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Three new phylogenetic and biological *Neurospora* species: *N. hispaniola*, *N. metzenbergii* and *N. perkinsii*

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Abstract: The recent recognition of provisional *Neurospora* phylogenetic species (PS) 1–3 gave us the opportunity to compare genetic isolation, which underlies phylogenetic species recognition (PSR), with reproductive isolation as criteria for recognizing new species. This investigation involved first finding new individuals of PS 1–3 from a search of the Perkins culture collection, then assessing genetic isolation by PSR for old and new members of PS 1–3 and finally comparing species recognition by genetic isolation as determined by PSR to species recognition by reproductive isolation as determined by biological species recognition (BSR) and geographic distribution. To aid the search for additional members of the PS we used the genetic variation originally used to discover *Neurospora* PS 1–3 to easily distinguish members of *Neurospora* PS 1–3 from the closely related species *N. crassa* and *N. intermedia*. To increase our chance of success the analysis was performed on *N. crassa* and *N. intermedia* isolates that were either not clearly assignable to species by BSR using tester strains or were from the same geographic locations as the known members of PS1–3. Eleven new members of *Neurospora* PS 1–3 were identified: one new PS1, nine new PS2 and one new PS3. To complement PSR we investigated reproductive isolation with BSR in PS1–3 and the two other most closely related species, *N. intermedia* and *N. crassa*, with intraspecific and interspecific crosses. PS1 and PS2 appear reproductively isolated because they successfully mated intraspecifically and not interspecifically. PS3 isolates successfully crossed with other PS3 isolates, however they also successfully crossed with *N. crassa*, as previously reported, indicating that genetic isolation can precede reproductive isolation. We compared phylogenetic, mating and geographical data to challenge the use of PSR as the main criterion in the formal description of species and, having failed to discredit the approach, describe the new species, *N. hispaniola* (PS1), *N. metzenbergii* (PS2) and *N. perkinsii* (PS3).

Key words: ascomycetes, biogeography, biological species recognition, fungi, phylogenetic species recognition

INTRODUCTION

Recently phylogenetic species recognition (PSR) of outbreeding *Neurospora* individuals has found at least 15 genetically isolated, species-level clades, where previous biological species recognition (BSR) using mating to tester strains had delimited just five reproductively isolated species (Dettman et al 2003a, 2006; Turner et al 2001). These 15 phylogenetic species (PS) are found in two sister clades. The first comprises four of the five described species, *N. crassa*, *N. sitophila* (Shear and Dodge 1927), *N. intermedia* and *N. tetrasperma* (Tai 1935) and three new species of *Neurospora* tentatively labeled PS 1, 2 and 3 (Dettman et al 2003a). The second clade comprises the fifth described species, *N. discreta* (Perkins and Raju 1986), and seven new species of *Neurospora* tentatively labeled PS 4–10 (Dettman et al 2006). The goal of our study was to determine whether species found with PSR could meet specific confidence criteria for use in the formal description of new *Neurospora* species. The criteria that had to be met were: (i) that the new species were distinct PS according to the PSR criteria set forth in Dettman (2003a); (ii) that the new species could mate successfully with other members of their species as determined through the mating tests required for biological species recognition (BSR); and (iii) that barriers to interspecific mating were either intrinsic as determined by BSR (Mayr 1996) or extrinsic as inferred from the presence of geographically distinct ranges that define allopatry. As a prelude to our testing of PSR we searched existing culture collections of *Neurospora* for new PS1, PS2 and PS3 specimens, using previously determined genetic variation. With the testing of PSR completed we formally described and named the three phylogenetic species.

When *N. crassa*, *N. sitophila*, *N. intermedia* and *N. tetrasperma* were described in 1927 and 1935, intra- and interspecific crosses showed clear differences and this information influenced the authors of the descriptions (Shear and Dodge 1927, Tai 1935) well before the publication of the Biological Species

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Concept in 1942 (Mayr 1942). Although morphology was the basis of the descriptions of all four species, since that time reproductive isolation measured by mating success has been shown to be a more reliable method for the identification of heterothallic *Neurospora* (Perkins and Raju 1986, Perkins 1988, Shear and Dodge 1927, Tai 1935). Morphology however continues to be useful for identifying *N. tetrasperma* because this pseudohomothallic species produces perithecia with asci containing four dikaryotic and binucleate spores, as opposed to the eight-spored asci found in all other *Neurospora* species (Turner et al 2001). Almost all strains in the extensive holdings of *Neurospora* collected from nature were assigned to species by matings to tester strains (Jacobson et al 2006, Perkins 1988, Turner et al 2001). This approach revealed the most recently described species of *Neurospora*, *N. discreta*, which was recognized and described based solely on crossing behavior that showed reproductive isolation from the other known species of *Neurospora* (Perkins and Raju 1986). Other accessions that failed to mate well with any tester strains were suspected of having a hybrid origin (Turner et al 2001).

Most recently species recognition by genetic isolation (PSR) was applied to outbreeding *Neurospora* species and compared to species recognition by reproductive isolation (BSR) using many crosses among individuals in a more thorough manner than could be achieved by crosses only to testers strains (Dettman et al 2003b). This study found that PSR was in general agreement with a broader BSR application and that both approaches recognized more species than were recognized by crosses to mating tester strains alone (Dettman et al 2003a, b). PSR found three new phylogenetic species (PS) that had been missed by crosses with tester strains. Moreover each putative hybrid was shown to belong to a single PS (i.e. by all single locus phylogenies) and there was no evidence that any were hybrids (Turner et al 2001). These new cryptic species would never have been found without PSR because of a lack of tester strains specific to the new *Neurospora* PS and because they do not have any distinguishing morphological features (Dettman et al 2003a, b).

The findings that *Neurospora* PS1 and PS2 contained a disproportionate fraction of what had been thought to be hybrid individuals and that all new species were narrowly endemic provided a strategy for discovering additional members of these new species within existing natural *Neurospora* collections (Turner et al 2001). We flagged for further study nearly 200 natural isolates that either were difficult to assign to a species due to equivocal crosses to mating testers or that were collected in geographic areas occupied by *Neurospora* PS1–3 or both. To evaluate this large number of candidates we developed a PCR and restriction enzyme digest screen to rapidly exclude genuine members of *N. crassa* or *N. intermedia*. Isolates passing the screen then were sequenced for the informative loci originally used to recognize the new species (Dettman et al 2003a) and subjected to phylogenetic analysis for assignment to species, first by the criterion of genetic isolation. To further understand the relationship of this first criterion of genetic isolation to the second criterion of intraspecific mating compatibility and intrinsic aspects of the third criterion of reproductive isolation, isolates found to belong to *Neurospora* PS 1–3 were mated among themselves and to individuals of *N. crassa* and *N. intermedia*. Where intrinsic barriers of the third criterion of interspecific reproductive isolation were not observed, biogeography was examined for potential extrinsic barriers to reproductive isolation, as inferred from allopatry. Emerging from the comparison of genetic and reproductive isolation was the finding that PSR and BSR recognize nearly the same groups and that PSR alone could be used as the principal criterion for the formal description of new fungal species.

MATERIALS AND METHODS

Neurospora *isolates dataset.*—We examined 188 wild *Neurospora* isolates from the Perkins collection (APPENDIX I). The isolates in our research were chosen for one of two reasons, ambiguity in mating tests and geographic location. The first 102 isolates were included because they did not mate well with tester strains, which commonly are used to identify outbreeding species in genus *Neurospora* (Perkins 1988). The next 86 samples were chosen based on their collection at localities where PS1 (Caribbean Basin), PS2 (Caribbean Basin, Madagascar) and PS3 (Sub-Saharan Africa) had been found.

