Female Fertility and Single Nucleotide Polymorphism Comparisons in *Cylindrocladium pauciramosum*

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ABSTRACT

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Cylindrocladium pauciramosum is well established in South America, and has recently been collected from nurseries in South Africa, Italy, and the United States. Isolates were compared with respect to the percentages of hermaphrodites and the respective mating types in the different samples. Based on these data, the effective population size could be determined for the different areas studied. All nurseries had mating type ratios significantly different from an idealized 1:1 ratio. In the South African nursery, the MAT-1 mating type was dominant, while the MAT-2 mating type dominated in other samplings. This is consistent with an introduction of a small starter population. High percentages of hermaphrodites also agreed with recent introductions into nurseries in Italy and the United States. Variability of DNA sequences of the 5′ end of the β-tubulin gene from a set of *C. pauciramosum* isolates from different geographic regions was low to high. Isolates from South Africa, the United States, and Australia had identical β-tubulin DNA sequences; this sequence was also found in the Italian sample, along with another unique group. Finally, a group of isolates obtained from South and Central America had the highest variation of all isolates investigated, and also included isolates that shared single nucleotide variations with another species, *C. candelabrum*. These findings suggest that *C. pauciramosum* most likely has a Central or South American center of origin.

Cylindrocladium pauciramosum C. L. Schoch & Crous was recently described as a member of the *Cylindrocladium candelabrum* Viégas species complex (26). *Cylindrocladium* spp. are associated with *Calonectria* De Not. teleomorphs (24). Species are distinguished based on morphological features of the anamorph, such as conidium, vesicle, and phialide morphology, as well as cultural characteristics. Morphological features of the teleomorph tend to be more conserved, and species identification based on these characters alone is usually not possible (9).

C. candelabrum is a well-known root and leaf pathogen of numerous hosts. This species has regularly been confused with *C. scoparium* Morgan (2,11,22). In order to distinguish these two species, *C. scoparium* was circumscribed as having ellipsoidal to pyriform vesicles, and isolates of *C. candelabrum* ellipsoidal to obpyriform vesicles (5). Mating studies have shown both these species to be distinct and heterothallic (5,26).

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Previous studies delineated four genetically isolated mating populations within the boundaries of what was accepted to represent *C. candelabrum* (26). DNA sequencing confirmed these to be separate entities and, consequently, four species were described. One of these species, described as *C. pauciramosum,* is known from isolates collected in Australia, Brazil, Colombia, Mexico, and South Africa.

Published records indicate that *C. pauciramosum* has been associated with diseases of plants in South Africa for several years as either *C. scoparium* (2,10,11,15) or *C. candelabrum* (5). Previous reports of a new disease attributed to *C. scoparium* from nurseries in Italy (22) were subsequently shown to be *C. pauciramosum* (23). In addition to this, another report confirmed the recent introduction of this fungus to California (14).

The phylogenetic relationship of *C. scoparium* to other heterothallic, small-spored *Cylindrocladium* spp. was recently investigated by means of DNA sequence comparisons (25,27). Although previous work in this regard could distinguish closely related species based on small differences in the sequence of the 5.8S rDNA and flanking internal transcribed spacers (ITS1 and ITS2; 26), the low number of informative characters made phylogenetic determinations difficult. The use of DNA sequences obtained from additional areas, like the β-tubulin gene and the HMG box of the *MAT-2* mating type gene, yielded higher variation and distinguished most species previously defined on other characters (25).

C. pauciramosum is self sterile. Female structures consist of protoperithecia, which can be spermatized by conidia or hyphae from an opposite mating type. A typical heterothallic ascomycete has been defined as a self-sterile hermaphrodite, capable of producing the female reproductive structures as well as male gametes (17). Generally, male functions can be performed by asexual spores, sexual spores, or hyphae. Observations in *Gibberella fujikuroi* have shown that the female function is lost regularly (16). These female sterile isolates can only act as males and were proposed to have a vegetative advantage during asexual reproduction, because no resources would be required for female reproductive structures. The opposite scenario was proposed for conditions favoring sex, resulting in a higher percentage of hermaphrodites (17). The ratios of the two mating types and of female steriles and hermaphrodites can be used to determine the importance of sexual replication and the effective population (N_e), giving an estimate of the size of a finite population as first proposed by Wright (33). These principles were reviewed by Caballero (4) and adapted for haploids (17). Recent studies (3,19) made use of these assumptions in order to gain information on the effective population size and sexual dynamics of mating populations in the *G. fujikuroi* complex.

