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Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurospora discreta* complex

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Abstract: Previous observations of morphological, reproductive and genetic variation have suggested that *Neurospora discreta*, as presently circumscribed, might represent a diverse complex of multiple species. To investigate this hypothesis we examined the phylogenetic relationships among 73 fungal strains traditionally identified as *N. discreta*. Strains were chosen from across the morphological, ecological and geographical ranges of the species. Sequence data were obtained from three unlinked nuclear loci, and phylogenetic species recognition was applied to the dataset using protocols that have been shown to be reliable for identifying independent lineages and delineating species of *Neurospora*. The results demonstrate that the present circumscription of *N. discreta* includes at least eight separate phylogenetic species. This research also reveals an abundance of previously unrecognized genetic diversity within the genus, characterizes the interspecific evolutionary relationships and contributes to a fuller understanding of species diversity in *Neurospora*.

Key words: genealogical concordance, phylogenetic species recognition, phylogeny

INTRODUCTION

Global surveys have shown that multiple species of *Neurospora* are abundant in tropical, subtropical and temperate regions around the world (Perkins et al 1976, Perkins and Turner 1988, Turner et al 2001, Jacobson et al 2004, Jacobson et al 2005). *N. discreta* is commonly encountered across the latitudinal and longitudinal range of the genus, making it the most broadly distributed *Neurospora* species. In the forests of western North America, *N. discreta* is the most abundant species, accounting for 95% of the isolates collected (Jacobson et al 2004).

A number of observations suggest that *N. discreta*, as presently circumscribed, might represent a diverse complex of several species. First, strains are distinctly variable in colony morphology when cultured in the laboratory. In addition, conidial pigmentation ranges from yellow to the typical *Neurospora* orange to a paler and more pink color (Perkins and Raju 1986, Jacobson pers obs). When *N. discreta* was first described, Perkins and Raju (1986) reported variation in ascospore morphology among crosses. Most *Neurospora* crosses produce longitudinally ribbed ascospores, but some crosses of *N. discreta* strains produce ribbed ascospores with slight pits or indentations. Second, inconsistency of sexual fertility in pairings among *N. discreta* strains is suggestive of a species complex: Some pairs of strains mate well whereas other pairs mate poorly (Perkins and Raju 1986, Jacobson pers obs). This pattern of reproductive compatibility is rare in other biological species (Turner et al 2001, Dettman et al 2003b) and has been observed, to a much lesser degree, only in *N. intermedia*. Third, abundant genetic diversity was found within *N. discreta*. In a phylogenetic study of outbreeding species of *Neurospora* (Dettman et al 2003a) the genetic distances between some *N. discreta* strains were greater than the genetic distances among other recognized *Neurospora* species.

In this report we assess the phylogenetic relationships among 73 fungal strains traditionally identified as *N. discreta*. Strains were chosen to represent the full morphological, ecological and geographical ranges of the species. Sequence data were obtained from three unlinked nuclear loci and analyzed with parsimony and Bayesian phylogenetic methods. To the dataset we applied previously described protocols for phylogenetic species recognition (PSR) that have been shown to be consistent with biological species recognition in *Neurospora* and, therefore, reliable for identifying independent lineages and delineating species in this genus (Dettman et al 2003a, b). By extending our studies of PSR to *N. discreta* we confirm the prediction that the present circumscription of *N. discreta* includes several separate phylogenetic species. The acknowledgment of this complex of multiple species provides an explanation for the aforementioned observations of morphological, reproductive and genetic variation. In addition this research reveals a wealth of previously unrecognized genetic diversity within the genus, contributes to a fuller understanding of species diversity in *Neuros-*

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pora and characterizes the evolutionary relationships among the species. This information is essential to comparative biology and should enhance the utility of *Neurospora* as an evolutionary model system.

MATERIALS AND METHODS

Individuals and species.—The 73 individuals characterized in this study (TABLE I) were collected, cultured and stored following standard protocols (Perkins et al 1976, Jacobson et al 2004). These strains were assigned to *N. discreta* because they produced more than 50% black ascospores when crossed with *N. discreta* species testers or because they mated more successfully with *N. discreta* testers than with any other species testers. Each strain, whether received from a culture collection or collected from the wild, was purified by single conidium subculture to ensure characterization of individual haploid genotypes. Each resulting culture was given a unique identification number starting with ''D''. The collection of single-conidium strains was redeposited in the FGSC and assigned new FGSC numbers (TABLE I). The *N. discreta* strains used in Dettman et al (2003a) were included in the present study.

This research focuses solely on *Neurospora* species that are outbreeding (heterothallic or pseudohomothallic), all of which produce abundant macroconidia. Multiple phylogenetic studies (Pöggeler 1999; Dettman et al 2001, 2003a; Cai et al 2006) have demonstrated that outbreeding *Neurospora* species collectively form a well supported monophyletic clade. Homothallic *Neurospora* species are not considered here.

Loci.—Sequence data from three unlinked nuclear loci were obtained from the sample of strains. The three microsatellite-containing loci, named DMG, TMI and TML, were characterized in a phylogenetic study by Dettman et al (2003a), which should be consulted for locus information, PCR amplification conditions and sequencing protocols. Previously determined sequences are available from TreeBASE accession M1574, whereas new TMI, DMG and TML sequences have been deposited respectively in GenBank under accession numbers DQ314301-314362, DQ314363-314427 and DQ314428-314490.