Conidium isolation.—All cultures used in the study are homokaryons subcultured from a single conidium isolated from cultures belonging to the David Perkins culture collection (Turner et al 2001). Single conidia were isolated by streaking conidia from a mass culture onto 10-fold diluted Vogel's medium agar plates (Vogel 1956). After incubation 18–24 h at ambient temperatures single, germinated conidia were transferred to establish cultures (Jacobson 1995). New isolates were deposited in the Fungal Genetics Stock Center (FGSC) (APPENDIX I).

DNA extraction, PCR and restriction enzyme digest.—DNA was extracted from all strains with the same methods described in Dettman et al (2003a). Polymerase chain reaction (PCR) was performed on genomic DNA extraction samples with TMI loci primers (Dettman et al 2003a). The TMI locus is one of four phylogenetically informative loci (DMG, QMA, TML, TMI) found on different linkage

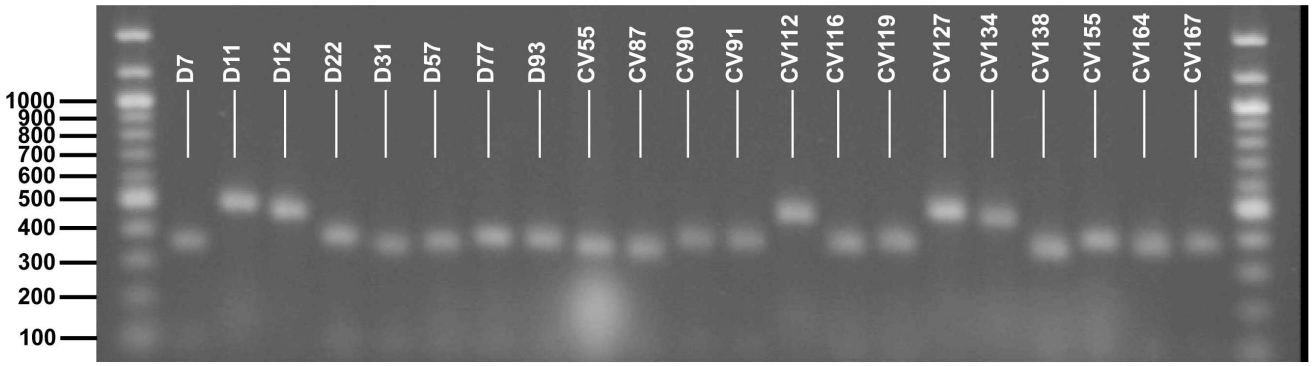

FIG. 1. NciI restriction digestions of the PCR amplified *Neurospora* TMI locus electrophoresed in agarose and stained with ethidum bromide. Controls: *N. crassa* (undigested) D11, D12, *N. intermedia* (digested) D7, D22, D31, PS1 (digested) D57, PS2 (digested) D93 and PS3 (digested) D77. Unknowns: Undigested and putative *N. crassa* CV112, CV127, CV134; digested and putative *N. intermedia* or putative new PS CV55, CV87, CV90, CV91, CV116, CV119, CV138, CV155, CV164, CV167. The 466 bp TMI locus is digested by Nci1 into a 344 bp fragment and a 102 bp fragment, which is too small be seen on this gel. TMI PCR lengths might be slightly larger than 446 bp (D22) because of microsatellites (Dettman et al 2003a). A 100 bp ladder (NEB) was used to measure PCR product lengths. The 102 bp segment is not clearly visible and is not needed for analysis.

groups in *Neurospora* sp. discovered, tested and described in Dettman et al (2003a). Reactions included 200 μM dNTP, 0.4 μM reverse and forward primers, 1× PCR buffer and 1.0 unit DNA polymerase in 50 μL reactions. An Eppendorf Mastercycler gradient thermocycler was used for DNA amplification with 94 C for 2 min during primary denaturation of DNA, 40 cycles 94 C (denaturation), primer specific annealing temperature 1 min (Dettman et al 2003a), 72 C for 1 min (extension) and 72 C for 7 min during final extension.

After PCR amplification of the TMI locus the samples were digested with 0.5 μL (10 units enzyme) NciI (New England Biolabs), 2.0 μL NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9 at 25 C), 7.5 μL filter-sterilized dH_2O and 10 μL TMI PCR product (20 μL total reaction). The reactions were left to digest overnight at 37 C, and 5 μL of each reaction were electrophoresed on a 1.5% agarose gel at 150 mA for 3 h (FIG. 1). Undigested bands (446 bp long) identified isolates as *N. crassa*.

PCR products digested at TMI position 102 (344 bp long) were selected for processing with a second restriction digest enzyme, BciVI (New England Biolabs), to identify *N. intermedia* isolates. BciVI is more sensitive to contaminating molecules so PCR products to be digested with this enzyme were cleaned of primers, single-strand PCR product and dNTP in one-quarter ExoSAP-IT (UBS) reactions as recommended by the manufacturer. Cleaned PCR products were digested 4 h at 37 C in a thermocycler with 0.2 μL BciVI (2 units enzyme), 2 μL NEBuffer 4, 7.8 μL filter sterilized dH_2O and 10 μL cleaned PCR product. BciVI was inactivated 20 min at 65 C. TMI PCR product left undigested by BciVI was identified as *N. intermedia*, and TMI PCR products that digested (240 bp) were identified as possibly coming from *Neurospora* PS1–3 (FIG. 2). Positive controls for the screen were well characterized individuals of *N. crassa* (D11, D12), *N. intermedia* (D7, D31), PS1 (D57), PS2 (D93) and PS3 (D77) (Dettman et al 2003a) (FIGS. 1, 2).

Sequencing of informative loci.—To assign screened candidates to phylogenetic species PCR amplifications of the TMI locus and three additional loci, DMG, QMA and TML, were sequenced in both directions using published protocols for amplification and BigDye Terminator 3.1 cycle sequencing (ABI) (Dettman et al 2003a). Scanning of the sequences was performed with an ABI Prism 3100 Genetic Analyzer. Sequence data were analyzed with Sequencher 4.2.2 (Gene Codes Corp.) and consensus sequences for all four loci for each candidate isolate were assembled. New sequences were deposited at GenBank and assigned accession numbers FJ535356–FJ535439.

Sequence alignment.—Sequences of each of the four loci generated here were aligned with Clustal W [13] with those used by Dettman et al (2003a). For each locus the aligned, combined data file was inspected visually to optimize the alignment and remove unalignable microsatellite positions (Dettman et al 2003a). The alignment was deposited at TreeBASE (www.treebase.org).