The goals of this study were, first, to determine the ratios of the two mating types in the newly recognized populations in Italy (22) and California (14), and, second, to compare this with a well-established South African population. A final aim was to obtain data relating to intraspecific variation of *C. pauciramosum*-based single nucleotide polymorphisms (SNPs) in a β-tubulin gene among isolates collected from a wide geographical area.

MATERIALS AND METHODS

Isolates. Isolates of *C. pauciramosum* were either obtained from symptomatic plant material or baited from soil samples (7). All isolates were identified using the morphological concepts, mating types, and keys as defined in earlier studies (7,26). Isolates studied are listed in Table 1.

South African isolates of *C. pauciramosum* were obtained from the culture collection at the Department of Plant Pathology at the University of Stellenbosch (STE-U; Table 1). These were collected throughout South Africa over a period from 1990 to 1995 and were obtained from diseased plant material as well as from soil. It was assumed that cultural preservation did not adversely affect mating ability because a recent subset from this collection produced successful crosses (26). An additional sample of 50 isolates was obtained from diseased crown and root tissues of individual cherry plants from a small nursery in Stellenbosch where this disease was recently recognized. Italian isolates were obtained from a number of nurseries in Sicily and Calabria (23). In all cases, isolates were randomly collected from hosts, one isolate per plant. In a similar manner, 50 isolates were collected from diseased crowns and roots of heath (*Erica capensis* Salter) from a single nursery in California.

Sexual compatibility. Two opposite mating tester strains (STE-U 416 = MAT-1, STE-971 = MAT-2) were selected for their high fertility during previous studies (25,26). Single isolates were grown on petri dishes containing 2% malt extract agar (MEA; Biolab, Midrand, South Africa) for 2 to 4 weeks until sporulation. Sterile water (1 ml) was added to each petri dish and conidia were dislodged with the help of a sterile glass rod. The conidial suspension was removed with a micropipette. Cultures were spermatized by applying the conidial suspension to petri dishes containing carnation leaf agar (CLA) with 2- to 4-week-old growth. Colonized CLA plates of the selected cultures were respectively spermatized with the two tester strains. Furthermore, the tester strains also were spermatized by each of the selected isolates. Plates were subsequently packed in stacks, sealed in plastic bags, and incubated on the laboratory bench at 22°C. Successful crosses were determined after 2 months of incubation and were regarded as those isolate combinations that produced perithecia with extruding, viable ascospores.

Statistical analysis. The effective population numbers were calculated according to Leslie and Klein (17). The effective population number based on mating type ($N_{e(mt)}$) was determined as $N_{e(mt)} = (4N_{MAT\text{-}1} N_{MAT\text{-}2})/(N_{MAT\text{-}1} + N_{MAT\text{-}2})$ with $N_{MAT\text{-}1}$ the number of MAT-1 strains and $N_{MAT\text{-}2}$ the number of MAT-2 mating-type strains. These are parameters to estimate genetic drift and inbreeding in populations. The inbreeding effective population ($N_{e(f)}$) is based on the probability of identity due to common ancestry and determined as $N_{e(f)} = (4N^2N_h)/(N + N_h)^2$, with N being the total number of individuals and N_h the total number of hermaphrodites.

Isolation of DNA, polymerase chain reaction amplification, and sequencing. Single conidial isolates selected for DNA comparisons (Table 2) were grown on MEA plates. Mycelial mats were removed from the plates by means of a sterile scalpel and ground to a powder with the help of liquid nitrogen and a mortar and pestle. Approximately 40 mg of ground mycelia was added to 2-ml microtubes containing 600 µl of extraction buffer. The extraction buffer consisted of 1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 100 mM EDTA. The subsequent protocol was followed as suggested for the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI). A 600-bp fragment was amplified with the use of primers T1 (21) and Bt2b (13). Polymerase chain reaction (PCR) fragments were sequenced and amplified as described previously (26). Newly acquired sequences were deposited at GenBank (Table 2).