Phylogenetic analyses.—Due to the presence of microsatellites and insertion/deletion gaps (indels), DNA sequences were aligned manually. Regions of ambiguous alignment and the microsatellite repeats themselves were excluded from phylogenetic analyses. However unambiguously alignable indels and substitution mutations within the microsatellite repeat arrays were phylogenetically informative, so they were coded as multistate characters and included in phylogenetic analyses.

For rooting purposes and genetic distance calculations, exemplar sequences from the seven other outbreeding *Neurospora* species were included. Species and strain numbers were: *N. crassa* subgroup NcA, D143; Phylogenetic Species 3, D74; *N. intermedia* subgroup NiA, D47; Phylogenetic Species 2, D120; Phylogenetic Species 1, D55; *N. sitophila*, D53, and *N. tetrasperma*, D145 (see Dettman et al 2003a, b for species designations). These sequence data are available from TreeBASE accession M1574. Unless otherwise stated, sequences from *N. crassa* D143 (FGSC 2489) were used as outgroup during phylogenetic analyses. Reported genetic distances are Kimura 2-parameter distances.

To avoid confinement at local optima in maximum parsimony (MP) searches, 10 replicates of random stepwise-addition heuristic searches (tree bisection-reconnection branch swapping; maximum of 5000 trees retained [MAXTREES]) were performed with PAUP (version 4.0b8a, Swofford 2001). When multiple MP trees were produced, the tree chosen for display in a figure was the one determined to be most likely using substitution models suggested by Modeltest (version 3.06, Posada and Crandall 1998). Weighted MP heuristic searches had characters weighted inversely proportional to the total number of phylogenetically informative sites contributed by the locus from which they came (relative weights: DMG = 1.00, TMI = 0.64, TML = 0.30). MP bootstrapping was performed with heuristic searches (100 replicates for individual loci, 300 replicates for combined analysis; simple stepwise addition; nearest neighbor interchange branch swapping [NNI]; MAXTREES = 5 000).

Combinability of single-locus datasets was determined by partition homogeneity tests using informative characters and random stepwise-addition MP heuristic searches with 1000 replicates (NNI; MAXTREES = 500). The null hypothesis of congruence was rejected if $p < 0.001$ (Cunningham 1997). These tests were conservative because all taxa were included, which allows for detection of incongruence both among and within clades.

Bayesian analyses were performed (MrBayes version 3.0b4, Huelsenbeck and Ronquist 2001) as an alternative to the more computationally intensive maximum likelihood analyses. To avoid over parameterization the predetermined likelihood models were used for Bayesian analyses. For example, gamma distributed site-to-site rate variation was implemented only if recommended by Modeltest. Each run consisted of three incrementally heated Markov chains run simultaneously, with default heating values and uniform priors. Markov chains were initiated from a random tree, run for 1 000 000 generations, and sampled every 200th generation. Samples taken before burn-in (<100 000 generations, 500 tree burn-in) were discarded and the remaining samples were used to determine posterior probability distributions. Each run was performed at least twice and consensus trees were compared: All replicate runs converged on congruent trees, suggesting entrapment in local optima did not occur.

Species recognition.—For phylogenetic species recognition, previously described protocols (Dettman et al 2003a) were applied to the *N. discreta* dataset. In brief a clade was recognized as an independent evolutionary lineage if it satisfied either of two criteria: (i) genealogical concordance, in which the clade was present in

TABLE I. Strain numbers, mating types, and geographic sources of *Neurospora discreta sensu lato* strains