Tree building and species identification.—Sequence data from the DMG, QMA, TMI and TML loci were aligned in one consensus file. Sequences of D104 and D138 from Dettman et al (2003a) were excluded because they were missing QMA sequences. With MrModeltest 3.1.7 (Nylander 2004) and PAUP 4.0 (Swofford, 2003) the appropriate nucleotide substitution model was chosen for the consensus sequence as a whole, which was input into MrBayes (Huelsenbeck et al 2001, Ronquist and Huelsenbeck 2003) (Bayesian inference) and Garli (Zwickl 2006) (maximum likelihood) for phylogenetic analysis. The consensus alignment and the chosen nucleotide substitution model were input into MrBayes for 1 million generations with a burn-in of 250 000 generations to produce a consensus tree with Bayesian posterior probabilities. The same alignment and nucleotide substitution model was input into Garli and run 1 million generations to determine the most likely tree. One hundred 10 000-

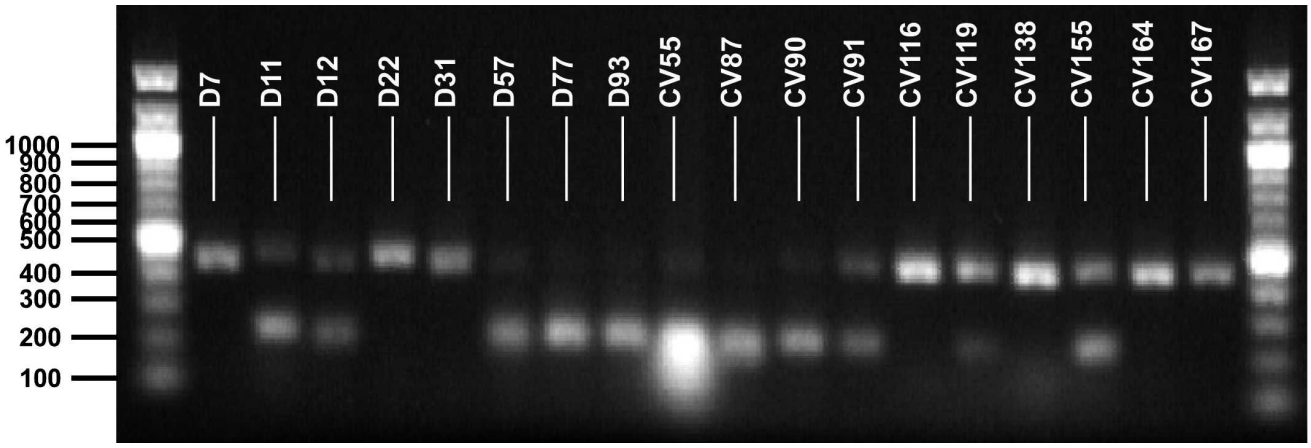

FIG. 2. BciVI restriction digestions of the PCR amplified *Neurospora* TMI locus electrophoresed in agarose and stained with ethidium bromide. Controls: *N. intermedia* D7, D22, D31; digested TMI indicating *N. crassa* (D11, D12), PS1 (D57), PS2 (D93) and PS3 (D77). Unknowns: Undigested and putative *N. intermedia*, CV116, CV138, CV164, CV167; digested and putative new PS, CV55, CV87, CV90, CV91, CV119, CV155. The 446 bp TMI locus was digested by BciVI into 240 bp and 206 bp fragments. Enzyme did not always completely digest TMI PCR product, but any PCR sampled that looked digested was queued for further analysis. CV87 eventually was identified as *N. sitophila* (RESULTS and DISCUSSION). A 100 bp ladder (NEB) was used to measure PCR product length.

generation bootstrap replicates were run to obtain 100 trees that were input into PAUP to determine bootstrap support numbers for the different clades. Species assignment of the putative PS isolates was determined by the placement of the unknown isolates in relation to other known isolates from Dettman et al (2003a).

Design of crossing matrix and mating of isolates.—Matings and evaluation of reproductive success were done following protocols of Dettman et al (2003b). The *Neurospora* strains used in the crosses were grown on synthetic crossing medium in the dark for 7 d at 24 C (Westergaard and Mitchell 1947). Reciprocal crosses were performed by collecting conidia from two cultures of opposite mating type and inoculating each culture with conidia from the mating partner. After 14 additional d at 24 C the slants were inspected visually and graded for reproductive success (FIG. 3).

RESULTS

RFLP screen for phylogenetic species.—To design our RFLP screen we used NEBcutter 2.0 (Vincze et al 2003) to search among the four loci used for PSR to find restriction endonuclease recognition sites that were invariant within *N. crassa*, *N. intermedia* and the combined PS 1–3 but variable among these three groups. We confirmed the predictions that NciI would digest TMI sequences not belonging to *N. crassa* at nucleotide position 102 (target sequence: 5′-CCCGG-3′) and that BciVI would digest TMI sequences not belonging to *N. intermedia* at nucleotide position 240 (target sequence: 5′-GTATCC-3′) using positive controls for *N. crassa*, *N. intermedia* and PS 1–3 (FIGS. 1, 2). In the screen NciI failed to digest PCR-amplified TMI from 41 of the 188 PCR samples, which were considered to be *N. crassa* and were excluded from further analysis. BciVI failed to digest PCR-amplified TMI from 124 of the remaining 147, which were considered to belong to *N. intermedia* and also were excluded from further analysis. The remaining 22 samples were candidates for membership in *Neurospora* PS1–3.

Phylogenetic species recognition.—Species assignment of the 22 individuals possessing both NciI and BciIV restriction sites and possibly belonging to *Neurospora* PS 1–3 required PCR amplification and sequencing of the loci used previously for PSR (DMG, QMA, TMI and TML; Dettman et al 2003a). The sequences were aligned with the corresponding sequences of the 145 individuals used by Dettman et al (2003a) to recognize phylogenetic species. The alignments of the four loci were compiled into one consensus alignment, which was used to build a maximum likelihood (ML) tree with Garli (Zwickl 2006) and a Bayesian inference (BI) tree with MrBayes (Huelsenbeck et al 2001, Ronquist and Huelsenbeck 2003). The phylogeny found by both methods is provided (FIG. 4). Both the ML tree and the BI tree were in agreement with Dettman et al (2003a) in that each species-level clade had well supported Bayesian posterior probabilities and maximum likelihood bootstrap proportions (FIG. 4).

Of the 22 strains sequenced 10 were identified as PS2 and one isolate each was identified as a member

PS1-3 Matings (BSR)

	matA-->	Nh CV55	Nh D58	Nm CV152	Nm CV155	Nm CV156	Nm D120	Np D78	Nc D107	Nc D62	Ni D122	Ni D36
	mata											
Nh	D55	**6/6**	**6/6***	1/1	6/1	1/5	0/4*	-*	0/3	-*	0/0*	-
Nm	CV89	6/0	6/1	**6/6**	**6/5**	**6/5**	**6/6**	5/5	5/6	4/5	4/1	1/5
Nm	CV119	1/1	1/2	**2/5**	**5/4**	**4/5**	**5/6**	6/6	6/5	6/6	1/1	6/5
Nm	CV148	0/5	5/1	**6/6**	**6/6**	**6/6**	**5/6**	5/5	4/4	4/4	1/5	5/6
Nm	D92	1/5	3/0*	**5/5**	**6/5**	**5/4**	**5/5***	3/3*	4/4*	3/3*	4/1	-*
Np	CV79	1/1	1/1	6/6	6/1	2/5	5/6	**6/6**	6/6	6/6	1/1	6/6*
Np	D77	1/4	3/0*	5/5	5/4	5/4	3/3*	**6/6***	5/5*	-*	3/0*	-*
Nc	D100	4/1	3/0*	5/4	5/4	2/6	3/3*	5/5*	**6/6***	**4/4***	3/2*	-*
Nc	D116	1/4	3/0*	6/6	6/5	4/1	3/3*	6/6*	**6/5***	**6/6***	0/0*	3/3*
Ni	D127	0/1	1/0*	2/2	2/1	1/2	3/3*	3/3*	3/1*	1/2*	**5/6***	**6/6***
Ni	D2	0/3	-*	-	2/1	1/1	-*	-*	3/3*	-*	**6/6***	**6/0***

6	>50% black ascospores
5	15-50% black ascospores
3&4	<1% black ascospores; & 1-15% black ascospores
2	perithecia developed ostioles, no ascospores ejected
0&1	sterile, no perithecia produced; & barren perithecia, no ostiole developed