Phylogenetic analysis. The isolates selected for phylogenetic analysis are listed in Table 2. Alignments of sequences were done with the computer package Malign version 2.7 (32), assessed manually, and

Table 1. Isolates used for female fertility and mating type ratio determinations

Isolate number	Location, province	Collector	Date	Host
Italian isolates				
DISTEF-G 3, 191, 192, 193, 194, 195, 196	Lamezia location 1, Catanzaro	G. Polizzi	Jun. 1997	*Polygala myrtifolia*
DISTEF-G 173	Lamezia location 2, Catanzaro	G. Polizzi	Nov. 1997	*Polygala myrtifolia*
DISTEF-G 75, 77, 79, 81, 127, 128	Barcellona location 1, Messina	G. Polizzi	Oct. 1997	*Callistemon* spp.
DISTEF-G 149, 155, 158, 160, 162, 168	Barcellona location 2, Messina	G. Polizzi	Oct. 1997	*Callistemon viminalis*
DISTEF-G 5, 6	Milazzo, Messina	G. Polizzi	Sept. 1997	*Callistemon citrinus*
DISTEF-G 62, 67, 69, 74	Milazzo, Messina	G. Polizzi	Oct. 1997	*Callistemon viminalis*
DISTEF-G 84, 87, 92, 96, 98, 104, 108, 115	Milazzo, Messina	G. Polizzi	Oct. 1997	*Acacia retinodes*
DISTEF-G 10, 20, 31, 32	Carruba, Catania	G. Polizzi	Sept. 1997	*Callistemon* Mauve Mist
DISTEF-G 9	Carruba, Catania	G. Polizzi	Sept. 1997	*Metrosideros robustus*
DISTEF-G 40	Carruba, Catania	G. Polizzi	Sept. 1997	*Eucalyptus viminalis*
DISTEF-G 46	Carruba, Catania	G. Polizzi	Sept. 1997	*Callistemon citrinus*
DISTEF-G 50, 53, 60	Carruba, Catania	G. Polizzi	Sept. 1997	*Myrtus communis*
DISTEF-G 124, 126	Carruba, Catania	G. Polizzi	Oct. 1997	*Arbutus unedo*
DISTEF-G 199	Praiola, Catania	G. Polizzi	Sept. 1994	*Myrtus communis*
DISTEF-G 186	Praiola, Catania	G. Polizzi	Oct. 1994	*Eucalyptus rostrata*
DISTEF-G 1, 2	Praiola, Catania	G. Polizzi	Jul. 1996	*Polygala myrtifolia*
DISTEF-G 21	Praiola, Catania	G. Polizzi	Sept. 1997	*Metrosideros robustus*
DISTEF-G 183	Grotte, Catania	G. Polizzi	Sept. 1994	*Eucalyptus rostrata*
DISTEF-G 175	Grotte, Catania	G. Polizzi	Oct. 1994	*Metrosideros robustus*
DISTEF-G 23	Grotte, Catania	G. Polizzi	Sept. 1997	*Myrtus communis*
South African isolates				
STE-U 247, 249, 256, 257, 271, 273, 274, 344, 346	Kwambonambi, Kwa-Zulu Natal	P. W. Crous	Feb. 1990	*Eucalyptus grandis*
STE-U 1239	Kwambonambi, Kwa-Zulu Natal	P. W. Crous	Oct. 1995	*Eucalyptus grandis*
STE-U 391	Pietermaritzburg, Kwa-Zulu Natal	P. W. Crous	Mar. 1990	*Eucalyptus nitens*
STE-U 575	George, Western Cape	S. Lambrecht	Feb. 1993	*Azalea* bushes
STE-U 971, 972	Knysna, Western Cape	P. W. Crous	Nov. 1994	Soil
STE-U 138, 143	Kruisfontein, Mpumalanga	P. W. Crous	Feb. 1990	Soil
STE-U 356, 358	Sabie, Mpumalanga	P. W. Crous	Feb. 1990	Soil
STE-U 286, 287, 288	Klipkraal, Mpumalanga	P. W. Crous	Feb. 1990	Soil
STE-U 379, 380	Witrivier, Mpumalanga	S. Lambrecht	May. 1990	*Azalea* sp.
STE-U 282 – 284,	Tzaneen, Northern Province	P. W. Crous	Feb. 1990	Soil
STE-U 416, 417	Tzaneen, Northern Province	P. W. Crous	Jun. 1990	Pine cuttings
STE-U 958, 959	Piet Retief, Northern Province	P. W. Crous	Nov. 1994	*Eucalyptus grandis*
STE-U 2575-2630	Stellenbosch, Western Cape	C. Linde	Apr. 1999	*Prunus* sp.
Californian isolates				
STE-U 1982-2030	California	S. Koike	1998	*Erica capensis*

deposited at GenBank and in TreeBase (SN612). Phylogenetic analysis of aligned DNA sequences was performed using a heuristic search with 500 random additions in PAUP* version 4.0b6 (30). Gaps were treated as missing data. Confidence intervals were determined using 1,000 bootstrap replications in a simple heuristic search, with maxtrees set to 1,000.