Strain number[a]						Collection location[b]	
D	Original FGSC	FGSC	Accession	Phylogenetic species	Mating type	Country	Site
D5		8765	P0047	PS4 subgroup A	a	Papua New Guinea	Tiaba-1
D17	3228	8777	P0851	*N. discreta sensu stricto*	A	U.S.A.	Kirbyville, Texas
D20		8780	P1406	PS7	A	U.S.A.	Homestead, Florida
D37	6789	8797	P2511	PS4 subgroup B	a	India	Bandipur
D54	6792	8814	P3016	PS4 subgroup B	a	Thailand	Kang Koi
D67		8827	P3660	PS6	a	Ivory Coast	Fougbesso
D71		8831	P3728	PS4 subgroup B	A	Ivory Coast	Golikro
D146		8906	W766	PS4 subgroup B	a	U.S.A.	Belen, New Mexico
D148		9940	P0048	PS4 subgroup A	a	Papua New Guinea	Tiaba-1
D149		9941	P0389	PS7	a	U.S.A.	Homestead-1, Florida
D150	3268	9942	P0390	PS7	a	U.S.A.	Homestead-1, Florida
D151	3319	9943	P0755	PS7	a	Guatemala	Santa Maria
D152	3229	9944	P0846	*N. discreta sensu stricto*	a	U.S.A.	Kirbyville, Texas
D153	6784	9945	P1692	PS4 subgroup A	a	Papua New Guinea	Wau-6
D154	6785	9946	P1859	PS4 subgroup A	A	Papua New Guinea	Marinville
D155		9947	P1911	PS4 subgroup A	a	Papua New Guinea	Sogeri Rd-1
D156	6786	9948	P1913	PS4 subgroup A	A	Papua New Guinea	Sogeri Rd-1
D157		9949	P1964	PS4 subgroup A	a	Papua New Guinea	Rouna-8
D158	6787	9950	P1966	PS4 subgroup A	A	Papua New Guinea	Rouna-8
D159	6788	9951	P1992	PS4 subgroup A	A	Papua New Guinea	Hiri
D160		9952	P2315	PS10	A	New Zealand	Brookby
D161		9953	P2324	PS9	a	New Zealand	Brookby
D162		9954	P2349	PS9	a	New Zealand	Waipu
D163	6790	9955	P3002	PS4 subgroup B	a	Thailand	Khao Yai-4
D164		9956	P3003	PS4 subgroup B	a	Thailand	Khao Yai-4
D165	6791	9957	P3004	PS4 subgroup B	a	Thailand	Pakchong-2
D166	6793	9958	P3388	PS10	A	Brazil	Serra Araca
D167		9959	P3602	PS6	A	Ivory Coast	Gahelile
D168		9960	P3603	PS6	a	Ivory Coast	Gahelile
D169		9961	P3619	PS6	a	Ivory Coast	Issia
D170		9962	P3621	PS5	a	Ivory Coast	Issia
D171		9963	P3635	PS4 subgroup B	A	Ivory Coast	Carrefour Couessesso
D172		9964	P3639	PS5	a	Ivory Coast	Carrefour Couessesso
D173	6794	9965	P3642	PS4 subgroup B	A	Ivory Coast	Gouana
D174		9966	P3644	PS4 subgroup B	a	Ivory Coast	Gouana
D175		9967	P3650	PS4 subgroup B	A	Ivory Coast	Fougbesso
D176		9968	P3722	PS4 subgroup B	A	Ivory Coast	Foro-Foro
D177		9969	P3723	PS4 subgroup B	a	Ivory Coast	Foro-Foro
D178		9970	P3796	PS4 subgroup B	A	Congo	Port du Djoue-2
D179		9971	P3836	PS8	A	Congo	Loudima
D180		9972	P3920	PS4 subgroup B	a	Gabon	Ekowong
D181		9973	P3927	PS4 subgroup B	A	Gabon	Ekowong
D182		9974	P3928	PS4 subgroup B	?	Gabon	Makokou-5
D183		9975	P3931	PS8	a	Gabon	Makokou-7
D184		9976	P4159	PS7	A	Mexico	Chichen Itza
D185		8567	W593	PS4 subgroup B	A	U.S.A.	Cobalt, Idaho
D186		8568	W594	PS4 subgroup B	a	U.S.A.	Cobalt, Idaho
D187		8565	W564	PS4 subgroup B	A	U.S.A.	Wells, Nevada
D188		8566	W565	PS4 subgroup B	a	U.S.A.	Wells, Nevada
D189		8572	W620	PS4 subgroup B	a	U.S.A.	Perma-2, Montana
D190		8991	W963	PS4 subgroup B	A	U.S.A.	Weaverville, California
D191		8992	W964	PS4 subgroup B	a	U.S.A.	Weaverville, California

TABLE I. Continued

Strain number[a]						Collection location[b]	
D	Original FGSC	FGSC	Accession	Phylogenetic species	Mating type	Country	Site
D192		8994	W1070	PS4 subgroup B	A	U.S.A.	Chelan Lake, Washington
D193		8995	W1071	PS4 subgroup B	a	U.S.A.	Chelan Lake, Washington
D194		8560	W512	PS4 subgroup B	a	U.S.A.	Kennedy Meadows, California
D195		8561	W514	PS4 subgroup B	A	U.S.A.	Kennedy Meadows, California
D196		9977	W862	PS4 subgroup B	a	U.S.A.	Perma-2, Montana
D197		9978	W853	PS4 subgroup B	a	U.S.A.	Tok, Alaska
D198		9979	W854	PS4 subgroup B	A	U.S.A.	Tok, Alaska
D199		9980	W855	PS4 subgroup B	A	U.S.A.	Tok, Alaska
D200		9981	W814	PS4 subgroup B	a	U.S.A.	Tok, Alaska
D201		9982	W744	PS4 subgroup B	a	U.S.A.	Pecos, New Mexico
D202		9983	W745	PS4 subgroup B	A	U.S.A.	Pecos, New Mexico
D203		9984	W751	PS4 subgroup B	a	U.S.A.	Los Alamos, New Mexico
D204		9985	W752	PS4 subgroup B	A	U.S.A.	Los Alamos, New Mexico
D215		9986	W1232	PS4 subgroup B	a	Portugal	Sangunhedo Boticas
D216		9987	W1233	PS4 subgroup B	A	Portugal	Sangunhedo Boticas
D217		9988	W1235	PS4 subgroup B	a	Portugal	Sangunhedo Boticas
D218		9989	W1254	PS4 subgroup B	A	Portugal	Monchique
D220		9990	W1269	PS4 subgroup B	A	Portugal	Monchique
D221		9991	W1289	PS4 subgroup B	A	Spain	Macanet de la Selva
D224		9992	W1303	PS4 subgroup B	A	Switzerland	Leuk
D225		9993	W1304	PS4 subgroup B	A	Switzerland	Leuk

[a] Cross reference of strain numbers from different collections. Consecutive D numbers were assigned as a convenient label for phylogenetic studies. Strains D5, D17, D20, D37, D54, D67, D71 and D146 were first used in Dettman et al (2003a). The entire collection was deposited in the Fungal Genetics Stock Center (FGSC) and given FGSC numbers. Accession numbers refer to either the Perkins (P) collection (now curated by FGSC) or the Jacobson (W) collection housed at Stanford University.