FIG. 3. BSR matrix of crosses between the new PS and previously identified *Neurospora* specimens. The grading criteria are the same as those used in Dettman et al (2003b). The boxes labeled in boldface signify intraspecific crosses. The majority of intraspecific crosses were successful displaying cohesion between BSR and PSR. Crosses with an asterisk are those taken from Dettman et al (2003b) to compare those crosses with our data. Reciprocal crosses were performed in every cell, and the data is ordered as follows: *mat a* (parent)/*mat A* (parent). Nh: *N. Hispaniola*; Nm: *N. metzenbergii*; Np: *N. perkinsii*; Nc: *N. crassa*; Ni: *N. intermedia*.

of PS1 or PS3 (FIG. 4). The 10 remaining individuals proved to be *N. sitophila*. Finding the false positive *N. sitophila* prompted us to seek false negative results among the strains excluded from further analyses by our screen. We sequenced the TMI loci of several isolates excluded by the restriction digestions as *N. crassa* (CV11, CV120, CV147) or as *N. intermedia* (CV54, CV72, CV76, CV83, CV99, CV111, CV170). None of the excluded individuals proved to be PS1, PS2 or PS3; however one was identified as belonging to *N. discreta sensu lato* (CV11).

Phylogenetic species BSR matrix.—To determine whether the new *Neurospora* PS1–3 specimens shared the same mating patterns as those reported by Dettman et al (2003b) we performed crosses among six of the new PS2 individuals, and PS1 and PS3, and two PS1, two PS2, two PS3, four *N. crassa* and four *N. intermedia* individuals used by Dettman et al (2003b). Matings were evaluated exactly as reported by Dettman et al (2003b), and we provided (FIG. 3) results for 130 new crosses and 68 crosses taken from Dettman et al (2003b). Intraspecific matings within PS1, PS2 and PS3 were successful and in most cases were scored as one of the two highest categories of reproductive success, 5 or 6. As in the previous study PS3 isolates were found to mate well with each other and also with individuals from *N. crassa* subclade A (Dettman et al 2003b).

TAXONOMY

Having conducted a more extensive search of culture collections and having discovered additional members of PS1, PS2 and PS3, we are confident in naming the three phylogenetic species. The original and newly discovered members of PS1, PS2 and PS3 all meet the criteria of genetic and reproductive isolation (intrinsic or extrinsic) necessary for a formal description. The additional specimens did not alter the *Neurospora* phylogeny of the original members, and their mating success with different *Neurospora* species was similar to that of the original members (Dettman et al 2003b).

Neurospora hispaniola Villalta, Jacobson et Taylor, sp. nov.

Fungus generatione sexuale inter individua, heterothallicus haploideus prius PS1 designatus, a speciebus Neurosporae alteris heterothallicis generatione sexuale inter individua distinguendus non per formam, sed per notionem speciei phylogenetici et quattuor locos geneticos DMG, DMA, TMI, et TML. Notae characteristicae nucleotidorum fixae in individuis notis speciei huius: positiones loci TMI 71 (T), 109 (G), 48 (T); loci TML 129 (T), 130 (G), 131 (G), 195 (T), 514 (G); loci QMA 70 (G), 290 (A), 415 (G).

Neurospora hispanola is an outbreeding heterothallic haploid fungus previously designated PS1. *N. hispaniola* is morphologically indistinguishable from the other heterothallic outbreeding species of *Neu-*

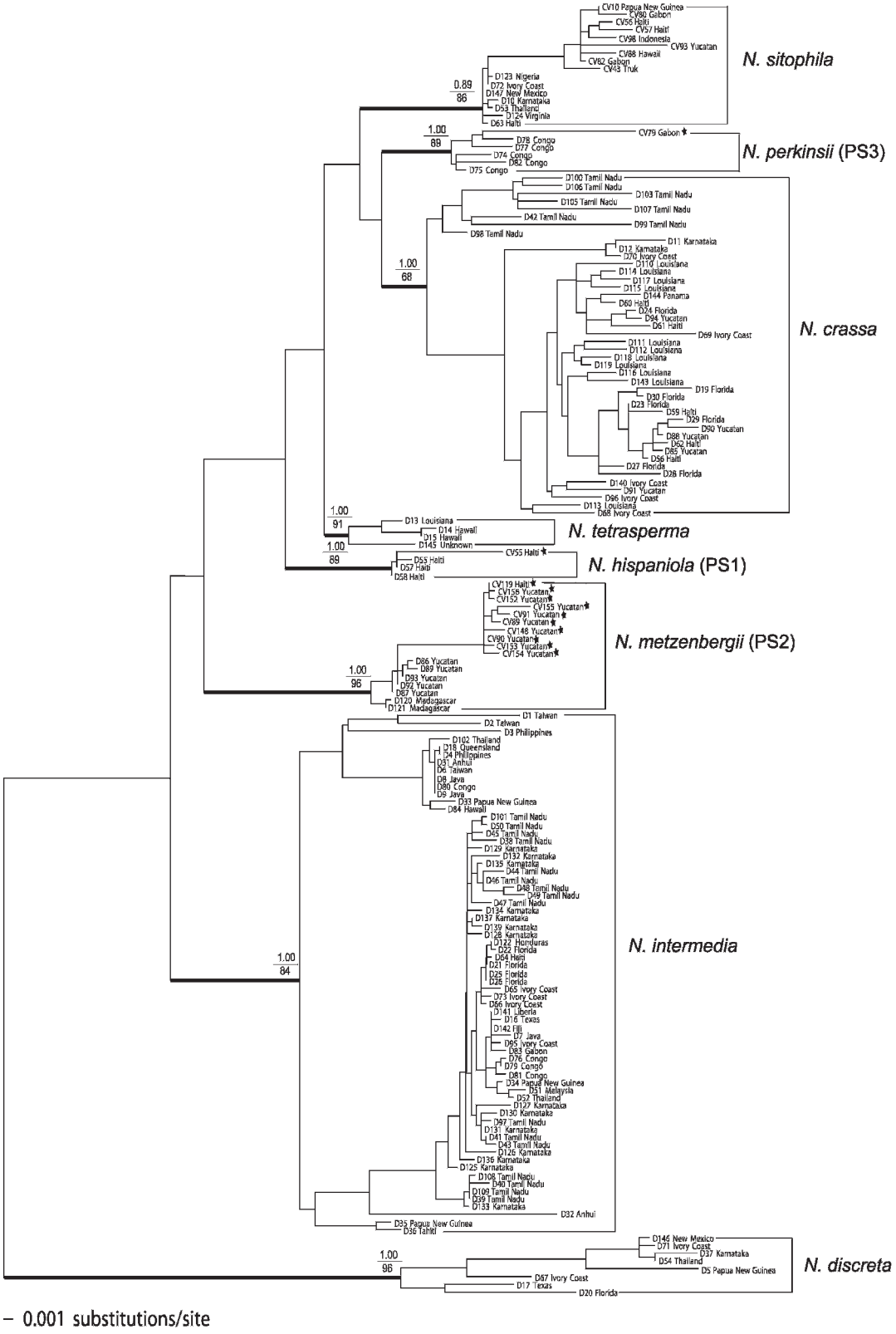

FIG. 4. These are the phylogenetic relationships among the outbreeding species of *Neurospora*, including the 12 newly characterized isolates of *N. hispaniola*, *N. metzenbergii* and *N. perkinsii* (denoted by black star) and the 145 specimens previously characterized by Dettman et al (2003a). The tree was constructed with MrBayes and the major branches defining each phylogenetic species (PS) are marked in boldface. The numbers above each major branch indicate confidence levels; Bayesian posterior probability/maximum likelihood bootstrap proportions.

rospora, but individuals can be assigned to *N. hispaniola* with the phylogenetic species recognition concept and these four loci: DMG, QMA, TMI and TML (Dettman et al 2003a). Diagnostic nucleotide characters that are fixed among the known individuals include TMI locus nucleotide positions 71 (T), 109 (G), 48 (T); TML locus nucleotide positions 129 (T), 130 (Dettman et al 2003b) (G), 131 (G), 195 (T), 514 (G); QMA locus nucleotide positions 70 (G), 290 (A), 415 (G).

