

The influence of genotypic variation on metabolite diversity in populations of two endophytic fungal species

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Abstract

The relationship between metabolite production and genotypic diversity in two endophytic fungi was investigated. We selected populations of *Cylindrocarpon destructans* and *Heliscus lugdunensis* from the roots of a single tree. A total of 49 isolates of both species were selected and classified by simple genotypic tests (random amplified polymorphic DNA analysis and rDNA-ITS sequencing). In a blind test, the ability of these fungi to produce natural products was tested by ethyl acetate extraction of hyphae and culture filtrates, followed by high-performance liquid chromatography analysis (HPLC). A positive relationship was found between genotype classification and the pattern of natural products produced by a given isolate. To test the robustness of this correlation, a discriminate selection procedure was carried out by collecting fungal isolates from a second site and selecting a sub-set of the population, on the basis of genotypic variability. This sub-set of fungal isolates produced greater numbers of unique metabolites than those selected indiscriminately.

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1. Introduction

Fungi are a well-known source of natural products and are the origin of important pharmaceuticals such as the immunosuppressant cyclosporin A and the statin family of cholesterol-lowering drugs (Dreyfuss and Chapela, 1994; Langley, 1997). The importance of natural products to the pharmaceutical industry is illustrated by the fact that 6 of the top 20 best-selling drugs in 1995 were of fungal origin, and out of 520 new pharmaceuticals approved between 1983 and 1994, 39% were natural products, or were derived from them (Langley, 1997; Wildman, 2003). Natural product screening has

therefore traditionally been an important component of the drug discovery process for many companies. Currently, however, there is less interest among large multinational drug companies in natural product screening. This is due to a variety of reasons including the greater time needed to screen biological material for natural product production, the desire of companies to work with single compounds in assays instead of the mixtures found in biological extracts, and the frequent re-finding of previously identified structures (Wildman, 2003). The availability of large chemical libraries and rapid combinatorial chemistry also provide powerful alternative routes to drug discovery. When considered together, these issues indicate that new strategies will be required in order to make natural product screening from fungi more efficient and cost-effective (Knight et al., 2003).

A fundamental constraint on identifying natural products from fungi is the initial selection process. Each year large numbers of fungal isolates are selected from a

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range of different habitats and geographic locations, in a more-or-less random manner (Dreyfuss and Chapela, 1994; Peláez et al., 1998; Schultz et al., 2002; Wildman, 1995). The blind nature of the selection process is a rate-limiting step in natural product discovery because it is rare that any of the compounds produced by fungi, indiscriminately selected from the natural environment, will show useful biological activities (Biabani and Laatsch, 1998; Dreyfuss and Chapela, 1994; Gloer, 1995; Langley, 1997; Möller et al., 1996; Monaghan et al., 1995; Talbot et al., 1996; Wildman, 1995).

One possible route to identify new secondary metabolites is to screen larger numbers of fungal species from increasingly diverse habitats (Dreyfuss and Chapela, 1994). For example, there are reports of novel secondary metabolites with unexpected biological activities from marine fungi (Biabani and Laatsch, 1998), coprophilous fungi (Dreyfuss and Chapela, 1994), insect-inhabiting fungi (Gloer, 1995) and endophytic species (Dreyfuss and Chapela, 1994; Peláez et al., 1998; Schultz et al., 2002). A second strategy is to use information, such as the use of a particular fungal species in traditional medicine to guide natural product isolation (Young, 1999). A third strategy, which has not yet been thoroughly investigated, is to develop methods to maximise the genetic diversity of organisms that are screened for natural products. One important way in which this could be achieved is to focus on determining genetic and metabolic diversity that exists within an individual species.

In this study we set out to determine whether a simple genotypic analysis could allow us to discriminate the most metabolically diverse individuals from a population of fungi. To do this, we adopted an experimental design in which two previously un-sampled sites were selected and two representative fungal species were isolated from each, to explore their competence to produce novel metabolites. We report the results of experiments designed to test the relationship between the genotype of a fungal strain and its production of secondary metabolites.

2. Materials and methods

2.1. Collection, isolation, and characterisation of fungal isolates

Fungal isolates were collected from living roots of a Common Alder tree (*Alnus glutinosa* [L.] Gaertn.) located either on the bank of the River Dart (UK Ordnance Survey Ref: SX713711: site 1) or the River Teign (Ref: SX8089: site 2), both in Dartmoor National Park, Devon, UK and are stored in the laboratory of N.J. Talbot. Alder roots were selected either from the surface of the river bed, or 1–2 cm below the surface, or from soil at the edge of the river, sufficiently high enough

from the water level to ensure that they had not been subject to periods of prolonged submersion (1–1.5 m). From each habitat, approximately 2.5 m of roots were collected with diameters of 0.1–1.0 cm. Endophytic fungi were isolated from roots as described previously (Fisher et al., 1991). Isolates of *Cylindrocarpon destructans* (Zins.) Scholten and *Heliscus lugdunensis* (Sacc. & Therry) were subsequently identified according to published morphological characteristics (Samuels and Brayford, 1990; Webster, 1959). Fungi were routinely grown at 18 °C on sterile 2% malt extract agar (MEA) (Oxoid, Basingstoke, UK). Two replicates of each original culture were made by isolating single spores. For *C. destructans*, a 1 cm² plug of agar from the edge of each colony was placed in 3 g L⁻¹ sterile (quarter strength) Ringers solution (Oxoid) and conidia suspended by vigorous vortexing for 0.5 min. For *H. lugdunensis*, the same process was followed except that conidial suspensions were incubated for 2–5 days at room temperature. For isolates of each species, a 100 µl aliquot of conidial suspension was transferred to 2% MEA using a sterile glass spreader to allow individual germinating conidia to be identified using a stereo-microscope (Nikon SMZ2T). After 18–24 h at 18 °C, two individual germinating conidia were transferred to 2% MEA using a sterile needle for further analysis.