[b] All strains were sampled from burned plant material, except D37 and D184, which were sampled from soil.

the majority (at least two out of three) of the single-locus genealogies, as revealed by a majority-rule consensus tree; (ii) genealogical nondiscordance, in which the clade was well supported in at least one single-locus genealogy, as judged by both MP bootstrap proportions and Bayesian posterior probabilities, and was not contradicted in any other single-locus genealogy at the same level of support. To identify such clades a tree possessing only branches that received MP bootstrap proportions $\geq$ 70% and Bayesian posterior probabilities $\geq$ 0.95 was chosen to represent each of the three loci, then a semistrict consensus tree was produced from these three trees. This criterion prohibited poorly supported nonmonophyly at one locus from undermining well supported monophyly at another locus. When deciding which independent evolutionary lineages should be ranked as phylogenetic species, exhaustive subdivision was applied; all individuals had to be placed within a phylogenetic species, and no individuals were to be left unclassified.

RESULTS

This study includes sequence data from 217 (97.8%) of the 222 locus-by-individual combinations, 193 of which represent new data. Summaries of the alignments for the single-locus and combined datasets are shown (TABLE II). The amount and form of molecular variation differed considerably among the three loci. Even when accounting for differences in mean sequence length, the TML locus provided the largest number of informative nucleotide characters. The relative levels of variation among loci observed in the present study corresponded well with those reported from several other species of *Neurospora* (Dettman et al 2003a), suggesting these three loci have been evolving in a similar fashion across the genus.

Single-locus datasets.—Maximum parsimony (MP) was used to determine the genealogies from the

TABLE II. Summary of the four DNA sequence alignments

	Alignment			
	DMG	TMI	TML	3 loci combined
Number of taxa	74	71	72	74
Alignment length (bp)	429	446	611	1486
Mean number of included nucleotides per sequence	395.01	445.99	544.61	1352.36
Informative nucleotide characters (ingroup only)	18	39	64	121
Informative coded characters	8	1	24	33

three single-locus alignments (DMG: tree length = 48 steps, consistency index [CI] = 0.938; TMI: tree length = 97 steps, CI = 0.959; TML: tree length = 194 steps, CI = 0.871). The MP bootstrap and Bayesian consensus trees were nearly identical so rather than presenting Bayesian trees, Bayesian posterior probabilities (PP) of branches are indicated on the MP trees (FIG. 1). Several well supported clades appeared in common among the single-locus genealogies.

Combined dataset.—To infer the organismal phylogeny, MP and Bayesian analyses were performed on the combined alignment of characters from all three loci (FIG. 2, MP tree length = 353 steps, CI = 0.870). Combined analysis was justified because partition homogeneity tests failed to detect significant incongruence in any of the three pairwise comparisons of loci. As expected, most clades supported by single-locus analyses, received even greater support in combined analyses. The three loci contributed different numbers of phylogenetically informative characters and therefore had different amounts of influence on the combined-analysis tree search. To account for these differences a weighted MP heuristic search was performed with characters weighted in inverse proportion to the number of informative sites contributed by the locus from which they came. Weighted MP trees (not shown) revealed the same clades and relationships among clades as the original MP tree (FIG. 2), further demonstrating that the phylogenetic signals from the independent loci were complementary.

Species recognition.—Phylogenetic species recognition was applied with the protocol described in Materials and Methods, introduced by Dettman et al (2003a). Bold branches (FIG. 2) indicate the clades that satisfied either of the two grouping criteria (genealogical concordance or nondiscordance) and thus were identified as independent evolutionary lineages. Triangles at nodes (FIG. 2 indicate the lineages that satisfied the ranking criterion (exhaustive subdivision) and thus were recognized as phylogenetic species.

Eight phylogenetic species were recognized within this group of 73 individuals, all of which originally were assigned to a single species. The two strains collected from Texas are the type strains for *N. discreta* (Raju and Perkins 1986) and thus form a phylogenetic species that represents *N. discreta sensu stricto*. The seven newly discovered *Neurospora* species were named Phylogenetic Species 4, 5, 6, 7, 8, 9 and 10 (PS4 to PS10 respectively), extending the naming convention of Dettman et al (2003a).

Phylogeny of N. discreta *species complex.*—The eight phylogenetic species typically were well supported in each of the single-locus trees (FIG. 1), with no major conflicts over the placement of individuals within species. The phylogenetic relationships among species were similar between the TMI and TML loci, with slight differences from the lower resolution DMG locus. The three-locus combined-analysis tree (FIG. 2) represents the best estimate of the phylogeny of the *N. discreta* species complex. Most of the internal branches that united multiple species received significant support, providing a relatively well resolved phylogeny.

The root of the *N. discreta* complex was located in different positions in different phylogenetic analyses. Single-locus trees place the root on the branch between PS9 and PS10, or leading to a member of PS10. The combined-analysis MP tree placed the root on the branch leading to a member of PS10 (D166). When outgroup representation was increased to seven species, the combined-analysis MP tree (and NJ tree) placed the root between PS9 and PS10, consistent with Bayesian analyses. This rooting position received the most support overall and therefore is displayed (FIG. 2).