HOLOTYPE: CV55 (FGSC 10403)

Distribution. Haiti.

Etymology. Species is named after Hispaniola where it was first collected by David Perkins.

Neurospora metzenbergii Villalta, Jacobson et Taylor, sp. nov.

Fungus generatione sexuale inter individua, heterothallicus haploideus prius PS2 designatus, a speciebus Neurosporae alteris heterothallicis generatione sexuale inter individua distinguendus non per formam, sed per notionem speciei phylogenetici et quattuor locos geneticos DMG, QMA, TMI, et TML. Notae characteristicae nucleotidorum fixae in individuis notis speciei huius: positiones loci TMI 315 (G), 396 (A); loci TML 393 (G), 456 (T), 458 (G); loci QMA 124 (A), 135 (A), 146 (A), 219 (A), 318 (A), 357 (T).

Neurospora metzenbergii is an outbreeding heterothallic haploid fungus previously designated PS2. *N. metzenbergii* is morphologically indistinguishable from other heterothallic outbreeding species of *Neurospora*, but individuals can be assigned to *N. metzenbergii* using the phylogenetic species recognition concept and these four loci: DMG, QMA, TMI, and TML (Dettman et al 2003a). Diagnostic nucleotide characters that are fixed among the known individuals include the TMI locus nucleotide positions 315 (G), 396 (A); TML locus nucleotide positions 393 (G), 456 (T), 458 (G); QMA locus nucleotide positions 124 (A), 135 (A), 146 (A), 219 (A), 318 (A), 357 (T).

Distribution. Yucatan Peninsula in Mexico, in Haiti and in Madagascar.

HOLOTYPE: CV89 (FGSC 10395)

Etymology. The species is named after Robert L. Metzenberg who was a leading *Neurospora* geneticist. He collected most of the *N. metzenbergii* samples while in Mexico.

Neurospora perkinsii Villalta, Jacobson et Taylor, sp. nov.

Fungus generatione sexuale inter individua, heterothallicus haploideus prius PS3 designatus, a speciebus Neurosporae alteris heterothallicis generatione sexuale inter individua distinguendus non per formam, sed per notionem speciei phylogenetici et quattuor locos geneticos DMG, QMA, TMI, et TML. Notae characteristicae nucleotidorum fixae in populatione: positiones loci TMI 66 (T), 154 (G), 159 (A), 237 (C), 434 (G).

Neurospora perkinsii is an outbreeding heterothallic haploid fungus previously designated PS3. *N. perkinsii* is morphologically indistinguishable from other heterothallic outbreeding species of *Neurospora*, but individuals can be assigned to *N. perkinsii* with the phylogenetic species recognition concept and these four loci: DMG, QMA, TMI and TML (Dettman et al 2003a). Diagnostic nucleotide characters all present and fixed in the population include TMI locus nucleotide positions 66 (T), 154 (G), 159 (A), 237 (C), 434 (G).

Distribution. All current specimens have been found in the Democratic Republic of Congo and Gabon.

HAPLOTYPE: CV79 (FGSC 10406)

Etymology. *N. perkinsii* is named after the *Neurospora* geneticist and biologist David D. Perkins who was so important to *Neurospora* researchers and who was responsible for the majority of the specimens in the Perkins collection, including *N. perkinsii* isolates.

DISCUSSION

We found additional members of *N. hispaniola*, *N. metzenbergii* and *N. perkinsii* by searching among collections of Neurospora for which mating tests were equivocal or had been collected in endemic areas and then screening to exclude *N. crassa* and *N. intermedia*. In formally describing species preliminarily identified by PSR we found that *N. hispaniola*, *N. metzenbergii* and *N. perkinsii* all were distinct phylogenetic species, according to the PSR criteria set forth in Dettman (2003a), and all were able to mate intraspecifically. Intrinsic interspecific mating barriers consistent with the biological species concept were found for *N. hispaniola* and *N. metzenbergii* but not for *N. perkinsii* when mated to *N. crassa* (FIG. 3). However *N. crassa* and *N. perkinsii* strains that successfully mated are genetically and geographically isolated, suggesting that the extrinsic barrier of allopatry keeps the two species reproductively isolated. The African *N. crassa* strains that mated successfully with *N. perkinsii* were from the Ivory Coast, which is at least 2000 km from Congo. It would be interesting to see whether any mating barriers are present between *N. perkinsii* and *N. crassa* collected from Congo because studies have shown that mating barriers may be stronger between species in sympatry than in allopatry (Dettman et al 2003b).

Adding the newly discovered *Neurospora* individuals to the existing phylogeny changed neither the phylogenetic relationships nor the distinct geographic ranges of the species. The narrow endemism found

in *N. hispaniola* (Haiti), *N. metzenbergii* (Yucatan and Madagascar) and *N. perkinsii* (Congo) remains, but our sampling was partially biased in favor of those regions. We did not find any wild hybrid individuals, as was the case in previous studies.

We found cases where *Neurospora* individuals had been incorrectly assigned to species by crosses to mating testers. For individuals that belong to *N. hispaniola*, *N. metzenbergii* and *N. perkinsii*, the assignment problems could be explained by a lack of tester strains for the new species, a problem that has been corrected (Dettman et al 2003b). In the cases of the *N. sitophila* or *N. discreta* (individuals that previously had been assigned to *N. crassa* or *N. intermedia*) the problem might be due to intraspecific variation in reproductive compatibility that cannot be represented by a few tester strains. In the case of *N. discreta sensu lato* we know that there is significant genetic differentiation and isolation in this species (Dettman et al 2003a, 2006). The necessary detailed studies have not been carried out for *N. sitophila*. We found no misassigned *N. tetrasperma* isolates, indicating that the four-spored morphology and pseudohomothallic mating is a reliable morphological character. However genetically isolated or differentiated groups exist within *N. tetrasperma* (Saenz et al 2003). Our screen could be improved to account for *N. sitophila* and *N. discreta* genotypes and thereby reduce the number of false positives. We were unable to detect any false negatives.

Although we found additional members of each species, more individuals of *Neurospora* species need characterization. Obviously *N. hispaniola and N. perkinsii* still are under-sampled and *N. metzenbergii* individuals are split between two well separated geographic regions, Yucatan and Madagascar. Genetic differentiation in this species correlates with geographic range (FIG. 3), but our data show that individuals from the two areas are not reproductively isolated. For example isolate D120 collected in Madagascar mates successfully with isolates from Yucatan. When more Madagascar individuals are collected and characterized as the result of new fieldwork the species status of this group might change if the presently observed genetic differentiation is unchanged. The need for more collection and characterization also applies to *N. sitophila*, where a sampling comparable to that provided by Dettman et al (2003a, 2006) for *N. crassa* or *N. intermedia* will be required to interpret the phylogenetic relationships of the 10 false positive *N. sitophila* isolates discovered here.