RESULTS

Effective population numbers based on ratios of mating types and female sterile hermaphrodites of the *C. pauciramosum* samples obtained from the selected areas are shown in Table 3. For the purpose of this study, mating capability of isolates was assumed not to be influenced by the host from which they were isolated, because species in the genus have been found not to be host specific and are essentially soilborne (26). More specific data about host specificity, epidemiology, mating, and other characteristics of these newly described species are still needed.

The values obtained in Table 3 reflect differences in the profiles for the various nurseries. Samples from various areas in South Africa, where the disease has been well established, tended to have a mating type ratio of approximately 1:1. All other samplings, representing areas where the disease has been recently introduced, yielded ratios that significantly favored one mating type. In the Stellenbosch nursery, the ratio favored the MAT-1 mating type, while the nurseries in Sicily had mostly MAT-2 isolates. Additionally, only the MAT-2 mating type was present in California. These figures differ appreciably from those obtained by other workers for species of the *G. fujikuroi* complex (3,17,19), where the highest mating type ratio was near 1:2. Effective population numbers based on mating types ($N_{e(mt)}$) of between 49 and 73% of the total count were inferred in the South African and Italian nurseries (Table 3). Higher effective population numbers were found based on the presence of hermaphrodites (Table 3), particularly for nurseries in California and Italy (98.4 to 99.8%). A lower effective population based on this criterion was found for the South African isolates.

β-tubulin DNA sequence comparisons. Sequence comparisons based on DNA sequences of the β-tubulin gene have previously been used to investigate phylogeny in *C. scoparium, C. pauciramosum,* and related species (26). In the present study, an investigation on the variation in *C. pauciramosum* was undertaken. *C. candelabrum, C. multiseptatum,* and *Fusarium subglutinans* were used as outgroups. As far as possible, six isolates of opposing mating type from disparate regions within a country were used for comparison. The groups indicated in a neighbor-joining dendrogram agreed with the clades seen in the 10 most parsimonious trees (MPTs) found under a heuristic search. A strict consensus tree obtained from these is presented in Figure 1. The only difference between the individual parsimonious trees was in the placement of isolate STE-U 1671. When this isolate was removed from the data set, a heuristic search resulted in only two MPTs. In one of these trees, the South American isolates of *C. pauciramosum* (clade 2) grouped with isolates of *C. candelabrum* (clade 1) and STE-U 1670, while in the other tree these clades remained separate. No change was observed in the groups delineated in Figure 1 when different outgroup rootings were employed.

From a comparison between *C. candelabrum* and *C. pauciramosum*, 27 SNPs were found out of a data set of 521 unordered characters. This is comparable to and consistent with the low amount of variation previously observed between species of *Cylindrocladium* in the ITS1 and 2 spacers opposite to the 5.8S ribosomal RNA gene (6,26). The low number of informative characters (in some cases, a single character) was reflected by the low bootstrap

Table 2. Isolates of *Cylindrocladium pauciramosum* and other species selected for sequence comparisons of a portion of the β-tubulin gene