2.2. Generation of random amplified polymorphic DNA markers

Liquid cultures of both *C. destructans* and *H. lugdunensis* were generated in complete medium (CM), as described previously (Talbot et al., 1993). Genomic DNA extractions were performed using a CTAB (hexadecyltrimethylammonium) procedure (Talbot et al., 1993). RAPD primers used were prepared by the University of British Columbia (Vancouver, Canada). Sequences of the primers used for analysis were UBC 63 (5'-TTCCCCGCCC-3'), UBC 64 (5'-GAGGGCGGGA-3'), UBC 65 (5'-AGGGGCGGGA-3'), UBC 66 (5'-GAGGGCGGGA-3'), UBC 67 (5'-GAGGGCGAGC-3'), UBC 70 (5'-GGGCACGCGA-3'), UBC 71 (5'-GAGGGCGAGG-3'), UBC 72 (5'-GAGCACGGGA-3'), UBC 73 (5'-GGGCACGCGA-3'), UBC 77 (5'-GAGCACCA GG-3'), UBC 78 (5'-GAGCACTAGC-3'), and UBC 79 (5'-GAGCTCGTGT-3'). The site 2 isolates were analysed using primers UBC 63 and UBC 67 (*C. destructans*) and UBC 65 and UBC 66 (*H. lugdunensis*). RAPD reactions were routinely prepared as described by Talbot et al. (1996). Briefly, amplifications were carried out in a Hybaid Omn-E thermal cycler (Hybaid Teddington, UK) using the following cycling conditions: 2 min at 94 °C, 5 cycles of 94 °C for 30 s 36 °C for 30 s, and 72 °C for 120 s, followed by 30 cycles of 94 °C for 20 s, 45 °C for 20 s, and 72 °C for 120 s. This was followed by incubation at 72 °C for 10 min. Reaction products were

fractionated in a 1.6% agarose gel. RAPD fragments selected for analysis were only those reproducibly amplified in replicate single spore re-isolate samples and in three full biological replications of the experiment. The presence or absence of RAPD fragments was recorded as either a '1' or '0' in order to generate a binary matrix which was analysed using Nei and Li's index of Genetic Similarity (Nei and Li, 1979). The similarity matrix was used to generate dendrograms using the unweighted pair-group method of analysis (UPGMA) in the program PAUP*4.0s (Swofford, 2000). A heuristic search was performed with branch swapping (tree bisection-recombination) using a simple addition sequence. Branch strengths were tested by 200 repetitions of the bootstrap algorithm with sequential addition, branch swapping, and tree-branch-reconnection.

2.3. Analysis of the nuclear internal transcribed spacer regions of the ribosomal RNA-encoding gene unit

Amplification of the ITS 1 region of the rRNA-encoding gene unit was performed as described by Sreenivasiprasad et al. (1996). Primers used were provided by Genosys (Cambridge, UK) and were based on standard primers for fungal phylogenetic analysis (White et al., 1990). Sequences of the primers used were ITS 1ext (5'-GTAACAAGGTTTCCGTAGGTG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCRs were performed as described previously (Seifert et al., 1995; Sreenivasiprasad et al., 1996). Reaction products were fractionated in 1.6% agarose and gel-purified using GENECLEAN II, (BIO 101, Palo Alto, CA) according to manufacturers' instructions. The ITS 1 region was sequenced using the primers ITS1 (5'-TCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White et al., 1990). DNA sequence analysis was performed by fluorescent dye terminator cycle sequencing (Amersham-Pharmacia, Bucks, UK) using an ABI 377 automated sequencer (Applied Biosystems, Cheshire, UK). Double stranded DNA sequences were aligned using DNA Strider 1.1 (Marck, 1989) and significant similarities determined using the gapped Blast 2.0 program (Altschul et al., 1997). Multiple sequence alignments were performed using CLUSTALW program (Thompson et al., 1994). Phylogenetic analysis was performed by maximum parsimony in the program PAUP*4.0s (Swofford, 2000). The consistency of phylogenetic trees was determined using the heuristic search program and branch strengths were tested by 200 repetitions of the bootstrap algorithm with branch swapping and tree-branch-reconnection.

2.4. Analysis of secondary metabolic variability

Metabolite profiles were produced by HPLC analysis of ethyl acetate extracts made both from fungal myce-

lium and from culture supernatants. Each fungal isolate was inoculated in triplicate into 25 ml (pH 6.0) dextrose broth (20 g L⁻¹ dextrose, 7.5 g L⁻¹ soya protein, 1 g L⁻¹ yeast extract, 0.5 g L⁻¹ KH₂PO₄, 0.05 g L⁻¹ MgSO₄·7H₂O, 0.02 g L⁻¹ CaCl₂·2H₂O, 0.01 g L⁻¹ NaCl, 0.1% (vol/vol) trace elements [6 g L⁻¹ NaNO₃, 0.5 g L⁻¹ KCl, 0.5 g L⁻¹ MgSO₄, 1.5 g L⁻¹ KH₂PO₄ (pH 6.5), and 1 g L⁻¹ agar] in 125 ml baffled shake flasks (BDH, Dorset, UK) containing 2 g of 0.5 mm diameter glass beads (Biospec, Bartlesville, USA) using a disposable inoculating loop (Technical Service Consultants, Heywood, Lancs, UK) for 3 days at 23 °C and 90 rpm. A 1.5 ml aliquot of each liquid culture was then used to inoculate 25 ml (pH 6.5) glucose medium (10 g L⁻¹ glucose, 2 g L⁻¹ yeast extract) in a fresh 125 ml baffled shake flask. Cultures were incubated for 7 days at 23 °C and 90 rpm, by which time the fungi had entered stationary phase. Liquid culture was removed into 50 ml Falcon tubes and processed by centrifugation at 10,000g for 20 min and the supernatant removed to a fresh 50 ml Falcon tube. To the remaining mycelium, 15 ml ethyl acetate was added, and to the supernatant 2 ml ethyl acetate was added, and the tubes were placed on a rotating platform for 1 h. After centrifugation at 10,000g for 20 min, the top layer was recovered and placed into a 15 ml Falcon tube, and dried under vacuum (Eppendorf Speed-Vac 5301). A 1.5 ml aliquot of methanol (Sigma) was added to culture filtrate extracts, and a 0.2 ml aliquot of methanol added to mycelial extracts. Three individual 100 µl aliquots were removed from each extract and placed in 0.2 ml reactivials (Sigma) before loading by auto-injection. HPLC utilized a Waters 2690 with Photodiode Array Detector 996 (Waters GmbH, Eschborn, Germany) and analysed using *Millennium 32*; Version 3.1. A Symmetry C18 column (2.1 × 150 mm for site 1 isolates, 2.1 × 50 mm for site 2 isolates) with 5 µm octadecyl silane particles was used (Waters GmbH, Eschborn, Germany). The pressure in the column ranged between 2000 and 2500 psi and the flow rate in was 0.6 ml min⁻¹. The range of absorbance was 200–700 nm (in 1.2 nm increment steps). In all cases the column was eluted with 0.1% acetonitrile (vol/vol) HPLC-grade water (Sigma), adjusted to pH 3.8 using 0.1% phosphoric acid (Sigma). Standards were a mixture of 1 mM acetophenone:propiophenone:valerophenone (1:1:1) (Sigma). The retention times of standards were recorded as 6.9, 8.6, and 11.2 min, respectively. Only peaks with a retention time between 4 and 15 min (site 1) and 1.5 and 7.5 min (site 2) were recorded. In the analysis of isolates from site 1, for the *C. destructans* mycelial-extract chromatograms, the threshold for peak identification was set at 0.001 absorbance units (AU). For the analysis of the *C. destructans* filtrate-extract chromatograms, the threshold was set at 0.002 AU. For *H. lugdunensis* mycelial extract chromatograms, the threshold was set at 0.01 AU. For *H. lugdunensis* filtrate

