

Gustavo Caetano-Anollés · Robert N. Trigiano
Mark T. Windham

Patterns of evolution in *Discula* fungi and the origin of dogwood anthracnose in North America, studied using arbitrarily amplified and ribosomal DNA

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Abstract The anthracnose epidemic caused by exotic filamentous fungi of the genus *Discula* threatens the future of the prized flowering (*Cornus florida* L.) and Pacific (*C. nuttalli* Aud.) dogwoods in North America. A cross-section of fungi that cause anthracnose in broad-leaf temperate trees was characterized using DNA amplification fingerprinting, sequence and secondary structure analysis of the internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA), and compatibility of hyphal anastomosis. ITS-inferred phylogenies rejected the null hypothesis of only one fungal lineage, by defining four monophyletic and well differentiated groups, corresponding to *Discula* sp., *D. quercina*, *D. umbrinella* and *D. destructiva*, with the last two species sharing a common and recent ancestor. In turn, they showed that the dogwood pathogen, *D. destructiva*, did not evolve directly from an indigenous population related to *Discula* sp. In this study, rDNA spacers that are generally considered important for protein synthesis but are selectively neutral, appeared functionally constrained and subject to selective sequence diversification. Results confirmed the high variability of *D. umbrinella* and the remarkable homogeneity and exotic nature of *D. destructiva* at the genetic level, clarified the taxonomy and phylogeny of *Discula*, and provided clues as to the origin and diversification of dogwood anthracnose-causing fungi.

Keywords *Discula* · Dogwood anthracnose · ITS sequence · Ribosomal DNA

Introduction

North American forest trees have been ravaged by a number of diseases, with severe ecological and societal impact, many of them caused by exotic fungal invaders (Campbell and Schlarbaum 1994). One example is the blight epidemic that decimated the American chestnut [*Castanea dentata* (Marsh.) Borkh.] following the introduction of the fungus *Cryphonectria parasitica* (Murrill) Barr from Asia in the early 1900s (Newhouse 1990). The recent anthracnose epidemic caused by filamentous coelomycetous fungi of the genus *Discula* (Hibben and Daughtrey 1988; Redlin 1991; Windham et al. 1994) now threatens the prized flowering (*Cornus florida* L.) and Pacific (*C. nuttalli* Aud.) dogwoods of North America in much of their natural range (Daughtrey et al. 1996). The epidemic has already devastated native tree populations in eastern and western deciduous forests. The severity and rapid onset and spread of the disease, the absence of conspecific fungi in herbarium specimens and of records of disease symptoms prior to the 1970s (Redlin 1991), together with the apparent lack of plant resistance and the genetic homogeneity (clonality; Trigiano et al. 1995) and fine population structure (Caetano-Anollés et al. 1996) of the pathogen, support the exotic invader hypothesis. Despite a proposal that the pathogen was introduced in infected *C. kousa* (Buerguer ex Miq.) from eastern Asia (Daughtrey 1993), its origin remains unknown. To understand the origin and progression of dogwood anthracnose in North America, there is a need to characterize more rigorously the species of *Discula* at the molecular phylogenetic level.

Differences in morphology, physiology and host-specificity have refuted the single species concept of anthracnose fungi, uniting species under 11 *Discula* epithets (reviewed by Redlin 1991). Within the genus,

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G. Caetano-Anollés (✉)
Division of Molecular Biology, Department of Biology,
University of Oslo, P.O. Box 1045, Blindern, 0316 Oslo, Norway
E-mail: gustavoc@mac.com

R.N. Trigiano
Department of Ornamental Horticulture and Landscape Design,
The University of Tennessee, Knoxville, TN 37901-1071, USA

M.T. Windham
Department of Entomology and Plant Pathology,
The University of Tennessee, Knoxville, TN 37901-1071, USA

D. destructiva Red. (Redlin 1991) and a low-incidence, undescribed *Discula* sp. (present in 7–8% of lesions; Windham et al. 1994) infect dogwoods, causing one of the 54 described tree diseases collectively termed anthracnoses. These fungi are not conspecific with those causing anthracnose in ash, oak and sycamore (Redlin 1991) and do not exhibit a sexual stage as do other *Discula* species [with teleomorphs in the genera *Apiogonomonium* and *Gnomoniella* (Gnomoniaceae, Diaporthales)]. They are currently considered asexually reproducing ascomycetes.

Eukaryotic ribosomal genes are arranged in tandem repeats with the 5.8S coding region flanked by internal transcribed spacers (ITS) important for the processing and maturation of nuclear ribosomal RNA (rRNA) (van der Sande et al. 1992; van Nues et al. 1994, 1995). However, ITS are generally considered to be under low evolutionary constraint and are therefore treated as non-functional sequences (Furlong and Maden 1983). They have been widely used in phylogenetic analysis (Hillis and Dixon 1991) and are informative and diagnostic in the molecular systematics of eukaryotes such as fungi (Vilgalys and Sun 1994). The present study characterizes a cross-section of fungi that cause anthracnose in broadleaf temperate trees, using a combination of sequence and secondary structure analysis of rDNA spacers and DNA amplification fingerprinting (DAF; Caetano-Anollés et al. 1991). The study clarifies the taxonomy and phylogeny of *Discula* and provides clues as to the origin and diversification of an exotic and highly clonal fungal population.

Materials and methods

Fungi and vegetative compatibility tests

Anthracnose fungi examined included *D. campestris* (Pass.) Arx., *D. fraxinea* (Peck.) Redlin and Stack, *D. platani* (Peck.) Sacc., *D. quercina* (Westd.) Arx. and *D. umbrinella* (Berk. and Br.) Morelet strains, and 23 *D. destructiva*, *Discula* sp. and undescribed (LkM-A and LkM-B) isolates collected (1988–1995) from lesions on flowering, Pacific and Chinese dogwoods and representing a cross-section of extant sub-populations (cf. Caetano-Anollés et al. 1996; Table 1). Fungal isolates were grown and maintained in potato dextrose V8 juice agar (Trigiano et al. 1995). Compatibility of hyphal anastomosis was tested in 60-mm agar plates. Groups of 9 and 12 isolates were paired in replicated bifactorial designs, by laying 7-mm plugs (from 1-week-old cultures) 20 mm apart from each other with the mycelium in contact with the agar surface. Mycelial interfaces were examined for compatibility and hyphal lysis using 40× total magnification after 2 weeks and 4 weeks.

rDNA amplification

Mycelial DNA was isolated from stationary liquid cultures (Trigiano et al. 1995), using the Puregene kit (Gentra Systems, Research Triangle Park, N.C.), and stocks were diluted to 0.5 ng/μl and 5 ng/μl. ITS were amplified by PCR using 0.5 units AmpliTaq Gold DNA polymerase/μl (Perkin-Elmer, Norwalk, Conn.), 0.5 ng template DNA/μl, 200 μM of each deoxynucleoside triphosphate (USB, Cleveland, Ohio), 1.5 mM MgCl₂, buffer (50 mM KCl,