Species, accession no.	GenBank no.	Host	Origin
F. subglutinans			
NRRL 22016	U34417	…	…
C. pauciramosum			
STE-U 143	AF320200	*Eucalyptus grandis*	Mpumalanga, South Africa
STE-U 344	AF320201	*Eucalyptus grandis*	Kwa-Zulu Natal, South Africa
STE-U 416	AF210869	*Eucalyptus grandis*	Northern Province, South Africa
STE-U 913	AF320202	Soil	Santa Catarina, Brazil
STE-U 925	AF210870	Soil	Santa Catarina, Brazil
STE-U 951	AF320203	Soil	Veracruz, Mexico
STE-U 971	AF320204	*Eucalyptus grandis*	Western Cape, South Africa
STE-U 972	AF210871	*Eucalyptus grandis*	Western Cape, South Africa
STE-U 1160	AF320205	Soil	Córdoba, Colombia
STE-U 1670	AF320206	*Eucalyptus sp.*	Bahia, Brazil
STE-U 1671	AF320207	*Eucalyptus sp.*	Bahia, Brazil
STE-U 1691	AF320208	*Fragaria* sp.	Queensland, Australia
STE-U 1692	AF320209	*Fragaria* sp.	Queensland, Australia
STE-U 1990	AF320210	*Erica capensis*	California, USA
STE-U 2030	AF320211	*Erica capensis*	California, USA
DISTEF-G 2	AF320212	*Polygala myrtifolia*	Catania, Sicily, Italy
DISTEF-G 6	AF320213	*Callistemon citrinus*	Messina, Sicily, Italy
DISTEF-G 60	AF320214	*Myrtus communis*	Catania, Sicily, Italy
DISTEF-G 62	AF320215	*Callistemon citrinus*	Messina, Sicily, Italy
DISTEF-G 84	AF320216	*Acacia retinodes*	Messina, Sicily, Italy
DISTEF-G 126	AF320217	*Arbutus unedo*	Catania, Sicily, Italy
DISTEF-G 127	AF320218	*Callistemon citrinus*	Messina, Sicily, Italy
DISTEF-G 128	AF320219	*Callistemon citrinus*	Messina, Sicily, Italy
DISTEF-G 192	AF320220	*Polygala myrtifolia*	Catanzaro, Calabria, Italy
DISTEF-G 196	AF320221	*Polygala myrtifolia*	Catanzaro, Calabria, Italy
C. candelabrum			
STE-U 1674	AF210857	*Eucalyptus* sp.	Brazil
STE-U 1677	AF210858	*Eucalyptus* sp.	Amazonas, Brazil
STE-U 1951	AF210859	Soil	Brazil
UFV 89	AF320199	Soil	Brazil
C. mexicanum			
STE-U 927	AF210863	Soil	Yucatan, Mexico
STE-U 941	AF210864	Soil	Campeché, Mexico
C. multiseptatum			
STE-U 1589	AF210865	*Eucalyptus* sp.	Indonesia
STE-U 1602	AF210866	*Eucalyptus* sp.	Indonesia

Table 3. Comparison of the distribution of mating type and hermaphrodites among isolates of *Cylindrocladium pauciramosum* from three geographic areas

		N_e (effective population)[a]		
Geographic origin	Ratio[b]	N_{fs}:N_h[c]	$N_{e(mt)}$[d]	$N_{e(f)}$[e]
South Africa: Stellenbosch nursery	48:8	31:25	49.0	85.3
Elsewhere in South Africa	21:23	29:15	99.7	75.8
United States: California nursery	0:50	4:46	0	99.8
Italy: various nurseries	13:41	12:42	73.1	98.4

[a] Effective population number based on the numbers of males (N_{fs}) and hermaphrodites (N_h) as percentage of the actual count.
[b] Mating type ratio as MAT-1:MAT-2; data from Schoch et al. (26).
[c] Ratio of female sterile: hermaphrodites in the population.
[d] Effective population number based on mating type ratios (17).
[e] Inbreeding effective number based on numbers of female steriles and hermaphrodites (17).

values seen in Figure 1. In order to show the variation at sequence level, the SNPs are presented as numbered boxes or circles in Figure 1. Variable characters were almost exclusively situated in the noncoding regions of the β-tubulin gene. Only two characters (base pairs 273 and 348) were inside the coding regions.

The *C. candelabrum* isolates obtained from Brazil (group 1; Fig. 1) had identical sequences. Although *C. candelabrum* is closely related to *C. pauciramosum*, these two species have been shown to differ biologically and genetically (26). The *C. candelabrum* isolates (clade 1) shared a