extract chromatograms, the threshold was set at 0.002 AU. In analyses of site 2 isolates, for *C. destructans*, the threshold was set at 0.015 AU for the mycelial extracts and 0.001 AU for culture filtrate-extract chromatograms. For *H. lugdunensis*, the threshold was set at 0.015 AU for mycelial extracts and 0.002 AU for culture filtrate-extract chromatograms. Peaks were recorded as 1 (present) or 0 (absent). The raw HPLC data and binary matrices derived from them are available (Seymour, 2000).

To determine whether individual metabolite peaks were likely to correspond to single metabolites, the UV spectrum along a peak was examined. Peaks from extracts of the same species which showed identical retention times and identical UV spectra along the peak (peak purity), were assumed to represent the same metabolite. Major peaks that consisted of more than one metabolite were excluded from further analysis. For the site 1 isolates, the secondary metabolite data sets were analysed by UPGMA to generate dendrograms using a Dollo function in which the probability of a forward change (gain of a metabolite peak) was weighted as a rare event, compared to the probability of a reversal (loss of metabolite peak), using the program PAUP*4.0s (Swofford, 2000). The consistency of phylogenetic trees was determined using the heuristic search program and branch strengths were tested by 200 repetitions of the bootstrap algorithm with branch swapping and tree-branch-reconnection. Further analysis of this data set is available (Seymour, 2000).

The difference between the proportions of isolates producing unique metabolites in each sampled fungal population was statistically assessed by using the expression:

$$d = \frac{k_1 - k_2}{\sqrt{\left[k(1-k) \left(\frac{1}{n_1} + \frac{1}{n_2} \right) \right]}}$$

where $k_1 = a_1/n_1$, a_1 is the number of isolates producing unique metabolites (a metabolite produced only by a given fungal individual) in site₁ and n_1 is the population of sampled fungi in site₁, $k_2 = a_2/n_2$ representing the same measurements for a second sampled population of fungi and $k = (a_1 + a_2)/(n_1 + n_2)$ (Bailey, 1995). The null hypothesis is that the two populations share equal numbers of isolates producing unique metabolites.

3. Results

3.1. Experimental design and selection of root-endophytic fungi

The main aim of this study was to investigate the relationship between genotypic variation and metabolic diversity in populations of endophytic fungi isolated

from the natural environment. The experimental design we adopted was to select a site and carry out an indiscriminate selection process in which isolates of the most prevalent fungal species were collected and genotypically classified. In a blind test, the ability of these isolates to produce metabolites production was then assessed and compared to the genotypic classifications. For isolation of fungi we selected a previously unsampled habitat—a single common Alder tree (*A. glutinosa*) on the banks of a river (see methods for location)—and isolated roots taken either from free-flowing water in the river or from soil on the river bank. From these root samples, endophytic fungi were isolated and cultured. The two most commonly identified species were *H. lugdunensis* and *C. destructans*, both of which are highly pigmented (orange) fungi present throughout root material. The aquatic hyphomycete *H. lugdunensis* was the predominant coloniser of roots from the riverbed (which we termed ‘aquatic’ roots), while *C. destructans* was among the commonest fungal species in root samples from soil (‘terrestrial’ roots). The two species are related and their sexual stages have been classified as *Nectria* species, *Nectria lugdunensis* and *Nectria radicola*, respectively (Samuels and Brayford, 1990; Webster, 1959). In total we collected 24 isolates of *H. lugdunensis* and 26 isolates of *C. destructans* (Table 1). Two re-isolations of each individual were prepared from single conidia to provide biological replicates for subsequent analysis.

3.2. Genetic diversity of *H. lugdunensis* and *C. destructans* populations

To assess genotype variation of the endophytic fungi, RAPD analysis was carried out using 10 random primers. Each primer typically gave 10–15 reproducibly amplified fragments in RAPD-PCR analysis of *C. destructans* or *H. lugdunensis* as shown in Fig. 1. This allowed the presence or absence of 114 amplicons to be used to compare *C. destructans* isolates, and 152 fragments for analysis of *H. lugdunensis*.

Cluster analysis allowed resolution of four putative *C. destructans* RAPD-groups that broadly distinguished two genotype groups (II and IV) among the isolates collected from terrestrial roots and two genotype groups composed of isolates collected from aquatic roots (I and III). A dendrogram of these relationships is given in Fig. 2A. RAPD analysis of *H. lugdunensis* revealed three putative groups (Fig. 2B). Quality assurance of RAPD (a technique where low annealing temperatures in the PCR can sometimes lead to spurious and sometimes non-reproducible amplification products) was carried out by routinely performing analysis on both mono-conidial re-isolates of each individual and demonstrating amplification profiles at least three times with uniform results. Mono-conidial re-isolate pairs normally

Table 1

Isolates of *C. destructans* and *H. lugdunensis* collected from roots of *A. glutinosa*, located at the edge of the River Dart in the Dartmoor National Park (site 1)

Isolated from	Species					
	<i>C. destructans</i>			<i>H. lugdunensis</i>		
Aquatic roots	CDA 10 ^a	CDA 75	CDA 181	HDA 10 ^b	HDA 56	HDA 100
	CDA 35	CDA 92	CDA 200	HDA 14	HDA 74	HDA 112
	CDA 46	CDA 159	CDA 204	HDA 20	HDA 87	HDA 144
	CDA 63	CDA 174	CDA 241	HDA 22	HDA 89	HDA 234
Terrestrial roots	CDT 34	CDT 134	CDT 190	HDT 49	HDT 138	HDT 199
	CDT 86	CDT 147	CDT 200	HDT 55	HDT 143	HDT 218
	CDT 115	CDT 159A	CDT 202	HDT 94	HDT 190	HDT 238A
	CDT 119A	CDT 159B	CDT 237	HDT 120	HDT 191	HDT 238B

^a‘CDA’ and ‘CDT’ refer to: *C. destructans* isolates collected from aquatic and terrestrial roots from the River Dart, respectively.

^b‘HDA’ and ‘HDT’ refer to: *H. lugdunensis* isolates collected from aquatic and terrestrial roots from the River Dart, respectively.

