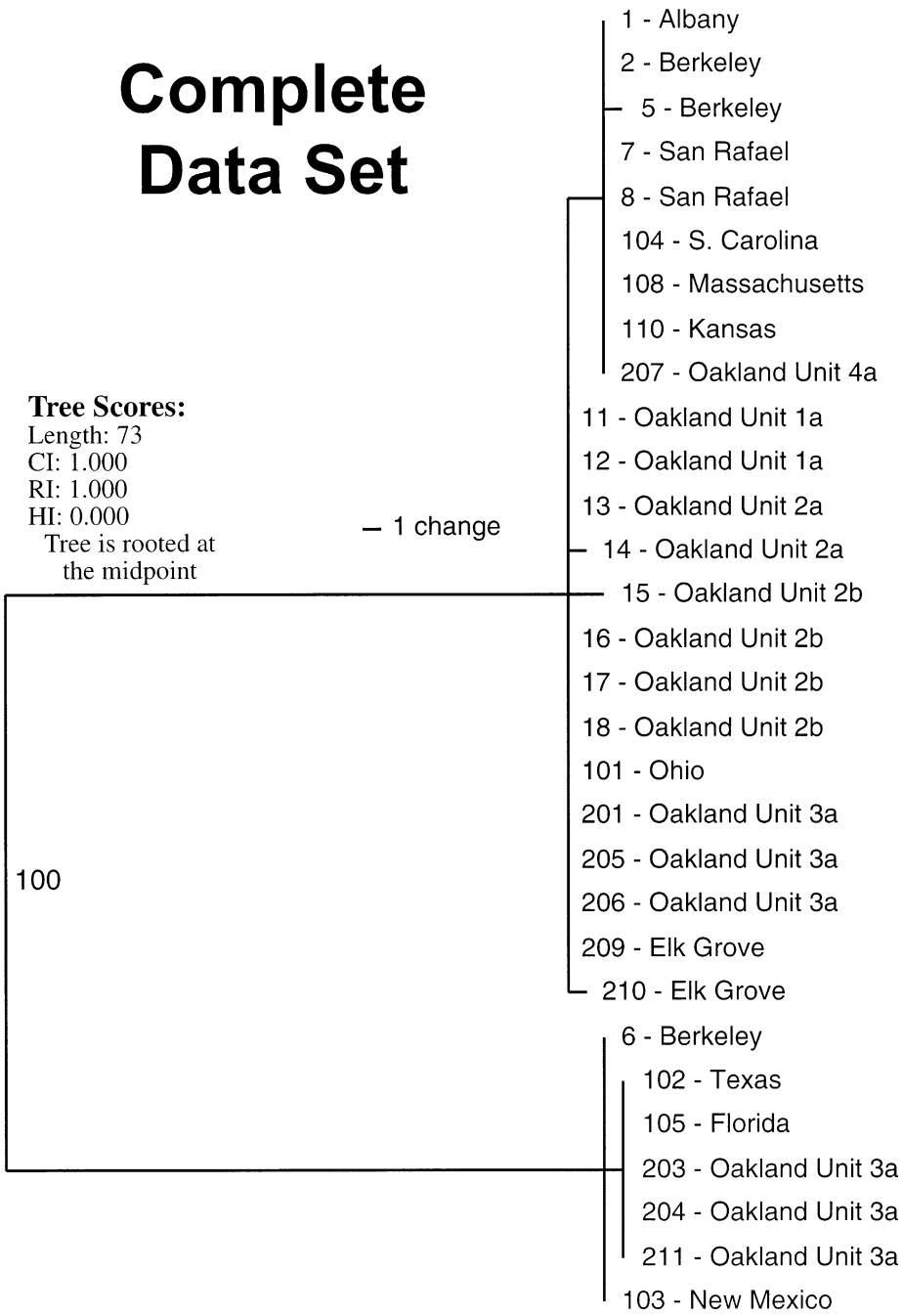

FIG. 4. Combined *chs1*, *tub2* and *tri5* phylogeny. Shown is the one of three most parsimonious trees based on combined gene sequence using a heuristic search with 1000 random sequence additions as described in the text, for which the data set is the most likely based on maximum likelihood analysis. Support for internal branches represents the percentage of 1000 bootstrap resampled data sets containing the branch. A scale for the number of nucleotide substitutions is given.

like the strong geographic distinction seen between the phylogenetic species in *Histoplasma capsulatum* (Kasuga et al 1999) and *Coccidioides immitis* (Koufopanou et al 1997, 1998, Fisher et al 2001). With further geographic sampling it will be possible to see if this lack of correlation is an effect of our limited sampling, or perhaps, of the spread of this fungus by human activity. With further sampling and markers it may also be possible to identify populations within the species, as has been done for *C. immitis* (Fisher et al 2001).

Each clade is fixed for one of the two alleles for ITS as previously described (Haugland and Heckman 1998). One clade (the larger group) has the ITS se-

quence of the type collection (ATCC 9182, GenBank AF081468). The other clade, therefore, represents the new phylogenetic species.

Due to the recent attention given to *S. chartarum*, a pressing question is raised by this study. Are the types and amounts of toxin produced by members of the two phylogenetic species different? We hope that our work will stimulate research in this area.

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