Genetic diversity.—To display the genetic distances among species, a combined-analysis neighbor-joining tree was constructed from examplar

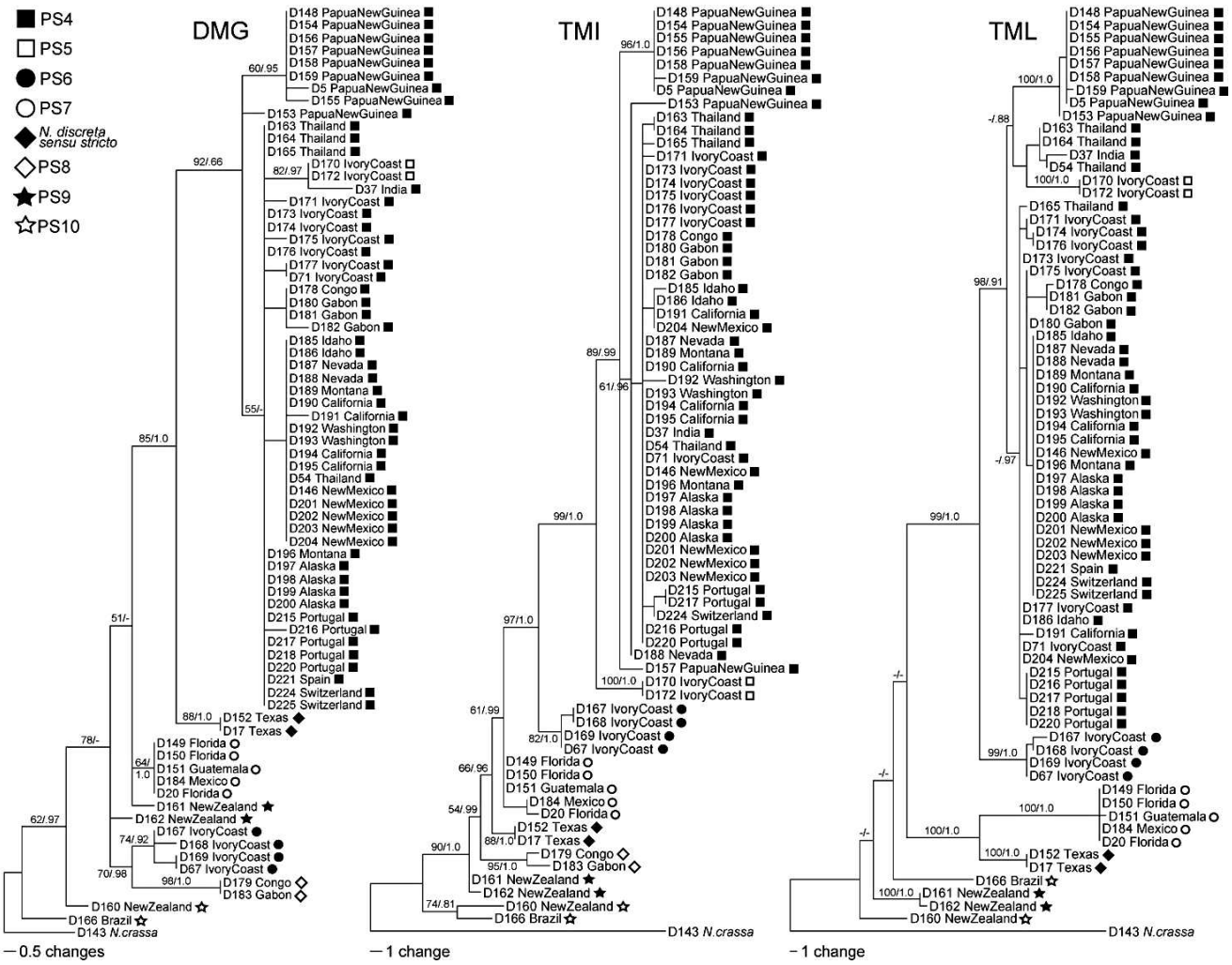

FIG. 1. Maximum parsimony phylograms produced from each of the three single-locus datasets (DMG, TMI and TML). Taxon labels indicate strain number and geographic source, followed by symbols which indicate species assignment based on phylogenetic species recognition, as indicated in the legend. Branch support values (maximum parsimony bootstrap proportions/Bayesian posterior probabilities, MPBP/PP) are displayed for major branches only. A dash indicates the support for branch was <50% MPBP or <0.50 PP.

sequences from the *N. discreta* complex and the seven other outbreeding *Neurospora* species (FIG. 3). The mean genetic distance among phylogenetic species within the *N. discreta* complex was 0.0269 (SE = 0.0014), whereas the same statistic was 0.0238 (SE = 0.0012) for the other clade of species, which includes *N. crassa*, *N. intermedia*, *N. tetrasperma*, *N. sitophila* and three recently described phylogenetic species, PS1-PS3. For maximum genetic distance between two phylogenetic species, the values were 0.0407 for the *N. discreta* complex (PS5 to PS10), and 0.0336 for the other group (PS2 to *N. crassa*). Thus the *N. discreta* species complex contains more species (eight) and more genetic diversity than is found in the other major clade of *Neurospora* species.