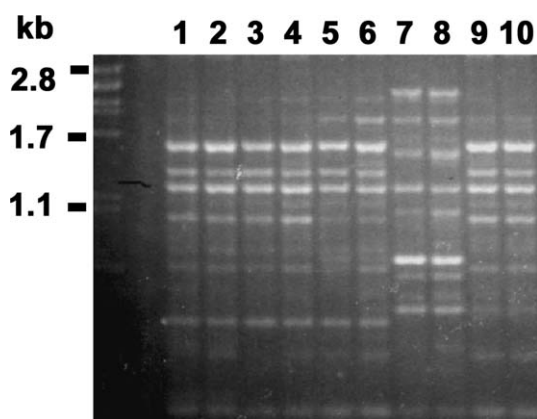


Fig. 1. Examples of RAPD profiles obtained for a selection of *H. lugdunensis* isolates generated using primer UBC63. Each lane shows the RAPD profile generated from genomic DNA of a single spore re-isolate of a given *H. lugdunensis* individual isolates from Alder tree roots. Lane 1, HDA10-1; lane 2, HDA10-2; lane 3, HDA14-1; lane 4, HDA14-2; lane 5, HDA20-1; lane 6, HDA20-2; lane 7, HDA22-1; lane 8, HDA22-2; lane 9, HDA56-1; and lane 10, HDA56-2. A size marker is shown on the left. kb, kilobase pairs.

generate identical RAPD profiles (Fig. 1) and therefore clustered closely with each other (Fig. 2). The only exceptions were the mono-conidial re-isolate pairs CDA159-1 and CDA159-2, and HDA14-1 and HDA14-2, although they were all found within the same RAPD group (Fig. 2). Analysis of the data sets presented here using maximum parsimony analysis, Dollo parsimony, and using the neighbour-joining method in PAUP*4.0s were also carried out and produced broadly similar groupings of isolates (Seymour, 2000).

The ITS1 region of the ribosomal RNA-encoding gene unit was sequenced from all isolates of *C. destructans* and *H. lugdunensis* to determine the extent of genetic variability among the isolates and to confirm that each isolate belonged to the predicted species, because all of the isolates were selected solely on the basis of morphological characters (O'Donnell and Cigelnik, 1997; Seifert et al., 1995). Four ITS1 alleles were identified within the population of *C. destructans* isolates

(Table 2) and a single ITS1 allele was present in all *H. lugdunensis* isolates. A dendrogram provided evidence that the species identified grouped in the expected manner among *Nectria* species (Fig. 3).

3.3. Natural product isolation from *H. lugdunensis* and *C. destructans* populations

To determine the variation in metabolite production from the sampled populations of *H. lugdunensis* and *C. destructans*, ethyl acetate extracts were made from mycelium and culture filtrates of each re-isolate. In a blind test, HPLC analysis was then used to generate metabolite profiles. For the purposes of our analysis we selected only major metabolite peaks that showed identical retention times and uniform UV spectra (peak purity) in extracts of both monoconidial re-isolates of a given individual. Examples of the metabolite profiles generated from extracts of two isolates of *H. lugdunensis* are given in Fig. 4. Metabolite extractions and HPLC analysis were repeated three times for each fungal isolate and a total of 26 secondary metabolite peaks was then recorded from the combined mycelial extract and the culture filtrate extract profiles for each species, and designated A–Z (Fig. 5). We termed any metabolite that was unique to a particular isolate, or small sub-set of isolates, as ‘novel.’ A binary presence/absence metabolite matrix was produced from this information for re-isolates of *C. destructans* and *H. lugdunensis*, which was used to produce a dendrogram using UPGMA (Fig. 5A). Three clusters were predicted within the *C. destructans* population (isolate CDA 75 could not be classified). The *H. lugdunensis* isolates were classified into two putative clusters (Fig. 5B).

3.4. Comparative analysis of genotypic relationships with secondary metabolite production

In order to determine whether a correlation could be detected between the genotype of a fungal strain and its