While the species described here have narrow geographic ranges, as many as five *Neurospora* species can be found in sympatry at the same location (Turner et al 2001) and on the same substrate (Powell et al 2003). However as mentioned above no hybrids have been collected in the wild although ample opportunity for hybridization exists (Dettman et al 2003a, 2006; Powell et al 2003; Turner et al 2001). *Neurospora* remains an ecological enigma because little is known about the life history or niches occupied by any of the species or about interactions with other organisms. Understanding the genetic distance of *Neurospora* species will be especially helpful in the era of high-throughput sequencing and comparative genomics, where having that information can determine what organisms require genome sequencing.

This and previous studies have found that identification by successful mating, BSR, is similar to PSR but that genetic isolation can precede reproductive isolation (Dettman et al 2003a, b, 2006). Our results show that BSR by mating to tester strains alone can be misleading due to a lack of testers or the absence of reproductive isolation. In the past decade phylogenetic species recognition has become a popular alternative to morphological species recognition (MSR) and biological species recognition (BSR) (Taylor et al 2000), as evidenced by studies involving *Coccidiodes* (Fisher et al 2002), *Neurospora* (Dettman et al 2003a, b, 2006), *Saccharomyces* (Ae et al 2006) and *Schizophyllum* (James et al 2001, Taylor et al 2006). The results above show that while PSR alone is powerful and accurate it also is important to the formal description of new species to account for reproductive isolation, biogeography and morphology. We hope that our characterization and naming of *Neurospora* species and the addition of additional strains to the *Neurospora* phylogenetic tree will add to the attractiveness of *Neurospora* as an interesting model organism in the study of evolutionary biology and ecology.

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APPENDIX I. Specimens used in RFLP analysis

Study ID	FGSC ID[1]	Perkins ID[2]	Original species[3]	Phylogenetic species	Mating type[4]	NciI[5]	BciVI[6]	Collection site
CV-1		P1563	*N. intermedia?*		A&a	+	−	Yalu, Papua New Guinea
CV-2		P1666	*N. intermedia?*		A&a	+	−	Kaindi Road, Papua New Guinea
CV-3		P1668	*N. intermedia?*		A&a	−		Kaindi Road, Papua New Guinea
CV-4		P1717	*N. intermedia?*		A&a	+	−	Goroka, Papua New Guinea
CV-5		P1751	*N. intermedia?*		A&a	+	−	Goroka, Papua New Guinea
CV-6		P1804	*N. intermedia?*		A&a	+	−	Baiyer River, Papua New Guinea
CV-7		P1828	*N. intermedia?*		a	+	−	Mt. Hagen, Papua New Guinea
CV-8		P1829	*N. intermedia?*		a	+	−	Mt. Hagen, Papua New Guinea
CV-9		P1982	*N. intermedia?*		A&a	+	−	Brown River Area, Papua New Guinea
CV-10	**10393**	**P2026**	***N. intermedia?***	***N. sitophila***	**A**	+	+	**Brown River Area, Papua New Guinea**
CV-11	10394	P2027	*N. intermedia?*	*N. discreta* (PS?)*	A	−		Brown River Area, Papua New Guinea
CV-12		P2745	*N. intermedia?*		A&a	+	−	Kampung Cempaka, Malaysia

APPENDIX I. Continued

Study ID	FGSC ID[1]	Perkins ID[2]	Original species[3]	Phylogenetic species	Mating type[4]	NciI[5]	BciVI[6]	Collection site
CV-13		P2840	*N. intermedia*?		A&a	+	−	Mantin, Malaysia
CV-14		P2853	*N. intermedia*?		A&a	+	−	Melaka, Singapore
CV-15		P2864	*N. intermedia*?		A&a	+	−	Singapore, Singapore
CV-16		P2871	*N. intermedia*?		A&a	+	−	Singapore, Singapore
CV-17		P2891	*N. intermedia*?		A&a	+	−	Bangkok Airport, Thailand
CV-18		P2922	*N. intermedia*?		A&a	+	−	Khao Eto, Thailand
CV-19		P2933	*N. intermedia*?		A&a	+	−	Khao Yai, Thailand
CV-20		P2944	*N. intermedia*?		A&a	+	−	Khao Yai, Thailand
CV-21		P2983	*N. intermedia*?		A&a	+	−	Klong Rangsit, Thailand
CV-22		P3006	*N. intermedia*?		A&a	+	−	Pakchong, Thailand
CV-23		P3032	*N. intermedia*?		A&a	+	−	Saraburi, Thailand
CV-24		P3038	*N. intermedia*?		A&a	+	−	Wang Noi, Thailand
CV-25		P3077	*N. intermedia*?		A&a	+	−	Singapore, Singapore
CV-26		P3083	*N. intermedia*?		A&a	+	−	Kuching, Borneo
CV-27		P3085	*N. intermedia*?		A&a	+	−	Kuching, Borneo
CV-28		P3111	*N. intermedia*?		A&a	+	−	Lokawi Camp, Borneo
CV-29		P3140	*N. intermedia*?		A&a	+	−	Kota Kinabalu, Borneo
CV-30		P3154	*N. intermedia*?		A&a	+	−	Songsong, Rota
CV-31		P3164	*N. intermedia*?		A&a	+	−	Songsong, Rota
CV-32		P3169	*N. intermedia*?		A&a	+	−	Rota, Rota
CV-33		P3176	*N. intermedia*?		A&a	+	−	Rota, Rota
CV-34		P3177	*N. intermedia*?		A&a	+	−	Rota, Rota
CV-35		P3182	*N. intermedia*?		A&a	+	−	Rota, Rota
CV-36		P3184	*N. intermedia*?		A&a	+	−	Rota, Rota
CV-37		P3185	*N. intermedia*?		A&a	+	−	Rota, Rota
CV-38		P3215	*N. intermedia*?		A&a	+	−	Merizo, Guam
CV-39		P3225	*N. intermedia*?		A&a	+	−	Taleysay, Guam
CV-40		P3227	*N. intermedia*?		A&a	+	−	Taleysay, Guam
CV-41		P3230	*N. intermedia*?		A&a	+	−	Taleysay, Guam
CV-42		P3233	*N. intermedia*?		A&a	+	−	Moen, Truk
CV-43	**10402**	**P3252**	***N. intermedia*?**	***N. sitophila***	**A**	+	+	**Moen, Truk**
CV-44		P3255	*N. intermedia*?		A&a	+	−	Moen, Truk
CV-45		P3288	*N. intermedia*?		A&a	+	−	Moen, Truk
CV-46		P3289	*N. intermedia*?		A&a	+	−	Moen, Truk
CV-47		P3300	*N. intermedia*?		A&a	+	−	Moen, Truk
CV-48		P3301	*N. intermedia*?		A&a	+	−	Moen, Truk
CV-49		P3309	*N. intermedia*?		A&a	+	−	Kolonia, Ponape
CV-50		P3321	*N. intermedia*?		A&a	+	−	Kolonia, Ponape
CV-51		P3324	*N. intermedia*?		A&a	+	−	Kolonia, Ponape
CV-52		P3338	*N. intermedia*?		A&a	+	−	Ponape, Ponape
CV-53		P3339	*N. intermedia*?		A&a	+	−	Ponape, Ponape
CV-54		P3340	*N. intermedia*?	*N. intermedia**	A&a	+	−	Ponape, Ponape
CV-55	**10403**	**P3431**	***N. intermedia*?**	***N. hispaniola* (PS1)**	**A**	+	+	**Leogane, Haiti**
CV-56	**10404**	**P3472**	***N. intermedia*?**	***N. sitophila***	**A**	+	+	**Bas Quarter, Haiti**
CV-57	**10405**	**P3473**	***N. intermedia*?**	***N. sitophila***	**A**	+	+	**Bas Quarter, Haiti**
CV-58		P3534	*N. intermedia*?		A&a	+	−	Ran Adjame, Ivory Coast
CV-59		P3537	*N. intermedia*?		A&a	+	−	Ran Adjame, Ivory Coast
CV-60		P3546	*N. intermedia*?		a	+	−	Yopougon, Ivory Coast
CV-61		P3547	*N. intermedia*?		A&a	+	−	Universite, Ivory Coast
CV-62		P3548	*N. intermedia*?		A&a	+	−	Universite, Ivory Coast
CV-63		P3574	*N. intermedia*?		a	−		Godilisheri Ecole, Ivory Coast
CV-64		P3708	*N. intermedia*?		A&a	−		Hermankono, Ivory Coast
CV-65		P3745	*N. intermedia*?		A&a	+	−	Agbanou, Ivory Coast
CV-66		P3756	*N. intermedia*?		A&a	+	−	Adiopodoume, Ivory Coast
CV-67		P3777	*N. intermedia*?		A	−		Brazzaville, Congo