DISCUSSION

The assignment of a newly collected outbreeding *Neurospora* individual into a known biological species is a straightforward process. After mating-type determination, the individual is crossed with species-specific tester strains of the opposite mating type. Through many years of experimentation, Perkins and colleagues (Perkins et al 1976, Perkins and Turner 1988, Turner et al 2001) have designated sets of tester strains that represent most of the known biological species. Mating success is assessed by the proportion of progeny that are viable (i.e. black ascospores). If an individual produces more than 50% black ascospores when crossed with a species tester, the individual is considered conspecific with that tester. Rarely an

individual will not produce more than 50% black ascospores with any tester; in this case it is assigned to the species with which it mates best. This method has proven quite effective over many decades and thousands of strains (Turner et al 2001, Dettman et al 2003b, Jacobson et al 2004). Based on these traditional mating tests, at the onset of this project, 73 of the *Neurospora* individuals included in this study were assigned to a single species, *N. discreta*. By applying phylogenetic species recognition using genealogical concordance, we have identified eight phylogenetic species within this group. One of these phylogenetic species is composed of the reference strains from the original description of *N. discreta* (Perkins and Raju 1986) and therefore is *N. discreta sensu stricto*.

Species in the *N. discreta* complex typically were supported by multiple single-locus genealogies and the three genealogies were very similar regarding the relationships among species, with the exception of PS5, PS9, and PS10. The distinction of PS5 from PS4 was significantly supported by one single-locus genealogy (TMI), but the mixing of PS5 within PS4 in DMG and TML genealogies was not significantly supported and therefore cannot override the strong signal from TMI. PS9 and PS10 each were monophyletic in two of three single-locus genealogies, but again support for the nonmonophyletic phylogenies was not significant.

Two subgroups were named within PS4 to highlight their genetic differentiation, which was apparent at all three loci. These two subgroups, A and B, were not recognized as two phylogenetic species because of the incongruent placement of strains D170 (PS5), D172 (PS5), and D153 (PS4 subgroup A). When PSR was performed with these three strains removed, the two subgroups were recognized as separate phylogenetic species. This result demonstrates how two relatively well differentiated clades linked by a few inconsistently placed strains can be conservatively grouped into one species.

We are not formally naming these newly discovered phylogenetic species at this time because most are composed of a limited number of strains and the patterns of sexual reproductive isolation among species, or compatibility within species, have not yet been characterized. Before naming any species, we want to discover more strains belonging to the smaller species and cross members of the *N. discreta* complex in a comprehensive fashion to investigate the mating relationships of these species. The information provided by the anecdotal observations of previous mating among strains or with species testers is not sufficient. We will continue to refer to this entire group as the ''*N. discreta* species complex'', which is composed of *N. discreta sensu stricto* and *Neurospora* Phylogenetic Species 4–10. Taxonomies with arbitrarily named fungal species are common (e.g. O'Donnell et al 2000a, Steenkamp et al 2002, Chaverri et al 2003, Rehner and Buckley 2005) and let researchers communicate effectively before the formal species nomenclature is settled. The point to emphasize is that major phylogenetic divisions are clearly present within the *N. discreta* complex, and that these divisions are as great as or greater than those among named species such as *N. crassa*, *N. intermedia*, *N. sitophila* and *N. tetrasperma*.

The phylogeny of *N. discreta* species complex was generally well resolved, with most internal branches receiving significant support in combined analyses (FIG. 2). The members of species, or terminal clades within species, tended to originate from the same geographic region. For example, PS6 and PS7 were composed respectively of strains from the Ivory Coast and the Caribbean Basin. However the relationship of some species in this complex to geography is not simple, as demonstrated by the range of origins of PS4 strains (Asia, Europe, Africa and North America) and the fact that strains collected from the Ivory Coast could belong to any of three species. When comparing samples collected in North America, a notable phylogeographic division was observed between strains from temperate western North America and those from the tropical Caribbean Basin. In fact western North American strains were consistently more closely related to strains collected from Europe. This same phylogeographic pattern was observed in *N. crassa* (Jacobson et al 2004, 2005), suggesting that the effects of geography on population divergence might be similar across multiple species of *Neurospora*.

The discovery of multiple species within the originally described *N. discreta*, and the understanding of the phylogenetic relationships among species, allows for the reconciliation of several previous observations of reproductive, morphological, and genetic diversity:

(i) Low internal consistency of mating success.—Some pairs of strains identified traditionally as *N. discreta* mate well, whereas other pairs mate poorly (Jacobson, pers obs). This pattern likely represents mating between conspecific or heterospecific strains, respectively, a distinction that could not be made before our phylogenetic analyses. In some cases an individual was placed within *N. discreta* because it mated more successfully with the *N. discreta* tester than with any other species tester, despite never satisfying the 50% black ascospore criterion with any species tester. When Perkins