10 mM Tris HCl, pH 8.3) and 0.3 μM of paired ITS1, ITS2, ITS3 and ITS4 primers (White et al. 1990). Reactions (10 μl) were subjected to a pre-amplification enzyme activation step of 9 min at 94 °C, followed by 35 amplification cycles of 1 min at 96 °C, 1 min at 57 °C, 1 min at 72 °C and a final extension step of 7 min at 72 °C, in a recirculating hot-air thermocycler. PCR products (10 ng/μl) were purified with Quiagen PCR columns (Quiagen, Chatsworth, Calif.) and sequenced using an ABI 373A DNA sequencer and the ABI Prism cycle sequencing kit with dye-terminator chemistry (Applied Biosystems, Foster City, Calif.).

Phylogenetic reconstruction and secondary structure analysis

Sequences were consistently aligned using the ClustalX program (ver. 1.64; Thompson et al. 1997), with gap and extension penalties of 10 and 0.05, respectively. ITS boundaries were determined by comparing the aligned sequences with previously published sequences from *Ophiostoma ulmi* (Buisman) Nannf. strain MH75 (U23424) and *Rhizoctonia solani* 521 (U19950). Phylogenetic relationships were estimated using PAUP* (Swofford 1999), PHYLIP (Felsenstein 1995) and PUZZLE (ver. 3.1; Strimmer and von Haeseler 1996). When using maximum parsimony as the optimality criterion in PAUP*, the branch-and-bound algorithm was used to find most parsimonious trees. Alternatively, the heuristic search procedure was used when analyzing large numbers of taxa, with tree bisection-reconnection branch swapping, random stepwise addition sequence, collapse option and ACCTRAN character-optimization. A group of fungal isolates related to *Discula* (Table 1) was sometimes defined as the outlying group. Phylogenetic reliability was tested by the nonparametric bootstrap method (Felsenstein 1985), using 2,000 replications. The structure of the phylogenetic signal in the data was evaluated using the skewness (g_1) of the length distribution of 10⁴ random trees (Hillis and Huelsenbeck 1992). Pairwise distances were computed from nucleotide sequences, based on the two-parameter nucleotide substitution model of Kimura (1980), and were used to assess differences in substitution rates within spacer regions. Relative rate differences were evaluated by linear regression analysis of paired values obtained from each distance matrix. RNA secondary structure of spacer regions was predicted by a free-energy minimization method (Zuker 1989), using the Mfold program (ver. 3.0) with updated thermodynamic parameters (Mathews et al. 1999). The ITS2 secondary structure was inferred from recurrent RNA-folding patterns, 5.8S rRNA constraints (cf. Furlong and Maden 1983) and the yeast spacer model (Yeh and Lee 1990).

DAF analysis

DAF reactions (20 μl) were optimized with Taguchi methods (Caetano-Anollés 1998) and contained 0.1 ng fungal DNA/μl, 8 μM primer, 200 μM of each deoxynucleoside triphosphate, 2.5 mM MgCl₂, 0.7 units AmpliTaq Stoffel fragment DNA polymerase/μl (Perkin-Elmer) and Stoffel buffer (10 mM KCl, 10 mM Tris·HCl, pH 8.3) and were amplified for 35 cycles of 0.5 min at 96 °C, 1 min at 50 °C and 1 min at 74 °C, followed by an extension step of 7 min at 72 °C. DAF primers had the following arbitrary sequences (5'–3'): GACGTAGG, GAAACGCC, CCGAGCTG and CCTGGTGG. Amplification products were separated by electrophoresis in polyester-backed 0.45-mm-thick 10%T:2%C polyacrylamide/7 M urea/5% glycerol slab minigels. Silver-stained bands (50–700 bp) were scored as unordered genetic characters (loci) and analyzed using NTSYS-pc (Rohlf 1992). Phenetic relationships were evaluated using Jaccard similarity coefficients and both cluster (UPGMA) and neighbor-joining algorithms and were confirmed by principal coordinate analysis (PCO).

Analysis by arbitrary signatures of amplification products

Sequence divergence estimates were obtained from arbitrary signatures of amplification products (ASAP; Caetano-Anollés and

Table 1 Anthracnose fungi. Pathogen hosts: *As* sugar maple (*Acer saccharum* Marsh.), *Cf* flowering dogwood (*Cornus florida* L.), *Ck* Chinese dogwood [*C. kousa* (Buerger ex Miq.) Hance], *Cn* Pacific dogwood (*C. nuttallii* Audubon), *Cs* European chestnut (*Castanea sativa* Mill.), *Fa* tall fescue (*Festuca arundinacea* Schreb.), *Fr* ash (*Fraxinus* sp.), *Fs* European beech (*Fagus sylvatica* L.), *Gm* soybean (*Glycine max* L.), *Ms* apple (*Malus sylvestris* Mill.), *Pl* American sycamore or plane tree (*Platanus occidentalis* L.), *Pv* bean (*Phaseolus vulgaris* L.), *Qg* Oregon white oak (*Quercus garryana* Dougl.), *Qr* English oak (*Quercus robur* L.), *Ua* American elm (*Ulmus americana* L.), *Vi* grape (*Vitis* sp.). In hyphal anastomosis, compatibility groups A–G are arbitrary and defined at the interspecific level. *nt* Not tested

Species	Pathogenic host	Strains (geographic origin)	Hyphal anastomosis group
<i>Discula campestris</i>	As	PA1 (Pennsylvania)	C
<i>D. destructiva</i>	Cf	AL151 (Alabama); GA1 (Georgia); NY322, NY329 (New York); SC101A (South Carolina); E10, E10B, E10F, E61, E70, E99, E102, E107 (Tennessee)	A
	Cn	Cornu3 (Idaho); M10B, M12e, M24e (Oregon)	A
	Ck	MA11 (Massachusetts)	A
<i>D. fraxinea</i>	Fr	NY1 (New York)	D
<i>D. platani</i>	Pl	TN1, TN2, TN5 (Tennessee)	E
<i>D. quercina</i>	Qg	OR1, OR2, OR3, OR4, OR5 (Oregon)	F, G
	Cf ^a	LkM-A, LkM-B (Tennessee)	A
<i>D. umbrinella</i>	Qr	LT068 (Great Britain), 515, 617 (Switzerland)	B
	Fs	P4 (Poland); LT135 (Switzerland)	B
	Cs	416 (Switzerland)	B
<i>Discula</i> sp.	Cf	NC2 (North Carolina); NY326 (New York); VA17b (Virginia)	G
Outgroup	Ua	<i>Ophiostoma ulmi</i> MH75	nt
	Vi	<i>Botrytis cinerea</i> FR (France)	–
	Ms	<i>B. cinerea</i> BV (Maryland)	–
	Ms	<i>Glomerella cingulata</i> (Tennessee)	–
	–	<i>Colletotrichum gloeosporioides</i> (Tennessee)	–
	–	<i>C. acutatum</i> (Tennessee)	–
	Gm	<i>Cercospora sojina</i> (Tennessee)	–
	Fa	<i>Rhizoctonia solani</i> TNK5 (Tennessee)	–
	Pv	<i>R. solani</i> 521	–