APPENDIX I. Continued

Study ID	FGSC ID[1]	Perkins ID[2]	Original species[3]	Phylogenetic species	Mating type[4]	NciI[5]	BciVI[6]	Collection site
CV-68		P3779	*N. intermedia*?		a	−		Brazzaville, Congo
CV-69		P3806	*N. intermedia*?		A&a	+	−	Lebanda, Congo
CV-70		P3832	*N. intermedia*?		A&a	+	−	Jacob, Congo
CV-71		P3861	*N. intermedia*?		A&a	+	−	Mantsoumba, Congo
CV-72		P3871	*N. intermedia*?	*N. intermedia**	A&a	+	−	Mantsoumba, Congo
CV-73		P3876	*N. intermedia*?		A&a	+	−	Mindouli, Congo
CV-74		P3883	*N. intermedia*?		A&a	+	−	Missafou, Congo
CV-75		P3892	*N. intermedia*?		A&a	+	−	Kinkala,Congo
CV-76		P3896	*N. intermedia*?	*N. intermedia**	A&a	+	−	Missafou, Congo
CV-77		P3904	*N. intermedia*?		a	+	−	Kinkala,Congo
CV-78		P3945	*N. intermedia*?		A&a	+	−	Liberville, Gabon
CV-79	**10406**	**P3947**	***N. intermedia*?**	***N. perkinsii* (PS3)**	**a**	+	+	**Liberville, Gabon**
CV-80	**10407**	**P3948**	***N. intermedia*?**	***N. sitophila***	**a**	+	+	**Liberville, Gabon**
CV-81		P3949	*N. intermedia*?		a	+	−	Liberville, Gabon
CV-82	**10408**	**P3952**	***N. intermedia*?**	***N. sitophila***	**a**	+	+	**Liberville, Gabon**
CV-83		P3953	*N. intermedia*?	*N. intermedia**	A	+	−	Libreville, Gabon
CV-84		P3954	*N. intermedia*?		A&a	+	−	Libreville, Gabon
CV-85		P3961	*N. intermedia*?		a	+	−	MadingoMarket, Dominican Republic
CV-86		P4077	*N. intermedia*?		A	−		Torani Canal, Guyana
CV-87		P4098	*N. intermedia*?	*N. sitophila*	a	+	+	Laie, Hawaii
CV-88	**10394**	**P4099**	***N. intermedia*?**	***N. sitophila***	**a**	+	+	**Laie, Hawaii**
CV-89	**10395**	**P4166**	***N. intermedia*?**	***N. metzenbergii* (PS2)**	**a**	+	+	**Macantoc, Mexico**
CV-90	**10396**	**P4171**	***N. intermedia*?**	***N. metzenbergii* (PS2)**	**a**	+	+	**Coba, Mexico**
CV-91	**10397**	**P4172**	***N. intermedia*?**	***N. metzenbergii* (PS2)**	**a**	+	+	**Coba, Mexico**
CV-92		P4173	*N. intermedia*?		a	−		Coba, Mexico
CV-93	**10409**	**P4181**	***N. intermedia*?**	***N. sitophila***	**A**	+	+	**Chemax, Mexico**
CV-94		P4388	*N. intermedia*?		A&a	+	−	Adiopodoume, Ivory Coast
CV-95		P4390	*N. intermedia*?		a	+	−	Adiopodoume, Ivory Coast
CV-96		P4411	*N. intermedia*?		a	+	−	Adiopodoume, Ivory Coast
CV-97		P4535	*N. intermedia*?		a	+	−	Bani, Dominican Republic
CV-98	**10398**	**P4594**	***N. intermedia*?**	***N. sitophila***	**a**	+	+	**Sulawesi Indonesia**
CV-99		P4665	*N. intermedia*?	*N. intermedia**	a	+	−	Kampang Pengakalan Kuin, Malaysia
CV-100		P4666	*N. intermedia*?		A&a	+	−	Kampang Pengakalan Kuin, Malaysia
CV-101		P4771	*N. intermedia*?		A	+	−	Madurai, India
CV-102		P4772	*N. intermedia*?		a	−		Madurai, India
CV-103		P1143	*N. intermedia*		A	+	−	Garrochales, Puerto Rico
CV-104		P1149	*N. intermedia*		A	+	−	La Prada, Puerto Rico
CV-105		P1157	*N. intermedia*		a	+	−	La Prada, Puerto Rico
CV-106		P1174	*N. intermedia*		a	+	−	Aguadilla, Puerto Rico
CV-107		P1175	*N. intermedia*		A	+	−	Aguadilla, Puerto Rico
CV-108		P1200	*N. intermedia*		a	+	−	San German, Puerto Rico
CV-109		P1215	*N. intermedia*		a	+	−	Betances, Puerto Rico
CV-110		P1216	*N. intermedia*		A	+	−	Betances, Puerto Rico
CV-111		P1250	*N. intermedia*	*N. intermedia**	A	+	−	Playa Cortada, Puerto Rico
CV-112		P1291	*N. crassa*		a	−		Colonia Paraiso, Puerto Rico
CV-113		P1295	*N. crassa*		A	−		Colonia Paraiso, Puerto Rico
CV-114		P3430	*N. intermedia*		A	+	−	Leogane, Haiti
CV-115		P3439	*N. intermedia*		a	+	−	Merger, Haiti
CV-116		P3442	*N. intermedia*		A	+	−	Puilboreau Mt., Haiti
CV-117		P3443	*N. intermedia*		a	+	−	Puilboreau Mt., Haiti
CV-118		P3450	*N. intermedia*		a	+	−	Pescail,Haiti
CV-119	**10399**	**P3453**	***N. crassa***	***N. metzenbergii* (PS2)**	**a**	+	+	**Pescail, Haiti**
CV-120		P3454	*N. crassa*	*N. crassa**	A	−		Pescail, Haiti
CV-121		P3455	*N. intermedia*		A	+	−	Pescail, Haiti