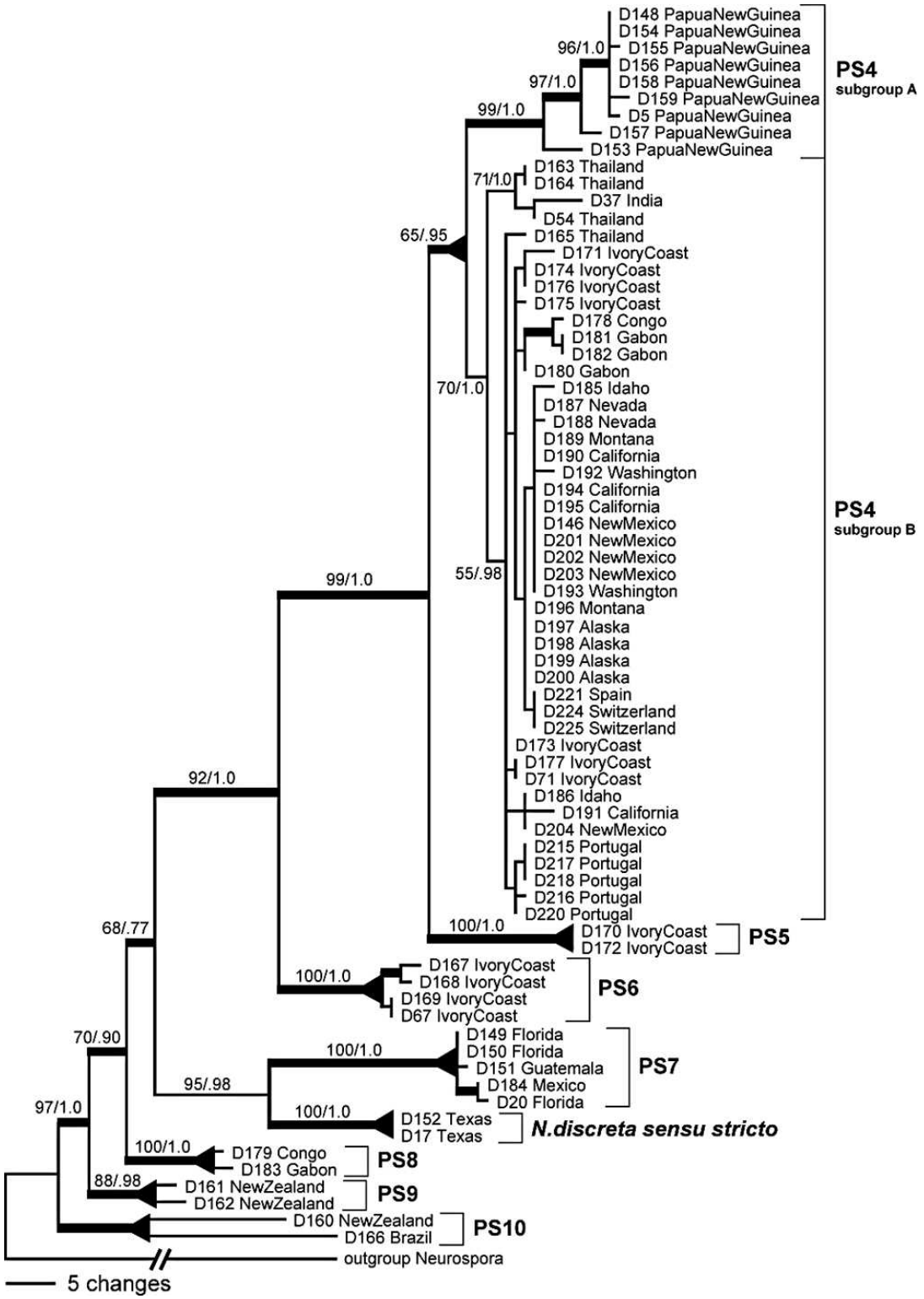

FIG. 2. Maximum parsimony phylogram produced from the DMG, TMI and TML loci combined. Taxon labels indicate strain number and geographic source. Labels to the right of phylogram indicate groups identified by phylogenetic analyses. Bold branches were supported concordantly by the majority of the loci or well supported by at least one locus but not contradicted by any other locus. Triangles at nodes indicate that all taxa united by (or distal to) it belong to same phylogenetic species. Branch support values (MPBP/PP) are displayed for major branches only. A dash indicates the support for the branch was <50% MPBP or <0.50 PP.

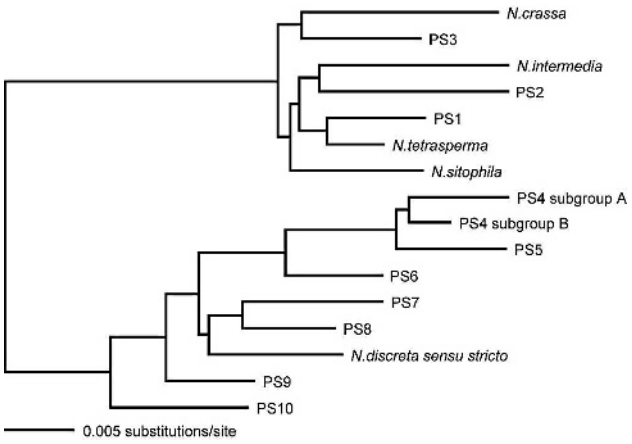

FIG. 3. Neighbor joining phylogram produced from the DMG, TMI and TML loci combined, including exemplars from each phylogenetic species of *Neurospora*.

and Raju (1986) described the species, they reported that strains from Florida and Guatemala mated well with the species testers and could be confidently assigned to *N. discreta*. These three strains (D149, D150 and D151 in this study) belong to PS7, which is closely related to the species testers themselves. In contrast strains from Papua New Guinea (D5 and D148) and New Zealand were not fully fertile with the testers and their assignment to *N. discreta* was provisional. Strains from Papua New Guinea and New Zealand are phylogenetically placed with PS4 subgroup A, PS9 or PS10, which are more distantly related to the species testers than PS7.