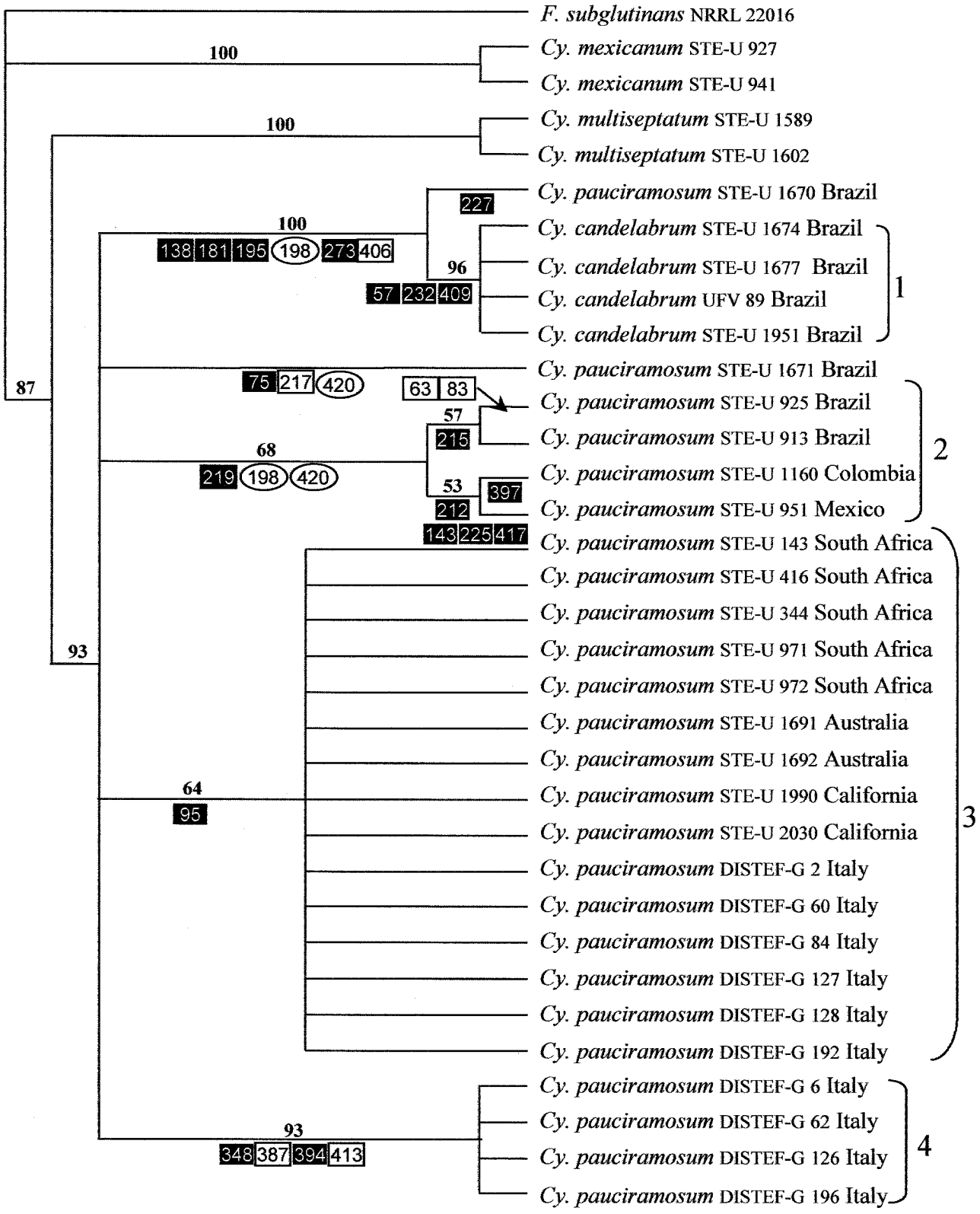

Fig. 1. A strict consensus tree of 10 most parsimonious trees obtained by a heuristic comparison of DNA sequences from the 5′ end of the β-tubulin gene. Clade stability was assessed by 1,000 bootstrap samplings (percentages shown above branches). Individual single nucleotide polymorphisms are shown beneath branches. Numbers denote base pair positions. Transitions are indicated with solid (black) shapes and tranversions with open (white) shapes. Unique derived characters for a specific group or isolate are indicated as squares and shared characters as circles.

total of nine SNPs, of which three (base pairs 57, 232, and 409) were unique for all isolates in this group. The remaining six SNPs were shared by group 2 with an isolate of *C. pauciramosum,* STE-U 1670. This isolate clustered with *C. candelabrum* in Figure 1 but still grouped separate and had one unique SNP (base pair 227). One SNP separated clades 1, 2, and STE-U 1670 (base pair 198).