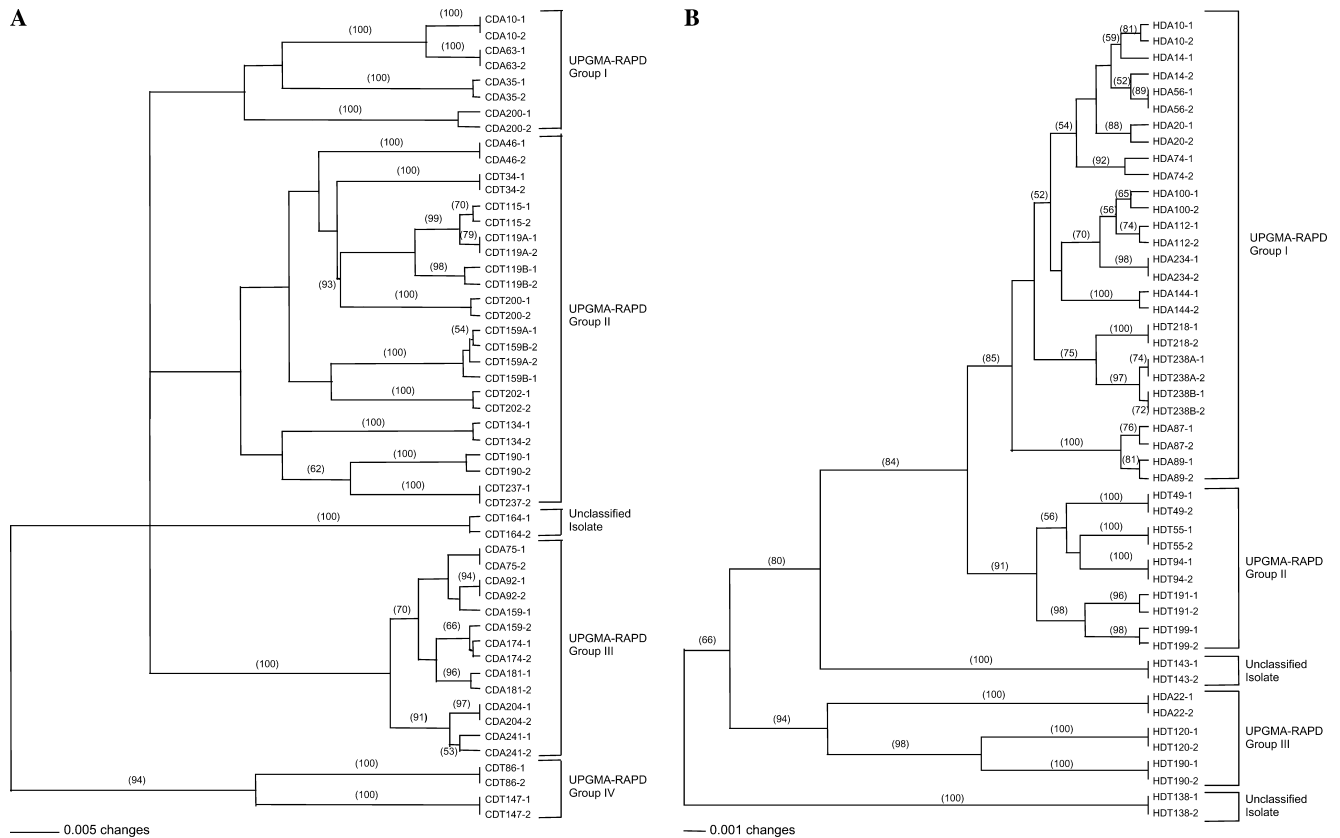


Fig. 2. Dendrograms derived from RAPD analysis of endophytic fungal isolates collected from roots of a single Alder tree (*A. glutinosa*) at site 1. The distance scale is given in terms of the number of changes. Numbers in parenthesis indicate the number of times (%) that groups of taxa were obtained in 200 bootstrap replications. Inferred UPGMA-RAPD groupings are shown on the right. (A) Dendrogram of *C. destructans* isolates. (B) Dendrogram of *H. lugdunensis* isolates.

Table 2
Classification of *C. destructans* and *H. lugdunensis* isolates from site 1 based on ITS1 sequence analysis

<i>C. destructans</i>						<i>H. lugdunensis</i>
Cd-1 ^a	Cd-2	Cd-3	Cd-4			HI-1 ^b
CDA 10	CDT 119A	CDA 75	CDA 181	CDT 134	CDT 86	There were no differences observed between isolates of <i>H. lugdunensis</i>
CDA 35	CDT 119B	CDA 92	CDA 200	CDT 190	CDT 147	
CDA 46	CDT 159A	CDA 159	CDA 204	CDT 237	CDT 164	
CDA 63	CDT 159B	CDA 174	CDA 241			
CDT 34	CDT 200					
CDT 115	CDT 202					

^a *Cylindrocarpon destructans* isolates showed four ITS1 allele types, Cd-1– Cd-4.

^b All *H. lugdunensis* isolates shared a common ITS1 allele.

ability to produce particular metabolites (its ‘chemotype’), a comparison was made between the RAPD-groups and metabolite clusters generated for each fungal species. We tabulated the data to identify fungal isolates that were related in genotype and chemotype, based on our analysis (Table 3). All of the *C. destructans* isolates within RAPD groups I and IV, for example, and the unclassified isolate CDT 164, were found to be contained within single secondary metabolite clusters. Similarly, the majority of isolates within the *C. de-*

structans RAPD groups II and III were found within single clusters (cluster 2 and cluster 3, respectively). Furthermore, isolates CDA 10 (RAPD group I, metabolite cluster 1) and isolate CDA 75 (RAPD group III) were found to produce secondary metabolite peaks that were unique to that RAPD group (metabolites ‘Z’ and ‘X,’ respectively) (Table 3A).

There was also a strong association between the predicted genotype groups and metabolite clusters predicted for the *H. lugdunensis* population (Table 3).



Fig. 3. Phylogram of the most parsimonious tree based on ITS 1 sequence analysis showing the relationship of isolates from site 1 to a number of related fungal species (identified by species name and GenBank Accession number). *C. destructans* isolates were of four ITS 1 allele types and *H. lugdunensis* all had a common ITS 1 allele. Numbers in parenthesis represent the percentage bootstrap value. Lengths of branches are shown. Gaps were treated as missing data. The tree was rooted using the midpoint method (Swofford, 2000). ITS sequences from *C. destructans* and *H. lugdunensis* have GenBank Accession Nos. AY039219–AY039223.

Exceptions did occur, however, such as isolates from RAPD group I, which were found within both metabolite clusters 1 and 2. The majority of these isolates produced between one and three secondary metabolites that were specific to a particular RAPD group (metabolites H, S, and W).

Our independent assessment of genotypic relatedness and secondary metabolite production provided evidence that fungal isolates, which were closely related genotypically, were more likely to produce similar metabolite profiles (chemotypes) than those that were not. There were, however, important limitations to our analysis. Novel metabolites identified in the analyses, for example, were only observed within 3 of the 7 RAPD groups resolved from the entire population of fungi examined. RAPD group I of *H. lugdunensis* proved to be particularly rich in production of novel metabolites and yet showed a large amount of variation with some isolates producing none of the novel metabolites produced by others. We also noted that in a total sample of 49 fungal isolates we only found two completely unique metabolites, which we defined as those produced by a single fungal isolate. Based on the observed positive (albeit

complex) relationship between metabolite production and genotypic relatedness, we decided to test whether selecting fungal strains based solely on their genotype would lead to identification of significantly more secondary metabolites than in an indiscriminate screen.

3.5. Discriminate selection of fungal isolates for secondary metabolite screening based on genotypic diversity

To test the relationship between metabolite production and genetic relatedness directly, we identified a second site 30 km from the initial collection point (see methods). Fungal isolates were collected from roots and monoconidial re-isolates were generated as before (see methods). Data are presented for individual fungal isolates only, although re-isolate pairs gave uniform results. A total of 98 *C. destructans* isolates were collected from aquatic roots, and 85 isolates from terrestrial roots. The isolates were designated CTA1-98 or CTT1-85 (*Cylin-drocarpon* Teign Aquatic or Terrestrial, respectively). RAPD profiles were generated for all isolates using 2 primers that had proved to be highly polymorphic from the initial screen. A visual examination of the RAPD profiles was then used to select a subset of 14 isolates that displayed distinctly different profiles from the remaining 169 isolates (see Fig. 6). For *H. lugdunensis* a total of 55 isolates were collected from aquatic roots and 2 isolates from terrestrial roots. These were designated HTA1-55 or HTT1-2 (*Heliscus* Teign Aquatic or Terrestrial, respectively). RAPD analysis was carried out using two primers and 10 out of 57 *H. lugdunensis* isolates were selected for further analysis (Table 4). Dendrograms were produced for the selected isolates of *C. destructans* and *H. lugdunensis* based on RAPD analysis with two primers (Fig. 6), although visual analysis had shown these were likely to be the most diverse individuals and therefore tree topologies showed relatively little bootstrap support. For the *C. destructans* isolates selected we found five putative RAPD-groups, one containing all of the four aquatic isolates (RAPD-Teign Group 1), and the remaining groups containing the terrestrial isolates (Fig. 7). Cluster analysis of the *H. lugdunensis* isolates revealed three putative RAPD-groups (Fig. 7).