^a These *D. quercina* isolates were obtained from diseased flowering dogwoods that were surrounded by white oak trees in Lookout Mountain (Chattanooga, Tenn.). Koch's postulates have not yet been fulfilled with these isolates

Gresshoff 1996), using formulae and assumptions previously described (Caetano-Anollés 1999). Sequence divergence was estimated from the number of ASAP polymorphisms and the total number of ASAP products.

Results

Cultural characteristics and somatic incompatibility

Except for *Discula* sp., none of the isolates could be distinguished unequivocally by growth habit in agar media (cf. Redlin 1991; Windham et al. 1994). Hyphal anastomosis tests (Fig. 1) showed marked somatic incompatibility between isolates of the different species, with the exception of selected strains of *D. quercina* and *Discula* sp. (notably, strains OR1 and VA17b; Table 1). Within an individual species, however, isolates were not always compatible with each other. In one experiment, incompatibility occurred in 45% of 12 *D. destructiva* pairwise combinations. Antibiosis occurred in 31% of isolate combinations tested; many isolates (especially *D. destructiva* and *D. fraxinea*) showed strong antibiosis.

DAF analysis of genome-wide genetic diversity

The levels of genome-wide diversity within representative isolates of individual *Discula* spp were evaluated by

amplification with four octamer primers known to generate high levels of polymorphic DNA in *D. destructiva*. DAF profiles contained an average of 37.3 ± 9.7 (mean \pm SD) character loci/primer, within the range 25–46 (an example of a DAF profile is shown in Fig. 2A). All primers produced polymorphic DNA, with an overall average of 20.6 ± 13.4 polymorphisms/primer. Although there were 8.0 ± 1.7 polymorphisms/primer within all *D. destructiva*, the mean number of character differences between any two isolates was 4.4 ± 1.3 per primer [compared to 1.0 ± 0.2 (Trigiano et al. 1995)]. Primer pre-selection therefore resulted in a 4-fold increase in strain discrimination. Diversity measured as a percentage of polymorphic characters within individual species increased in the order: *D. destructiva* (21.4%) < *D. quercina* (23.9%) < *Discula* sp. (56.2%) < *D. umbrinella* (89.2%) < *D. fraxinea*/*D. campestris*/*D. platani* (95.8%). This trend was also evident when the data were subjected to phenetic analysis, confirming the homogeneity of *D. destructiva* and *D. quercina* and suggesting a close relationship between isolates from Lookout Mountain in Tennessee (LkM) and *D. quercina* (Fig. 2B). DAF of bulked fungal DNA samples from the different species produced profiles with characters that were all informative (averaging 54.2 ± 11.1 per primer). Numerical analysis (PCO) showed that LkM isolates were clearly distantly related to *D. destructiva*, being instead phenetically close to *D. quercina* (Fig. 2C).

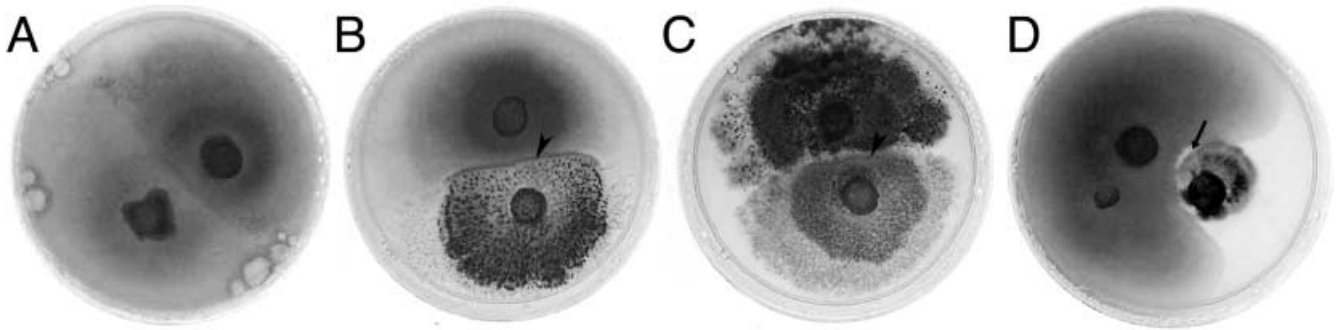


Fig. 1 A–D Hyphal anastomosis tests in *Discula*. Strains were inoculated in plugs and grown on agar plates. Mycelial interfaces were examined for hyphal lysis, barrage reactions and antibiosis. The figure illustrates the compatible interaction between *D. quercina* OR1 and OR3 isolates (A), barrage reactions (arrow heads) between *D. quercina* OR5 and *Discula* sp. VA17b (B) and between *D. fraxinea* and *Discula* sp. VA17b (C) and an antibiosis ring (arrow) between *D. quercina* OR1 and *D. destructiva* TN16 (D). The barrage reaction between incompatible strains results from regeneration of hyphae after the death of mycelial cells

Sequence variation in rDNA spacers

PCR-amplified ITS sequences were obtained from anthracnose-causing fungi and from an outlying group of distantly related fungal pathogens (Table 1). All sequences were easily aligned; and alignments showed acceptable quality scores, even when outgroup sequences were included. An alignment of *Discula* ITS sequences can be retrieved from the TreeBASE repository (<http://herbaria.harvard.edu/treebase/>) under study and matrix accession numbers S574 and M867, respectively. The length of ITS1 and ITS2 sequences ranged over 148–213 bp and 149–225 bp, respectively. *Discula* sequences ranged over 184–193 bp and 162–169 bp, respectively. Nucleotide substitution in the ITS1 and ITS2 spacers was calculated for a subset of 25 isolates that included all fungal groups. The estimates of substitution rates were generally higher for ITS1 than for ITS2 sequences. Regression analysis of pairwise ITS1 and ITS2 distances gave a positive linear relationship ($y = 0.54x$, $R^2 = 0.780$, $df = 298$, $P < 0.0001$) between the levels of sequence divergence in each data set, showing that the frequency of nucleotide substitution in ITS1 was approximately double that of ITS2 on a per nucleotide basis. Similar tendencies have been reported in other fungi (e.g., Boysen et al. 1996).