APPENDIX I. Continued

Study ID	FGSC ID[1]	Perkins ID[2]	Original species[3]	Phylogenetic species	Mating type[4]	NciI[5]	BciVI[6]	Collection site
CV-122		P3471	*N. crassa*		a	−		Bas Quartier, Haiti
CV-123		P3479	*N. crassa*		A	−		Berard, Haiti
CV-124		P3488	*N. intermedia*		A	+	−	Haut Du Cap, Haiti
CV-125		P3509	*N. intermedia*		a	+	−	Gran Sous, Haiti
CV-126		P3962	*N. crassa*		a	−		Jaco, Costa Rica
CV-127		P3963	*N. crassa*		A	−		Jaco-1, Costa Rica
CV-128		P3979	*N. crassa*		a	−		Agudas, Costa Rica
CV-129		P3981	*N. crassa*		A	−		Agudas, Costa Rica
CV-130		P3984	*N. crassa*		A	−		Esterillo Este, Costa Rica
CV-131		P3992	*N. crassa*		a	−		Esterillo Este, Costa Rica
CV-132		P3993	*N. crassa*		a	−		Esterillo Este, Costa Rica
CV-133		P3994	*N. crassa*		A	−		Esterillo Este, Costa Rica
CV-134		P4003	*N. crassa*		a	−		Esterillo Este, Costa Rica
CV-135		P4021	*N. crassa*		A	−		Covolar, Costa Rica
CV-136		P4035	*N. crassa*		a	+	−	Puerto Ayachucho, Venezuela
CV-137		P4052	*N. intermedia*		a	+	−	Mt. Ayanganna, Guyana
CV-138		P4055	*N. intermedia*		a	+	−	Big Emma, Guyana
CV-139		P4056	*N. intermedia*		a	+	−	Imbaima Dai, Guyana
CV-140		P4063	*N. intermedia*		A	+	−	Canje River, Guyana
CV-141		P4065	*N. crassa*		A	−		Digitima Creek, Guyana
CV-142		P4068	*N. intermedia*		a	+	−	Digitima Creek, Guyana
CV-143		P4070	*N. intermedia*		A	+	−	Ekwarun, Guyana
CV-144		P4082	*N. intermedia*		A	+	−	Lookout Village, Guyana
CV-145		P4087	*N. crassa*		A	−		Maripasoula, French Guiana
CV-146		P4088	*N. crassa*		a	−		Maripasoula, French Guiana
CV-147		P4108	*N. crassa*	*N. crassa**	A	−		Chemax, Mexico
CV-148	**10410**	**P4112**	***N. intermedia***	***N. metzenbergii* (PS2)**	**a**	+	+	**Chemax, Mexico**
CV-149		P4125	*N. crassa*		a	−		Kabah, Mexico
CV-150		P4127	*N. crassa*		A	−		Kabah, Mexico
CV-151		P4149	*N. intermedia*		A	−		Sayil, Mexico
CV-152	**10411**	**P4153**	***N. intermedia***	***N. metzenbergii* (PS2)**	**A**	+	+	**Uxmal, Mexico**
CV-153	**10412**	**P4156**	***N. intermedia***	***N. metzenbergii* (PS2)**	**a**	+	+	**Uman, Mexico**
CV-154	**10400**	**P4167**	***N. intermedia***	***N. metzenbergii* (PS2)**	**a**	+	+	**Macantoc, Mexico**
CV-155	**10401**	**P4168**	***N. intermedia***	***N. metzenbergii* (PS2)**	**A**	+	+	**Macantoc, Mexico**
CV-156	**10413**	**P4176**	***N. intermedia***	***N. metzenbergii* (PS2)**	**A**	+	+	**Coba, Mexico**
CV-157		P4180	*N. crassa*		a	−		Chemax, Mexico
CV-158		P4503	*N. intermedia*		a	+	−	Lookout Village, Guyana
CV-159		P4529	*N. intermedia*		A	+	−	Bani, Dominican Republic
CV-160		P4531	*N. intermedia*		a.	+	−	Bani, Dominican Republic
CV-161		P4538	*N. intermedia*		A	+	−	Bani, Dominican Republic
CV-162		P4539	*N. intermedia*		a	+	−	Bani, Dominican Republic
CV-163		P4540	*N. intermedia*		a	+	−	Bani, Dominican Republic
CV-164		P4541	*N. intermedia*		A	+	−	Bani, Dominican Republic
CV-165		P4542	*N. intermedia*		a	+	−	Bani, Dominican Republic
CV-166		P4543	*N. intermedia*		a	+	−	Bani, Dominican Republic
CV-167		P4544	*N. intermedia*		A	+	−	Bani, Dominican Republic
CV-168		P4547	*N. intermedia*		A	+	−	Bani, Dominican Republic
CV-169		P4552	*N. intermedia*		a	+	−	Bani, Dominican Republic
CV-170		P4553	*N. intermedia*	*N. intermedia**	A	−		Bani, Dominican Republic
CV-171		P4581	*N. crassa*		A	−		Arena Reser, Trinidad
CV-172		P4582	*N. intermedia*		a	+	−	Walter Air Force Base, Trinidad
CV-173		P4583	*N. intermedia*		a	+	−	Caroni Swamp, Trinidad
CV-174		P4584	*N. intermedia*		A	+	−	Caroni Swamp, Trinidad
CV-175		P4585	*N. crassa*		A	−		Caroni Swamp, Trinidad
CV-176		P4586	*N. intermedia*		A	+	−	Caroni Swamp, Trinidad

APPENDIX I. Continued

Study ID	FGSC ID[1]	Perkins ID[2]	Original species[3]	Phylogenetic species	Mating type[4]	NciI[5]	BciVI[6]	Collection site
CV-177		P4588	*N. crassa*		A	−		Orinoco Delta, Venezuela
CV-178		P4590	*N. intermedia*		a	+	−	Ile St. Joseph, French Guiana
CV-179		P4591	*N. intermedia*		A	+	−	Ile St. Joseph, French Guiana
CV-180		P4595	*N. intermedia*		a	−		Caroni Swamp, Trinidad
CV-181		P4694	*N. crassa*		a	−		Old Man Bay, Grand Cayman BWI
CV-182		P4723	*N. crassa*		A	−		Old Man Bay, Grand Cayman BWI
CV-183		P4765	*N. intermedia*		a	+	−	Colonia Paraiso, Puerto Rico
CV-184		P4773	*N. intermedia*		a	+	−	Friendship Village, Guyana
CV-185		P4776	*N. intermedia*		A	+	−	Friendship Village, Guyana
CV-186		P4782	*N. crassa*		a	−		Old Man Bay, Grand Cayman BWI
CV-187		P4783	*N. crassa*		a	−		Old Man Bay, Grand Cayman BWI
CV-188		P4784	*N. crassa*		A	+	−	Old Man Bay, Grand Cayman BWI

[1] FGSC ID are only present for samples that were identified with PSR with the 4 polymorphic loci.

[2] Perkins ID correspond to the numbers assigned to specimens belonging to the Perkins culture collection. The full culture collection catalog can be obtained from the FGSC via e-mail (questions@fgsc.net).

[3] Originally all the isolates used in the study were identified by BSR with tester strains of varying *Neurospora* species. Some specimens have question marks after their identification, meaning that the specimen could not be clearly identified but the name given appears to be the best assumption.

[4] A&a mating type means that when the specimen was placed in the Perkins collection it was saved as a mixture of both A and a mating types. All specimens in our study came from a single conidial isolate.

[5] In the NciI column the plus sign means the TMI PCR product was digested by NciI and a minus sign means it was not digested.

[6] In the BciVI column the plus sign means the TMI PCR product was digested by BciVI and a minus sign means it was not digested. If the cell is shaded in gray in the BciVI column it means that the digest was not performed for the particular TMI PCR sample.

* Specimen was identified by PSR only with the TMI loci as a control to ensure that RE digests were working correctly. A question mark is present for CV11 because while the specimen is likely *N. discreta* we did not analyze it further to determine what species clade it belongs to.

Specimens in boldface were those that had all 4 loci sequenced and were placed in the FGSC collection.