These anecdotal observations on a limited number of matings suggest that strains most closely related to the *N. discreta* testers are likely to be most sexually compatible with them. Therefore it is likely that many strains originally were described as *N. discreta* simply because they were more closely related to *N. discreta sensu stricto* than to any other species known to exist at the time of identification. This pattern is consistent with the positive correlation between phylogenetic divergence and reproductive isolation clearly demonstrated in other *Neurospora* species (Dettman et al 2003b). We did not cross strains of the *N. discreta* complex in a systematic manner, so a rigorous comparison of phylogenetic divergence and reproductive isolation could not be made. However the occurrence of different phylogenetic species in sympatry (e.g. PS4–PS6 from the Ivory Coast) indicates that barriers to mating in nature are prevalent enough to maintain the distinctness of species.

(ii) Morphological diversity.—Clear morphological differences have been observed among strains assigned to *N. discreta*. Perkins and Raju (1986) noted that the species testers differed markedly from other strains in macroconidial pigmentation, protoperithecial production and ascospore ornamentation and size. Because Perkins and Raju actually were working with at least four phylogenetic species when *N. discreta* was described, this variation might represent variation among phylogenetic species within the *N. discreta* complex. We compared macroconidial pigmentation and colony morphology from a subsample of 44 strains, including representatives from all eight phylogenetic species. Differences in pigmentation and morphology were evident, however they were not consistently species-specific and in some cases variation could be just as great within species as between species (data not shown). In addition, although the extremes of conidial morphology were apparent, the slight gradations along the spectrum of macroconidial morphology were not easily quantified in a reliable manner. Other recent studies in filamentous Ascomycetes also have demonstrated that morphological characters may possess limited taxonomic utility (e.g. O'Donnell et al 2004, Rehner and Buckley 2005) or no phylogenetic information (e.g. Steenkamp et al 2002, Chaverri et al 2003). We do not know if protoperithecial production, ascospore ornamentation or ascospore size track the phylogeny of the *N. discreta* complex better than macroconidial pigmentation because these characters were not investigated.

Regarding ecological diversity, the *N. discreta* strains from western North America and southern Europe were collected exclusively from burnt woody shrubs and trees. In contrast, other strains from tropical or subtropical regions were sampled from a variety of substrates, including burned grasses, herbaceous plants, shrubs, twigs and soil (Turner et al 2001). The western North American and European strains formed three minor clades within PS4 subgroup B, but they were not significantly differentiated from other strains within the subgroup. Thus no clear evidence for clade-specific ecological specialization or habitat preference was found.

(iii) Genetic diversity.—A previous phylogenetic study of *Neurospora* (Dettman et al 2003a) showed that *N. discreta*, despite a small sample size, contained well supported internal subdivisions and high genetic diversity relative to other species. No known characteristics of *N. discreta* could explain such diversity. With sample size increased to 73 strains, the phylogenetic analyses presented here confirm the high diversity and demonstrate

that it is a result of variation among multiple rather than a single species. Although the *N. discreta* species complex contains more genetic diversity than the other seven outbreeding species combined (FIG. 3), genetic variation within species is similar across the genus (Dettman et al 2003a).

This study demonstrates the power of phylogenetic species recognition to reveal cryptic diversity that may be missed by traditional methods of biological species recognition. Similar conclusions have been drawn in a number of fungal groups (e.g. Vilgalys and Sun 1994, Hibbett et al 1995, Aanen et al 2000, O'Donnell et al 2000b, Taylor et al 2000, Harrington et al 2002, Dettman et al 2003b). An even larger number of studies have shown that multiple, well differentiated phylogenetic species may display little or no distinguishing morphological variation among them (e.g. O'Donnell et al 2004, Rehner and Buckley 2005). Typically, morphological character states are shared among species or have overlapping ranges, rendering them useless for species diagnosis (e.g. Steenkamp et al 2002, Chaverri et al 2003). Because morphological and biological species recognition are not applicable to all organisms, and phylogenetic species recognition appears to be the most effective method for revealing species diversity, many authors are choosing to include information on fixed nucleotide differences in formal species descriptions (Fisher et al 2002, O'Donnell et al 2004).

Since the species' description in 1986 (Perkins and Raju 1986) only a single paper focusing mainly on *N. discreta* has been published (Jacobson et al 2004). That study expanded our knowledge of the incidence, distribution and ecology of *N. discreta*. The present study expands our knowledge of the genetic and species diversity within the *N. discreta* complex and how it relates to the other *Neurospora* species. Over the past two years, *N. discreta* has developed from a rarely collected species believed to be confined to moist tropical and subtropical regions into a common, globally distributed, highly diverse species complex that represents the majority of known diversity in this well studied fungal genus.

Just as more strains must be collected and studied before species can be named, more will be needed before questions can be addressed regarding the biogeography of *N. discreta* and PS4–PS10. For example, did the entire species complex originate in the southern hemisphere, and are central Africa and the Caribbean present-day centers of diversity? The one clade with the most individuals, PS4 subgroup B, is also the one with the greatest latitudinal distribution of any *Neurospora* species, from central Africa (Ivory Coast, Gabon, Congo), through Europe and western North America, as far north as Alaska. Studies of potential adaptation of these strains to different environmental parameters might be as productive as those of *Drosophila* (Oakeshott et al 1982, Loeschcke et al 2000, Ayrinhac et al 2004, Sezgin et al 2004) or other animals (Bradshaw et al 2000, Palo et al 2003, Lindgren and Laurila 2005, Mathias et al 2005), which have been studied along smaller latitudinal gradients.

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