In order to determine whether a greater number of novel secondary metabolites would be obtained based on a discriminate selection of fungal isolates, the novel secondary metabolite profiles produced by isolates of *C. destructans* and *H. lugdunensis* in each RAPD group were recorded (Fig. 7). A total of 60 metabolites were identified from the combined mycelium and culture filtrate HPLC data for the selected isolates of *C. destruc-tans* (numbered 1–60). In total the discriminate selection of 14 isolates yielded 16 ‘novel’ metabolites (found in single, or a sub-set of isolates) from the 60 metabolite peaks analysed. In analysis of *H. lugdunensis* a total of

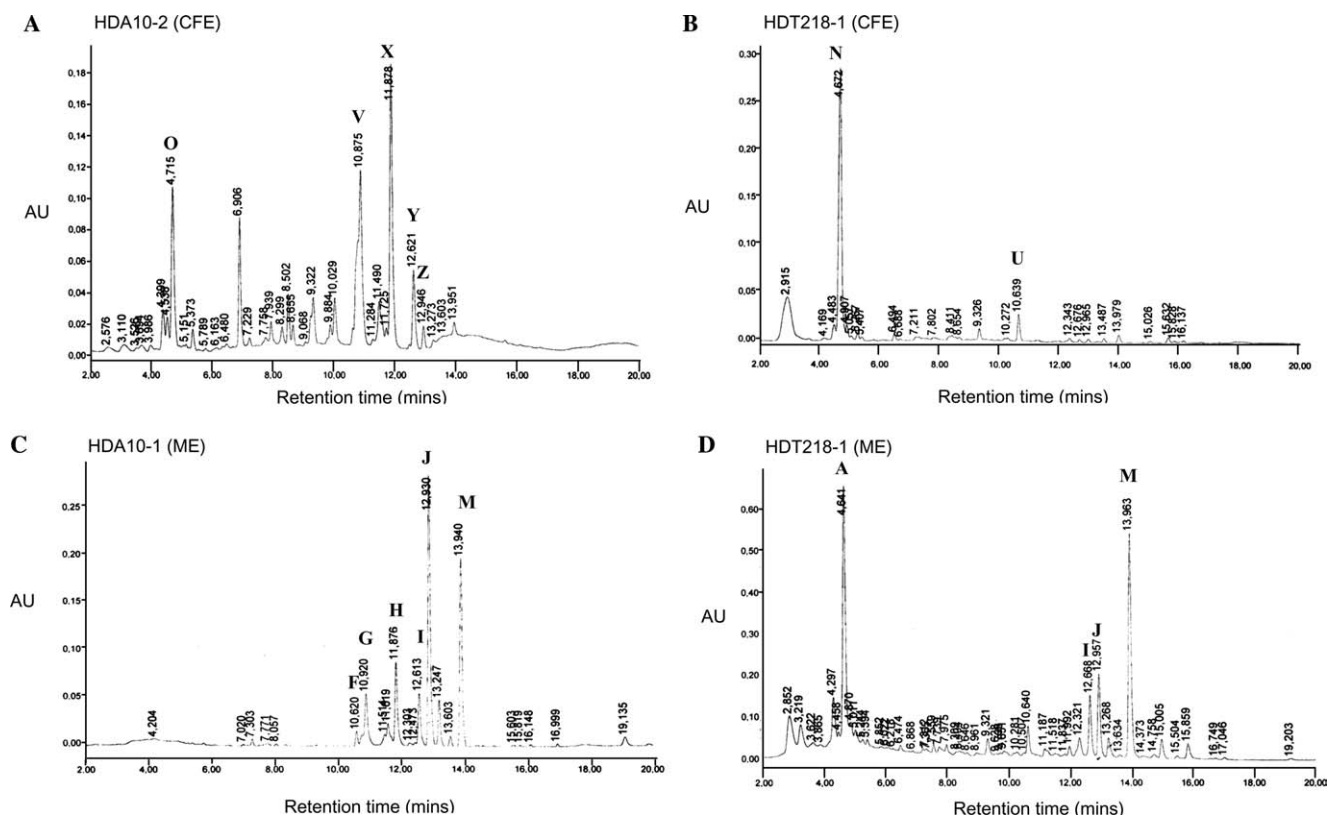


Fig. 4. Examples of secondary metabolite profiles from isolates of *H. lugdunensis* from roots of a single Common Alder tree (*A. glutinosa*) at site 1. Fungi were grown to stationary phase in yeast extract-glucose medium for 7 days at 23 °C. Ethyl acetate/methanol extracts were made from culture filtrates (CFE) or mycelium (ME), metabolites separated by HPLC, and detected using a photodiode array detector. Absorbance units (AU) are shown on the y-axis and retention time on the x-axis. The experiment was repeated three times and the average peak for a given retention time was calculated and used for metabolite classifications. Correlation of major peaks with a selection of the metabolite assignments presented in Fig. 5, are shown.

41 metabolites was resolved from for the combined mycelium and culture filtrate extracts. The discriminate screen led to identification of 6 novel metabolites from the 10 isolates selected (Fig. 7).

The metabolic diversity of the fungal isolates selected from site 2 using a discriminate selection procedure could be measured by determining the number of unique metabolites produced. These are 'novel' metabolites that are produced by a single fungal individual. The 24 fungal isolates selected thus yielded 22 unique secondary metabolites. This compares to the 2 unique metabolites produced from the 49 fungal isolates selected indiscriminately from the river Dart site. The discriminately sampled population contained a greater proportion of isolates that produced unique metabolites ($d = 7.49$, $p < 0.01$) (Bailey, 1995). However, this measurement is not completely valid because of the difference in size of the two sampled populations; it is more likely by chance that a smaller population will contain individuals, which produce metabolites that are not produced by others in the population. To correct for this, the statistical test was carried out by comparing the 24 individuals in the discriminate sample with 24 individuals that were ran-

domly selected from the larger indiscriminately selected population. Again, the evidence suggested that the discriminately sampled population contained a greater proportion of isolates with unique metabolites ($d = 6.07$, $P < 0.01$). Three repetitions of this selection procedure gave results of $d = 6.4$, $d = 5.78$, $d = 6.4$ ($p < 0.01$ in each case). We conclude that the selected population of fungal isolates chosen based on their genotypic diversity produced more novel metabolites than if we had selected a similar number of isolates indiscriminately.