Phylogenetic analysis of spacer regions

Molecular phylogenies were inferred from the two ITS regions, using distance and maximum parsimony methods. Inferred trees were fully resolved at the species level, with most internal branches well supported by bootstrap analysis. Figure 3 shows a phylogenetic tree generated by maximum parsimony. *Discula* isolates were placed

in four monophyletic groups that were well supported (97–100%) and contained the dogwood pathogen *D. destructiva* (group a), the tree pathogens *D. umbrinella* (hosts: European beech and English oak), *D. fraxinea* (ash), *D. platani* (plane tree) and *D. campestris* (sugar maple; group b), the Oregon white oak pathogen *D. quercina* and *D. umbrinella* 416 (isolated from European chestnut; group c), and the dogwood anthracnose-causing *Discula* sp. (group d), respectively. The two undescribed strains isolated from dogwood lesions (LkM isolates) that were found closely related to *D. quercina* by DAF analysis, grouped together with other *D. quercina* isolates. The shallow branching of the *D. destructiva* and *D. umbrinella* groups suggests that these species belong to sister clades that are of recent origin. In contrast, *D. quercina* and *Discula* sp. constitute distant and more ancestral groups. A phylogenetic tree inferred from the ITS2 spacer obtained from a larger number of isolates and rooted using an outlying group of distantly related fungi (Fig. 4) showed the same topology as the unrooted tree obtained from both ITS spacers.

Phylogenetic relationships among the different *Discula* species were also resolved using neighbor-joining and maximum-likelihood (using both the MLE criterion in PAUP* and quartet puzzling; not shown). The reconstructed trees had topologies that strongly supported the genetic relationships described in Figs. 3 and 4. Similarly, a subset of 15 ITS2 sequences was analyzed by ASAP fingerprinting with six mini-hairpin primers. Cluster (UPGMA) analysis of these data produced dendrograms that were congruent with the sequence-inferred trees (not shown).

RNA secondary structure modeling of the ITS2 region

RNA-folding algorithms that search for the state of minimal free energy (Zuker 1989; Mathews et al. 1999) were used to predict a secondary structure for the *D. destructiva* ITS2 region (Fig. 5). The initial objective was to determine whether sequence variability within isolates occurred in functionally unconstrained regions. Consensus substructural features were first used as scaffolds for higher-order domains. A total of 17 RNA foldings with 1–5 minimum free-energy states defined

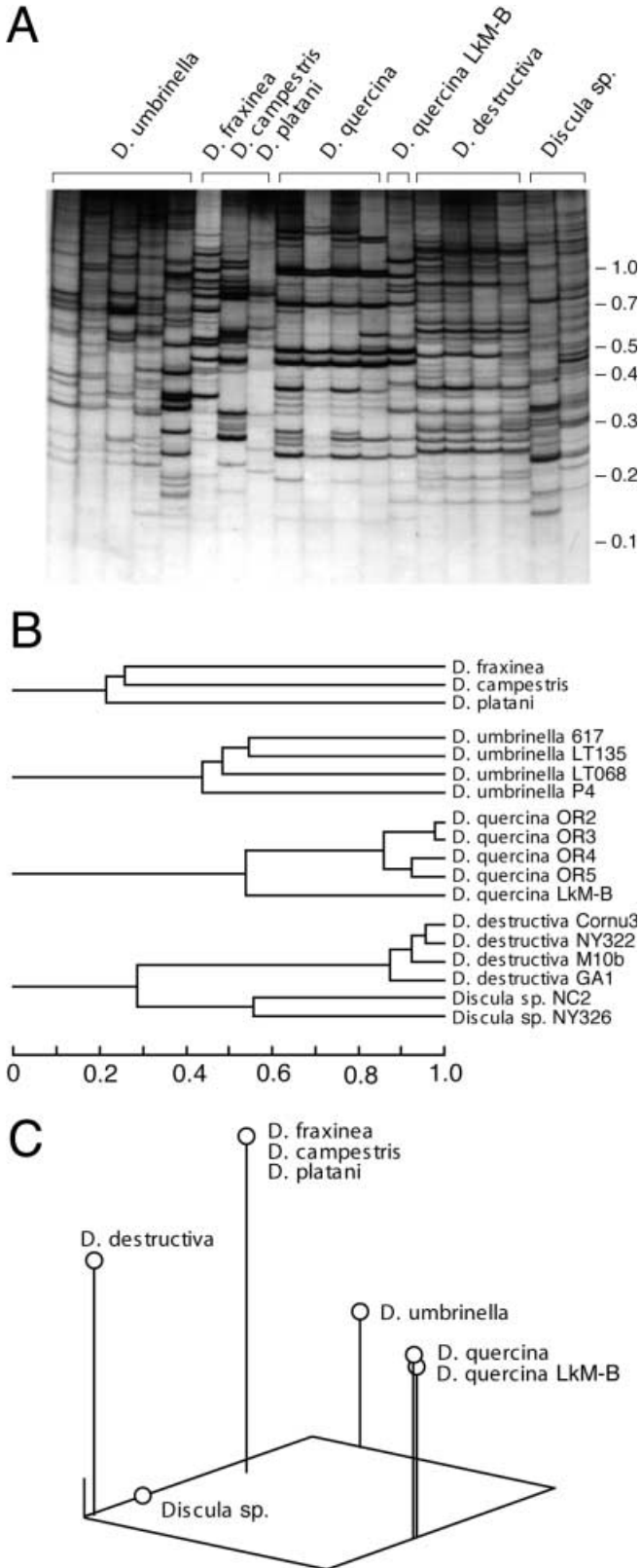


Fig. 2 A–C DNA amplification fingerprinting (DAF) analysis of *Discula* isolates. DAF profiles were obtained with pre-selected octamer primers. DNA fingerprints were generated using primer GAAACGCC from representative isolates (**A**) of *D. umbrinella* 617, LT135, LT068, P4 and 416 (lanes 1–5), *D. fraxinea*, *D. campestris* and *D. platani* TN1 (lanes 6–8), *D. quercina* OR2, OR3, OR4, OR5 and LkM-B (lanes 9–13), *D. destructiva* Cornu3, NY322, M10B and GA1 (lanes 14–17) and *Discula* sp. NC2 and NY326 (lanes 18–19). Molecular markers are indicated in kilobases. Data from individual or bulked fungal isolates representing the different species were subjected to cluster (**B**) or principal coordinate analysis (**C**), respectively. Bulked fungal samples contained equal amounts of DNA from the individual isolates. Dendrogram scales indicate relative genetic similarity of isolates, based on correlation coefficients of cluster groups. Note how Lookout Mountain (LkM) isolates are clearly distantly related to *D. destructiva* but are phenetically close to *D. quercina*