Variation was found for other individual *C. pauciramosum* isolates from South America and Mexico. Three SNPs supported clade 2. In addition to base pair 198 mentioned above, base pair 420 was shared with STE-U 1671. Although most of the variation occurred among Latin American isolates, no variable characters were shared with any of the isolates from the other geographic regions (South Africa, Australia, and Italy).

Isolates in clade 3 selected from the South African, Italian, and California populations had identical sequences but clustered together with no bootstrap support. This group differs by one unique character at base pair 95. A different group of Italian isolates had four unique base pair substitutions in close proximity to each other (clade 4) and had high (93%) bootstrap support (Fig. 1).

DISCUSSION

The results presented here showed fundamental differences in the profiles of the populations sampled. The nursery populations may have been founder populations and it is likely that some could have gone through recent population bottlenecks. This is reflected in the varying ratios of mating types found in the different nurseries and different geographic areas. The only population that approached a 1:1 mating type ratio was the South African sample, where the disease has been well established. These isolates were collected over a wide area over several years. All the Italian samples had the same mating type bias, suggesting a single source; namely, the nursery in Carruba, which supplies the other local nurseries. The low effective population, based on the number of hermaphrodites in this nursery, could be due to a persistent occurrence of the pathogen in this nursery under selective conditions for asexuality.

The high ratios of hermaphrodites in the samples agree with the recent introductions reported in the literature (22,23). However, the percentage of hermaphrodites found in the various nurseries is consistent with a mother population that is sexually reproductive (3,17). One would expect the percentage of hermaphrodites to drop if a single mating type persists in a nursery. However, the application of good nursery practices entails the immediate removal of diseased material and has the potential to create new bottlenecks due to small starter populations on the remaining plants. The influx of new diseased material containing the opposite mating type could further influence population dynamics.

Plant pathogens are commonly brought into the nursery by means of infected plant material or soil. The most important survival strategy of *Cylindrocladium* spp. is microsclerotia, which can survive for periods of up to 15 years and longer in soil (29,31). Under suitable climatic conditions, germination and subsequent infection of roots and leaves can occur (1,28). The conidia form on infected plant material and are splash dispersed between closely spaced plants (20). In the case of sexual reproduction, the ascospores can also be an additional source of inoculum and are generally wind dispersed (8). The profiles of the mating type distributions seen in this study are consistent with the effects seen for a small starter population, probably by asexual propagules in plants and soil. The fact that only one mating type was found in California, as well as the strong bias towards one mating type in the Italian sample, emphasizes that sexual reproduction may play a small role in these populations.

Genetic variation based on DNA sequence data also was detected between different isolates of *C. pauciramosum*. Although the gene tree based on partial sequences of the β-tubulin gene may not accurately reflect species phylogeny (12,18), recent analyses of different loci have produced concordant phylogenies for *C. pauciramosum* and closely related species (25). Thus, if the assumption that the variation in the β-tubulin gene DNA sequences reflects recent evolutionary history is correct, we can see a correlation of clades with the geographic origin of isolates.

Although isolates have previously been shown to belong to a single biological species (25,26), shared β-tubulin characters were found among isolates of *C. pauciramosum* and *C. candelabrum*, underlining their close phylogenetic relationship.

The high variation among South and Central American isolates of *C. pauciramosum* is consistent with an endemic population, but the small sample size precludes a more thorough assessment. Isolates from California, South Africa, Italy, and Australia differed slightly from the Latin American isolates in β-tubulin sequences. The identical sequence data obtained from the South African isolates suggests that this population was introduced. DNA sequences obtained from isolates collected from Australia, South Africa, Italy, and California were also identical and could indicate a common source of inoculum. There is anecdotal evidence of importation of South African nursery material into Italian nurseries. The occurrence of another DNA sequence from Italy complicates this issue, however. This allows for the possibility of more than one introduction of this species into Italy.

Because of the small sample sizes and use of only one locus, it must be emphasized that this is a first approximation of the variation present in geographic populations of *C. pauciramosum.* A more detailed study of genetic and mating markers will allow more comprehensive conclusions to be drawn. In spite of this, these results emphasize the importance of correct identification of the members of morphologically closely related species in the *C. candelabrum* complex in order to aid phytosanitation and quarantine practices.

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