4. Discussion

Identification of secondary metabolites from fungi is normally carried out in a random, speculative manner in which fungi are sampled from diverse environments, simple extractions made to detect low molecular weight compounds, and assays developed to select for interesting biological activities (Dreyfuss and Chapela, 1994; Talbot et al., 1996). This study sought to investigate the relationship between genetic variation and metabolic

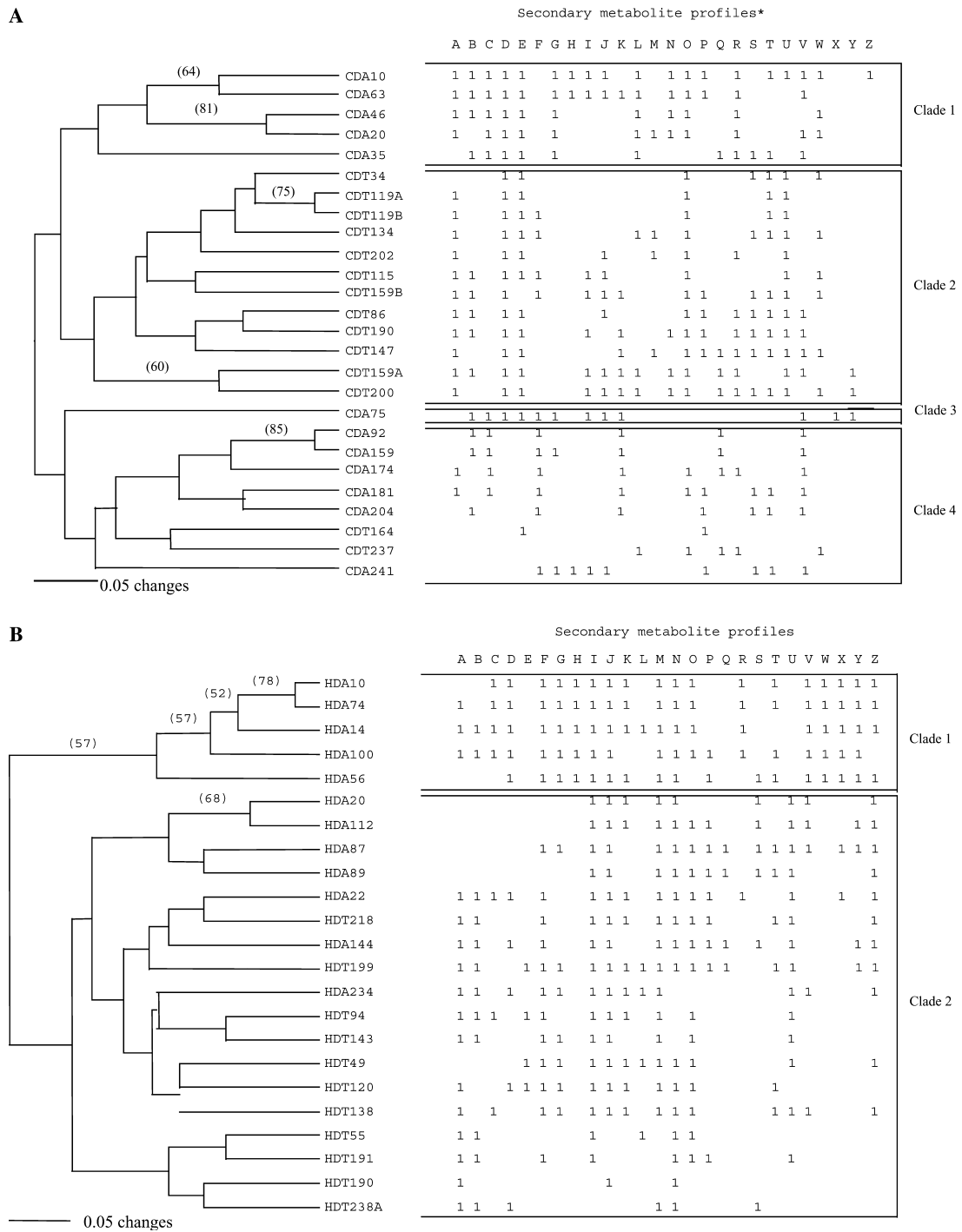


Fig. 5. Dendrograms produced by UPGMA showing clustering of fungal isolates based on the presence or absence of secondary metabolites extracted from mycelium and culture filtrates of (A) *C. destructans* and (B) *H. lugdunensis* isolated from roots of a single Common Alder tree (*A. glutinosa*) at site 1. The presence of the 26 secondary metabolite peaks scored are shown as '1.' The absence of metabolite peaks is signified by a blank space.

diversity in two endophytic fungal species. The production of secondary metabolites by fungi is regulated developmentally, in response to nutrient availability, or as a result of changes in the environment, and therefore is likely to be closely associated with the particular habitat occupied by a fungus and its stage of develop-

ment. We reasoned that by carrying out genotype analysis we might be able to pre-select the most diverse individuals from a particular habitat, thus allowing fewer fungal isolates to be used for metabolite profiling. This would allow a much more detailed analysis to be carried out on a smaller number of fungal isolates,

Table 3

Comparative analysis between RAPD groups and metabolite clades for isolates of (A) *C. destructans* and (B) *H. lugdunensis* using Dollo UPGMA on metabolite data matrices

(A) <i>C. destructans</i>					(B) <i>H. lugdunensis</i>		
RAPD Groups	Metabolite groups ^a				RAPD Groups	Metabolite groups ^a	
	Clade 1	Clade 2	Clade 3	Clade 4		Clade 1	Clade 2
Group I	CDA10 ^Z CDA35 CDA63 CDA200				Group I	HDA10 ^{H, W} HDA14 ^{H, S} HDA56 ^{H, S, W} HDA74 ^{H, W} HDA100 ^{H, W}	HDA20 ^S HDA87 ^S HDA89 ^S HDA112 ^S HDA144 ^S HDA234 HDT218 HDT238A ^S
Group II	CDA46	CDT 34 CDT 115 CDT 119A CDT 119B CDT 134 CDT 159A CDT 159B CDT 190 CDT 200 CDT 202		CDT 237	Group II		HDT49 HDT55 HDT94 HDT191 HDT199
Group III		CDT 164					HDA22 HDA120 HDT138 HDT143 HDT190
Group IV		CDT 86 CDT 147					
Group V			CDA75 ^X	CDA92 CDA159 CDA174 CDA181 CDA204 CDA241			

^a Letters appearing after isolate names as superscripts, refer to secondary metabolites produced only by isolates classified in a particular genotype group.

utilizing different growth conditions, substrates, and different developmental stages in metabolite profiling studies.