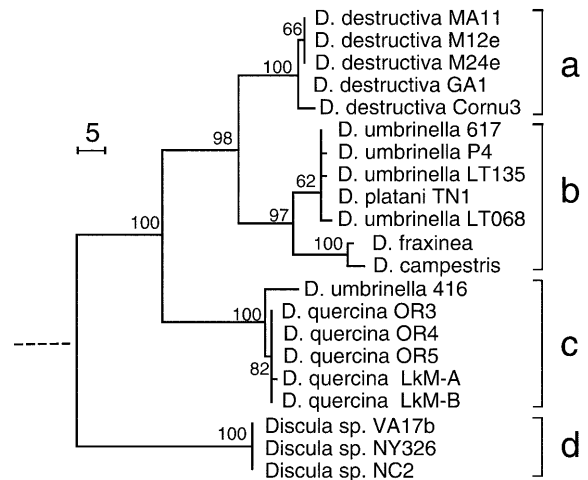


Fig. 3 Phylogenetic analysis of rDNA spacers in anthracnose-causing fungi. Trees were inferred from internal transcribed spacer (ITS) sequence data, using unconstrained maximum parsimony as the optimality criterion in PAUP*. Monophyletic groups (a–d) are indicated in representative phylograms. A branch-and-bound search generated six equally most parsimonious ITS trees of 137 steps [consistency index (CI)=0.869, retention index (RI)=0.957, rescaled consistency index (RC)=0.831; $g_1=-0.876$, $g_2=0.972$; permutation tail probability (PTP) test, $P=0.001$], that differed in the length of two branches in group *b*. Bootstrapping values (in %) are shown for those nodes with bootstraps of over 50%. The unrooted tree is rooted by the mid-point method. Phylogenetic relationships were similarly resolved using neighbor-joining algorithms, quartet puzzling, or the maximum-likelihood criterion in PAUP*

five general, knot-free, structural categories within the 18 *D. destructiva* sequences examined, with minimum free-energies (ΔG°) ranging from -20.9 kcal/mol to -46.3 kcal/mol (37 °C). Structures shared two domains

(II and V; Fig. 5) which were used to constrain recurrent folding resubmissions that forced canonical base-pairing of the 3' end of the 5.8S rRNA with the 5' end of the 28 S rRNA. The resulting secondary structure resembled the experimentally deduced eukaryotic ITS2 model described for *Saccharomyces cerevisiae* (Yeh and Lee 1990). *D. destructiva* ITS2 structures had ΔG° values averaging $-42.5(\pm 1.5)$ kcal/mol. These structures contained all *S. cerevisiae* domains, except for domain VI, which was absent. A number of features were common with the yeast model: (1) non-canonical

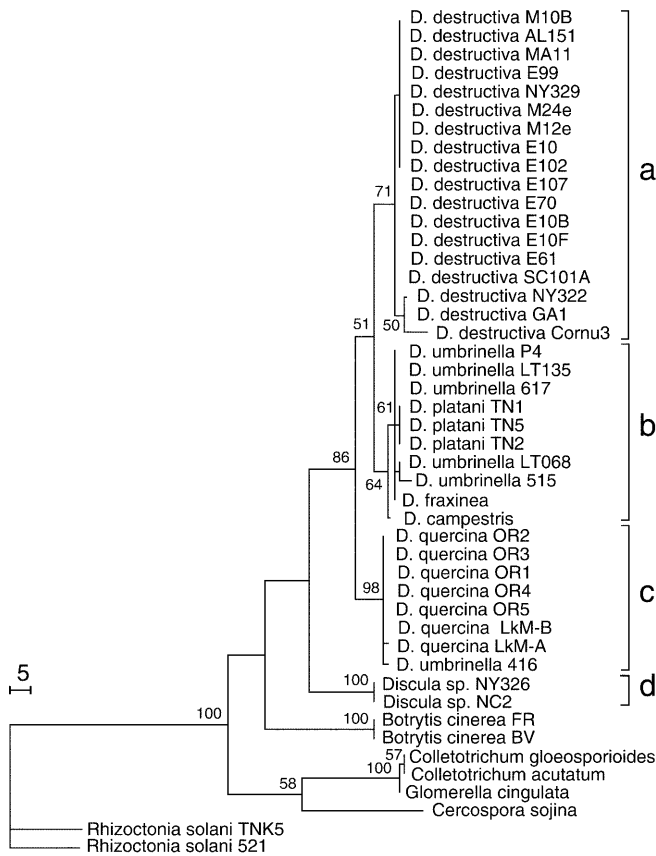


Fig. 4 Phylogenetic analysis of rDNA ITS2 spacers in anthracnose-causing fungi. Trees were inferred from ITS2 sequence data using unconstrained maximum parsimony as the optimality criterion in PAUP*. Using 100 heuristic searches with random addition sequence produced five trees of 276 steps [CI=0.736, RI=0.868, RC=0.638; $g_1 = -1.280$, $g_2 = 2.447$; PTP test, $P = 0.001$] differing in the topologies of group *a* taxa. Unrooted trees were rooted with the outgroup method. *Bootstrapping values* (in %) of 2,000 replications using fast heuristic searches are shown for those nodes with bootstraps of over 50%

base pair at the foot of domain II with conserved sequence tracts, (2) comparable stability of the stem and hairpin loop of domain II ($\Delta G^\circ = -6.2$ kcal/mol), (3) four interior loops and comparable length (19 bp) and stability of domain III ($\Delta G^\circ = -16.6$ kcal/mol), (4) comparable stability ($\Delta G^\circ = -13.2$ kcal/mol) of domain V, despite it being shorter, (5) existence of three interior loops in the stem of domain V, perhaps important for C2 cleavage, and (6) a similar sequence of the hairpin loop in domain V, a feature known to be important for rRNA processing (van der Sande et al. 1992; van Nues et al. 1995). An important difference was a shift of the C1, C2 and C3 processing sites towards domain I. Variable nucleotides and indels were located outside conserved elements crucial for rRNA processing and ribosome biogenesis (Fig. 5). The variability observed within these elements were compensating base pair changes that did not alter stem length or stability.

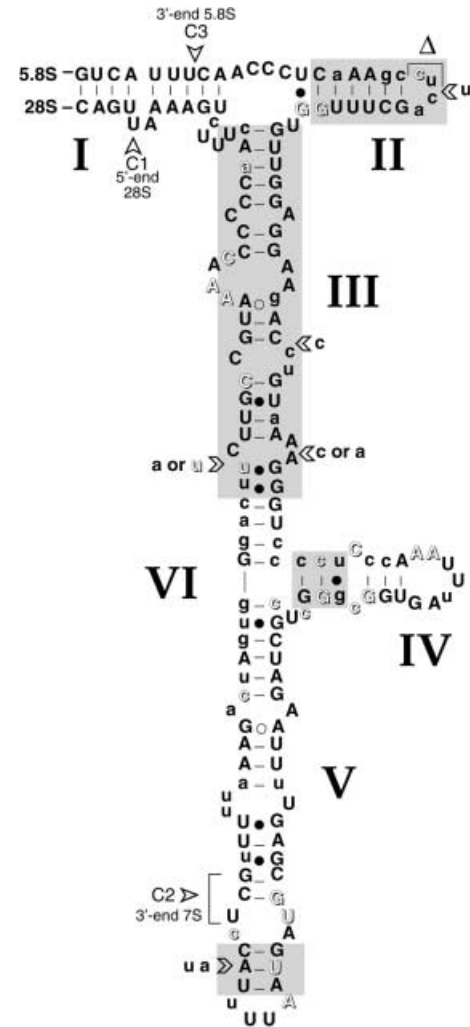


Fig. 5 Secondary structure model for the ITS2 rDNA region of *D. destructiva* M24e. Regions of primary structure conservation are in upper case with outlined nucleotides being those conserved across all fungal species examined. Nucleotides in lower case are variable within dogwood anthracnose-causing isolates, with those outlined or in italics being variable within the *D. destructiva* group or within other *Discula* spp., respectively. Insertions points and a deletion (Δ) are also shown. Structural domains analogous to those of the *Saccharomyces cerevisiae* ITS2 structure (Yeh and Lee 1990) are indicated in roman numerals (note domain VI missing). Putative cleavage sites (C1–C3) for rRNA processing are indicated with arrowheads. Regions homologous to those in *S. cerevisiae* in which structural alterations were found to interfere strongly with rRNA processing (van Nues et al. 1995) are shaded

Genome-wide and spacer rRNA nucleotide substitution levels in *D. destructiva*

Nucleotide genome-wide substitution levels in *D. destructiva* were obtained from published ASAP data (Caetano-Anollés et al. 1996), using a method that estimates the divergence in the sequence of invariant DAF loci (Caetano-Anollés 1999). ASAP is a cascade amplification strategy (Caetano-Anollés and Gresshoff 1996) that can fingerprint individual PCR products or complex DAF profiles, produce allelic signatures from

monomorphic DAF products and provide a measure of their sequence divergence. ASAP polymorphisms were linearly correlated with the divergence in the sequence of amplification products (cf. Caetano-Anollés 1999), providing reliable estimates of genetic distance. ASAP analysis with eight mini-hairpin primers of heptamer-amplified templates (16–24 kb/fingerprint) produced 267 character loci (Caetano-Anollés et al. 1996). Out of these, an average of 37.2 ± 6.8 loci was polymorphic in the pairwise comparison of nine *D. destructiva* isolates. This gave a genome-wide substitution estimate per nucleotide of 0.013 ± 0.002 (mean \pm SD). Note that ASAP primers were not pre-selected and therefore they targeted sites in the DAF profiles at random.

Nucleotide substitution levels in rDNA spacers were obtained directly from the DNA sequence, following Kimura's two-parameter model. When a collection of 18 *D. destructiva* isolates was examined, pairwise nucleotide substitution levels in the ITS2 spacer ranging over 0–0.066 and averaging 0.009 ± 0.001 (mean \pm SD). Only half of the isolates showed nucleotide variation; and these were strains isolated in the last 10 years, mostly from populations in recently colonized areas [South Appalachian mountains, SA-1 and SA-B; North West, NW-2 (cf. Caetano-Anollés et al. 1996)]. In particular, an isolate obtained from a disjunct population of *C. nuttallii* in Idaho (Cornu3) was particularly divergent, exhibiting seven uniquely characteristic nucleotide changes (out of 12) in the ITS2 sequence. In contrast, all *D. destructiva* ITS1 sequences examined were invariant.

Discussion

Nucleic acid-scanning can resolve taxa efficiently at the subspecies level (Caetano-Anollés 1996); and, as such, it has been applied successfully to the study of *Discula* populations (Haemmerli et al. 1992; Trigiano et al. 1995; Caetano-Anollés et al. 1996). DAF analysis with arbitrary primers known to generate high levels of polymorphic DNA in *D. destructiva* confirmed both the high genome-wide diversity of *Discula* sp. (Trigiano et al. 1995; Caetano-Anollés et al. 1996) and *D. umbrinella* (Haemmerli et al. 1992) and the remarkable genetic homogeneity of *D. destructiva* (Trigiano et al. 1995; Caetano-Anollés et al. 1996; Fig. 2). Again, the genetic homogeneity of *D. destructiva* is consistent with the existence of a founder effect (i.e., a genetic bottleneck that has limited the genetic diversity in this species to a restricted number of founder genotypes) and of a restricted gene flow from native to introduced populations proposed earlier (Trigiano et al. 1995; Caetano-Anollés et al. 1996).

Phylogenies were here reconstructed from rRNA ITS spacers, nuclear regions that are often sufficient to delimit species at the molecular level. Phylogenies inferred from the two ITS spacers using distance and character state methods, showed trees fully resolved at the species level, with most internal branches well supported by

bootstrap analysis (Figs. 3, 4). *Discula* spp clustered in four monophyletic groups (a–d), which corresponded to *Discula* sp., *D. quercina*, *D. umbrinella* and *D. destructiva*, in relative order of divergence. These observations reject the null hypothesis of only one evolutionary lineage and support a species concept based on lineages with monophyletic groups representing phylogenetically derived species groups. Generally, these groupings were consistent with host-specificity and somatic compatibility, sometimes fulfilling a cohesion species concept defined by reproductive, ecological and molecular attributes (Harrison 1991; Templeton 1998). The marked divergence of the *D. destructiva* and *Discula* sp. ribosomal lineages rejects the hypothesis that the dogwood pathogen evolved directly from an indigenous fungal population related to *Discula* sp. Instead, *D. destructiva* appears to be an exotic fungus with a recent ancestor somehow related to the diverse *D. umbrinella* group.

Molecular phylogenies also showed conflicting patterns. These were nevertheless expected, as individual loci may not accurately reflect the phylogenetic history of closely related species. Within the diverse *D. umbrinella* lineage (b), three other species (*D. platani*, *D. fraxinea*, *D. campestris*), representing pathogens of American origin (ash, plane tree and sugar maple), grouped with the other fungi originally isolated from European trees (Figs. 3, 4). Of these species, *D. platani* could not be resolved from *D. umbrinella*. Similarly, one *D. umbrinella* isolate from European chestnut (isolate 416) was not resolved from the Oregon white oak pathogen *D. quercina* (Fig. 3). However, this isolate was distantly related to *D. quercina* by DAF analysis (e.g., Fig. 2). Overall, these results are not surprising, since many broadly distributed fungal species are often not resolved by ITS sequence data. For example, ITS sequence analysis in the oyster mushroom (*Pleurotus*) showed that several intersterility groups that defined biological species and were confined to different continents were clustered together (Vilgalys and Sun 1994). In our study, ITS sequences were unable to clearly delimit *Discula* spp defined into lineage groups by host-specificity, when these represented recent divergences [except for the *D. destructiva* lineage (a)]. In this case, DAF profiles, host specificity and vegetative compatibility attributes were better tools for species identification than the ribosomal gene spacers.

Convergent evolutionary mechanisms and non-sexual transfer of genetic elements between separate evolutionary lineages, such as pathogenicity determinants (e.g., islands; Syvanen 1994), can complicate a species phylogeny of the organisms in question and violate a species concept primarily defined by host-specificity. A clear example is that of *D. destructiva* and *Discula* sp., species that are phylogenetically distant (a and d lineages), yet express the same host-specificity. Similarly, strains LkM-A and LkM-B, that were isolated from dogwood anthracnose lesions, shared a common ribosomal origin (Figs. 3, 4) and were related by DAF

analysis (Fig. 2) to *D. quercina*. These two undescribed isolates, originally presumed to be *D. destructiva*, are here tentatively named *D. quercina*, pending confirmation of their pathogenicity in dogwood. Since Koch's postulates have not yet been satisfactorily proven with LkM isolates, convergent evolution or nonsexual horizontal transfer of dogwood anthracnose determinants cannot be invoked at this time to explain their putative host-specificity. In contrast, the low incidence of dogwood anthracnose disease associated with *Discula* sp. could be the result of genetic exchange across phylogenetic species.

We favor the possibility of transfer of genetic elements by cytoplasmically replicating double-stranded RNA (dsRNA) viruses common in fungi, present in *D. destructiva* (McElreath et al. 1994) and usually transmitted through hyphal anastomosis. The presence of these viruses has been causally linked to the hypovirulence of *Cryphonectria parasitica* (Choi and Nuss 1992) and could also constitute important disease factors in *Discula*. The possibility of horizontal transfer of virulence determinants is of great concern for the future control of the dogwood disease in North America and warrants rigorous experimental challenge. In *D. destructiva*, dsRNA can be present in different segments of up to 12 kb in length (McElreath et al. 1994) and short segments may be of different origin in western and eastern isolates (Yao et al. 1997). Unfortunately, a causal relationship between dsRNA and pathogenicity in dogwoods has not yet been established. The possibility of horizontal transfer or hybridization and introgression events leading to changes in host-specificity implicated by the existence of dogwood anthracnose-causing *Discula* sp., *D. quercina* and *D. destructiva* fungi is supported by their compatibility in anastomosis experiments. In this context, natural compatibility barriers could determine which *Discula* lineages could overcome host-specificity.

Phylogenetic analysis disclosed diversity in the sequence of the ITS2 rDNA region of *D. destructiva*, a feature conspicuously absent in ITS1. The diversity encountered in isolates spanning the entire range of dogwood anthracnose on average exceeded that observed in *D. umbrinella* and was mostly restricted to strains recently isolated and from epidemic boundary areas (Fig. 4). ITS2 variants could represent the existence of different alleles from founder events resulting from the separate introduction of the pathogen on the east and west coasts of North America. This possibility, intimated from unique alleles in the western Cornu3 ITS2 sequence, is consistent with a recent ASAP-based study of fine population structure in *D. destructiva* (Caetano-Anollés et al. 1996) and dsRNA fragment analysis (Yao et al. 1997). Alternatively, ITS2 sequence diversity could arise from: (1) the existence of paralogous sequences in the rRNA array, resulting from either natural interspecific hybridization (Brasier et al. 1999) or an escape from concerted evolution (O'Donnell and Cigelnik 1997), (2) extraordinary

mutation events such as the rise of mutator strains (Sniegowski et al. 1997) or rapid co-evolutionary processes in the bottlenecked population (Thompson 1998), or (3) deterministically biased selection processes (Sniegowski and Lenski 1995). A wider and more detailed analysis of the rDNA spacers in *D. destructiva* is therefore needed to accurately trace the origin and diversification of these variant molecules.

Maximum nucleotide substitution levels in the ITS2 region of *D. destructiva* were five times higher than a genome-wide substitution value obtained from published ASAP data, using a procedure that estimates the divergence in arbitrarily amplified loci (Caetano-Anollés 1999). ITS2 is therefore changing at a rate that is comparable (within an order of magnitude) to the rest of the genome. It was therefore of interest to explore how constrained was the evolution of this rDNA spacer. Spacer sequences act as important "scaffolds" in the formation of functional ribosomal subunits (Theissen et al. 1993). In yeast, both sequence and structure of ITS2 are crucial for pre-RNA processing, the formation of fully functional 60S ribosomal units and the regulation of cellular growth (van der Sande et al. 1992; van Nues et al. 1995). We modeled the secondary structure of the ITS2 region in *D. destructiva* (Fig. 5) and found it closely resembled the structure unambiguously resolved in *S. cerevisiae* (Yeh and Lee 1990). Regions of sequence conservation in *Discula* coincided with those in which structural alterations by *in vivo* mutational analysis interfered strongly with yeast rRNA processing and ribosome biogenesis (van der Sande et al. 1992; van Nues et al. 1995). Conversely, entire domains (VI and most of IV) that play no role of consequence in yeast rRNA processing (van Nues et al. 1995) were either missing (domain VI) or were highly variable in *Discula* (Fig. 5). Generally, mutations were restricted to ITS2 regions homologous to those functionally unconstrained, with only a few substitutions in regions known to be important for cellular growth [e.g., V Δ 25 in domain V (van Nues et al. 1995)]. This suggests that neutral or advantageous mutations drifted to fixation, while those that were deleterious were selectively eliminated from this rDNA spacer.

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References

- Boysen M, Borja M, Moral C del, Salazar O, Rubio V (1996) Identification at strain level of *Rhizoctonia solani* AG4 isolates by direct sequence of asymmetric PCR products of the ITS regions. *Curr Genet* 29:174–181

- Brasier CM, Cooke DEL, Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proc Natl Acad Sci USA* 96:5878–5883
- Caetano-Anollés G (1996) Scanning of nucleic acids by in vitro amplification: new developments and applications. *Nature Biotechnol* 14:1668–1674
- Caetano-Anollés G (1998) DAF optimization using Taguchi methods and the effect of thermal cycling parameters on DNA amplification. *Biotechniques* 25:472–480
- Caetano-Anollés G (1999) High genome-wide mutation rates in vegetatively propagated bermudagrass. *Mol Ecol* 8:1211–1222
- Caetano-Anollés G, Gresshoff PM (1996) Generation of sequence signatures from DNA amplification fingerprints with mini-hairpin and microsatellite primers. *Biotechniques* 20:1044–1056
- Caetano-Anollés G, Bassam BJ, Gresshoff PM (1991) DNA amplification fingerprinting using short arbitrary oligonucleotide primers. *Bio/technology* 9:553–557
- Caetano-Anollés G, Trigiano RN, Windham MT (1996) Sequence signatures from DNA amplification fingerprints reveal fine population structure of the dogwood pathogen *Discula destructiva*. *FEMS Microbiol Lett* 145:377–383
- Campbell FT, Schlarbaum SE (1994) Fading forests. North American trees and the threat of exotic pests. (Natural Resources Defense Council Report) Natural Resources Defense Council, Washington, D.C.
- Choi GH, Nuss DL (1992) Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science* 257:800–803
- Daughtrey M (1993) Dogwood anthracnose disease: native fungus or exotic invader? In: McKnight BN (ed) *Biological pollution: the control and impact of invasive exotic species*. Indiana Academy of Science, Indianapolis, pp. 23–33
- Daughtrey ML, Hibbens CR, Britton KO, Windham MT, Redlin SC (1996) Dogwood anthracnose – understanding a disease new to North America. *Plant Dis* 80:349–358
- Felsenstein J (1985a) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Felsenstein J (1995b) Phylogenetic inference package (PHYLIP), version 3.75c. Department of Genetics, University of Washington, Seattle
- Furlong JC, Maden BEH (1983) Patterns of major divergence between the internal transcribed spacers of ribosomal DNA in *Xenopus borealis* and *Xenopus laevis*, and of minimal divergence within ribosomal coding regions. *EMBO J* 2:443–448
- Haemmerli UA, Brändle UE, Petri O, McDermott JM (1992) Differentiation of isolates of *Discula umbrinella* (telomorph: *Apiognomonia errabunda*) from beech, chestnut, and oak using randomly amplified polymorphic DNA markers. *Mol Plant-Microbe Interact* 5:479–483
- Harrison RG (1991) Molecular changes at speciation. *Annu Rev Ecol Syst* 22:281–308
- Hibben CR, Daughtrey ML (1988) Dogwood anthracnose in northeastern United States. *Plant Dis* 72:199–203
- Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453
- Hillis DM, Huelsenbeck JP (1992) Signal, noise, and reliability in molecular phylogenetic analysis. *J Hered* 83:189–195
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Mathews DH, Sabina J, Zuker M, Turner DH (1999) Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. *J Mol Biol* 288:911–940
- McElreath SD, Yao JM, Coker PS, Tainter FH (1994) Double-stranded RNA in isolates of *Discula destructiva* from the eastern United States. *Curr Microbiol* 29:57–60
- Newhouse JR (1990) Chestnut blight. *Sci Am* 263:106–111
- Nues RW van, Rientjes MJM, Sande CAFM van der, Zerp SF, Sluiter C, Venema J, Planta RJ, Raué HA (1994) Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. *Nucleic Acids Res* 22:912–919
- Nues RW van, Rientjes MJM, Morré SA, Molle E, Planta RJ, Venema J, Raué HA (1995) Evolutionary conserved structural elements are critical for processing of internal transcribed spacer 1 from *Saccharomyces cerevisiae* precursor ribosomal RNA. *J Mol Biol* 250:24–36
- O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet Evol* 7:103–116
- Redlin SC (1991) *Discula destructiva* sp. nov., cause of dogwood anthracnose. *Mycologia* 83:633–642
- Rohlf FJ (1992) Numerical taxonomy and multivariate analysis system (NTSYS-pc), version 1.8. Exeter Software, Setauket, N.Y.
- Sande CAFM van der, Kwa M, Nues RW van, Heerikhuizen HJ van, Raué HA, Planta RJ (1992) Functional analysis of the internal transcribed spacer 2 of *Saccharomyces cerevisiae* ribosomal DNA. *J Mol Biol* 223:899–910
- Sniegowski PD, Lenski RE (1995) Mutation and adaptation: the directed mutation controversy in evolutionary perspective. *Annu Rev Ecol Syst* 26:553–578
- Sniegowski PD, Gerrish P, Lenski RE (1997) Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* 387:703–705
- Strimmer K, von Haeseler A (1996) Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol Biol Evol* 13:964–969
- Swofford DL (1999) Phylogenetic analysis using parsimony and other programs (PAUP*), version 4.0. Sinauer Associates, Sunderland, Mass.
- Syvanen M (1994) Horizontal gene transfer: evidence and possible consequences. *Annu Rev Genet* 28:238–261
- Templeton AR (1998) The role of molecular genetics in speciation studies. In: DeSalle R, Schierwater B (eds) *Molecular approaches to ecology and evolution*. Birkhäuser, Basel, pp 131–156
- Theissen G, Thelen L, Wagner R (1993) Some base substitutions in the leader of an *Escherichia coli* ribosomal RNA operon affect the structure and function of ribosomes – evidence for a transient scaffold function of the rRNA leader. *J Mol Biol* 233:203–218
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins H (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Thompson JN (1998) Rapid evolution as an ecological process. *Trends Ecol Evol* 13:329–332
- Trigiano RN, Caetano-Anollés G, Bassam BJ, Windham MT (1995) DNA amplification fingerprinting provides evidence that *Discula destructiva*, the cause of dogwood anthracnose in North America, is an introduced pathogen. *Mycologia* 87:490–500
- Vilgalys R, Sun BL (1994) Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *Proc Natl Acad Sci USA* 91:4599–4603
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand J, Sninsky J, White TJ (eds) *PCR protocols: a guide to methods and applications*, Academic Press, San Diego, pp 315–322
- Windham MT, Erbaugh EK, Montgomery-Dee ME, Trigiano RN (1994) Frequency of *Discula destructiva* Redlin and an undescribed *Discula* species from dogwood tissue. *Phytopathology* 84:778
- Yao J-M, McElreath SD, Tainter FH (1997) Double-stranded RNA in isolates of *Discula destructiva* from the Pacific Northwestern United States and British Columbia, Canada. *Curr Microbiol* 34:67–69
- Yeh L-CC, Lee JC (1990) Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA from *Saccharomyces cerevisiae*. *J Mol Biol* 211:699–712
- Zuker M (1989) On finding all suboptimal foldings of an RNA molecule. *Science* 244:48–52