In carrying out a study of this kind it was soon apparent that we were investigating a complex set of variables including the geographic environment a fungal isolate was collected from, its local habitat (terrestrial or aquatic roots), the individual species concerned, its genotype variation, and its capacity to produce diverse secondary metabolites. The experimental design we adopted utilized two measures of genotype variation (RAPDs and ITS sequencing), and examined two species from two geographic sites. To assess metabolite production we used the presence of metabolite peaks in an HPLC profile as a simple measure of diversity. Such a measure has clear limitations, but was picked because it is normally the first form of evaluation of metabolite

production during selection of natural products. We reasoned that the presence or absence of metabolite peaks of identical retention time and uniform UV spectra from isolates of the same fungal species, would also provide a reasonable measure of the metabolite diversity exhibited within a fungal population. Although there are limitations in this experimental design, because of the number of geographic sites selected, and relatively small number of fungal isolates examined, the data generated have provided some evidence to support the use of genotype analysis as a means of reducing the genetic and chemical redundancy associated with natural product screening (Knight et al., 2003).

The first observation we made during the study was that very large numbers of fungal individuals occupy habitats such as tree roots. The roots taken from a single *Alnus* tree in this study yielded 68 genera of fungi that

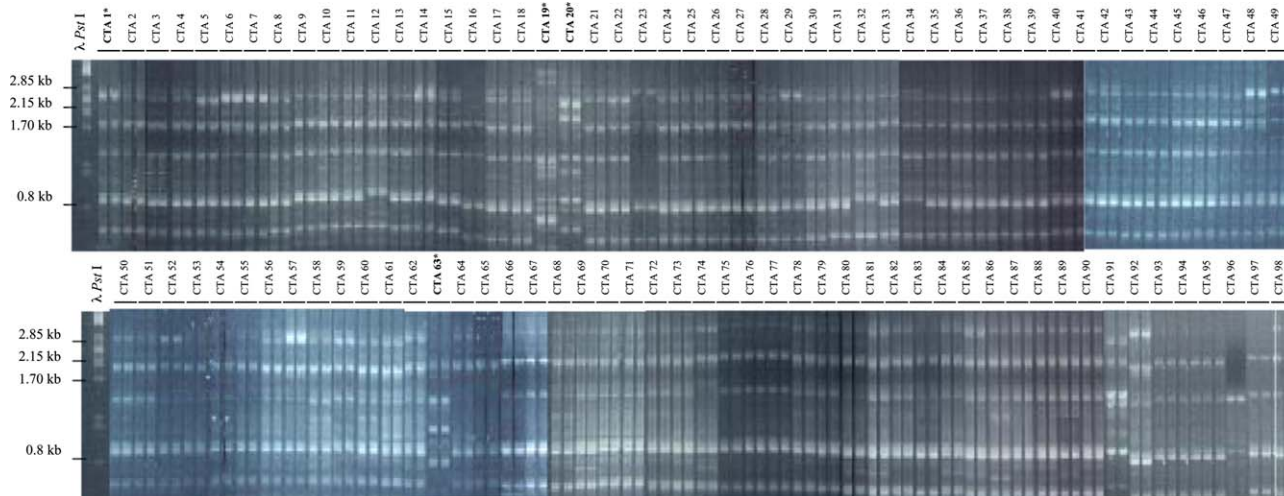


Fig. 6. Examples of RAPD profiles generated from *C. destructans* isolates from site 2 using primer UBC 66. These data were used as the basis of a discriminate selection procedure for natural product profiling. Isolates identified with an asterisk are examples of those selected for natural product analysis on the basis of their genotypic variability. Each lane shows the RAPD profile generated from genomic DNA of a single spore re-isolate of a given fungal individual. The size marker is lambda DNA digested with *Pst* I.

Table 4
Isolates of *C. destructans* and *H. lugdunensis* collected from the roots of *Alnus glutinosa*, located at the edge of the River Teign in Dartmoor National Park (site 2), discriminately selected by RAPD analysis for metabolite screening

Isolated from	Species			
	<i>C. destructans</i>		<i>H. lugdunensis</i>	
Aquatic roots	CTA 1 ^a	CTA 20	HTA 1 ^b	HTA 26
	CTA 19	CTA 64	HTA 5	HTA 33
			HTA 12	HTA 36
			HTA 25	HTA 43
Terrestrial roots	CTT 1	CTT 42	HTT 1	
	CTT 15	CTT 48	HTT 2	
	CTT 23	CTT 80		
	CTT 27	CTT 82		
	CTT 36	CTT 85		

^a‘CTA’ and ‘CTT’ refer to: *C. destructans* isolates collected from aquatic and terrestrial roots from the River Teign, respectively.

^b‘HTA’ refers to: *H. lugdunensis* isolates collected from aquatic and terrestrial roots from the River Teign, respectively.

could be readily identified, in addition to large numbers of unidentifiable mycelial fungi that could also be cultured (data not shown). This highlights the diversity of fungi occupying very specific environments and the large untapped resource of fungi available for metabolite screening. Among the root-colonising endophytes we selected two of the abundant species, one of which predominated in terrestrial roots and the other in roots taken from free-flowing water. When analysed genotypically, we found that populations of each species (*C. destructans* and *H. lugdunensis*) could be classified into between 3 and 4 major groups. Sequencing of the ITS region confirmed that all the isolates represented the

same fungal species (Bruns et al., 1991) and revealed four ITS1 alleles in isolates of *C. destructans* and a single *H. lugdunensis* ITS1 allele. Classifications made by ITS1 sequencing also corresponded well with RAPD group assignments, for example isolates within the ITS 1-group IV, CDT 86, 147 and 164, clustered together in RAPD-group V.

Secondary metabolite analysis revealed considerable variation among isolates from our initial collection site. Isolates showing the greatest similarity in secondary metabolite profiles were often isolated from the same habitat, either aquatic or terrestrial roots, perhaps indicating conservation of particular metabolite production in these ecologically isolated populations of fungi. Comparative analysis also revealed that the genotype of an isolate of *H. lugdunensis* or *C. destructans* often showed strong similarity to its pattern of metabolite production. This is consistent with previous studies where such a relationship has been investigated (Kubicek et al., 2003; Talbot et al., 1996). However, as in previous studies we found that the relationship was complex and some isolates that were genotypically similar still showed considerable metabolite variability. This may be due to genetic divergence that is undetected by RAPD profiling, yet has a pronounced effect on particular metabolic pathways, but there are also developmental and environmental factors that strongly influence metabolic diversity. Clearly, one would predict a close correspondence between an organism’s genotype and its phenotypic characteristics (one of which is the capacity to produce secondary metabolites), but in practice this relationship has not been straightforward to show and has not been usefully applied for selection of novel metabolites from fungi. We were

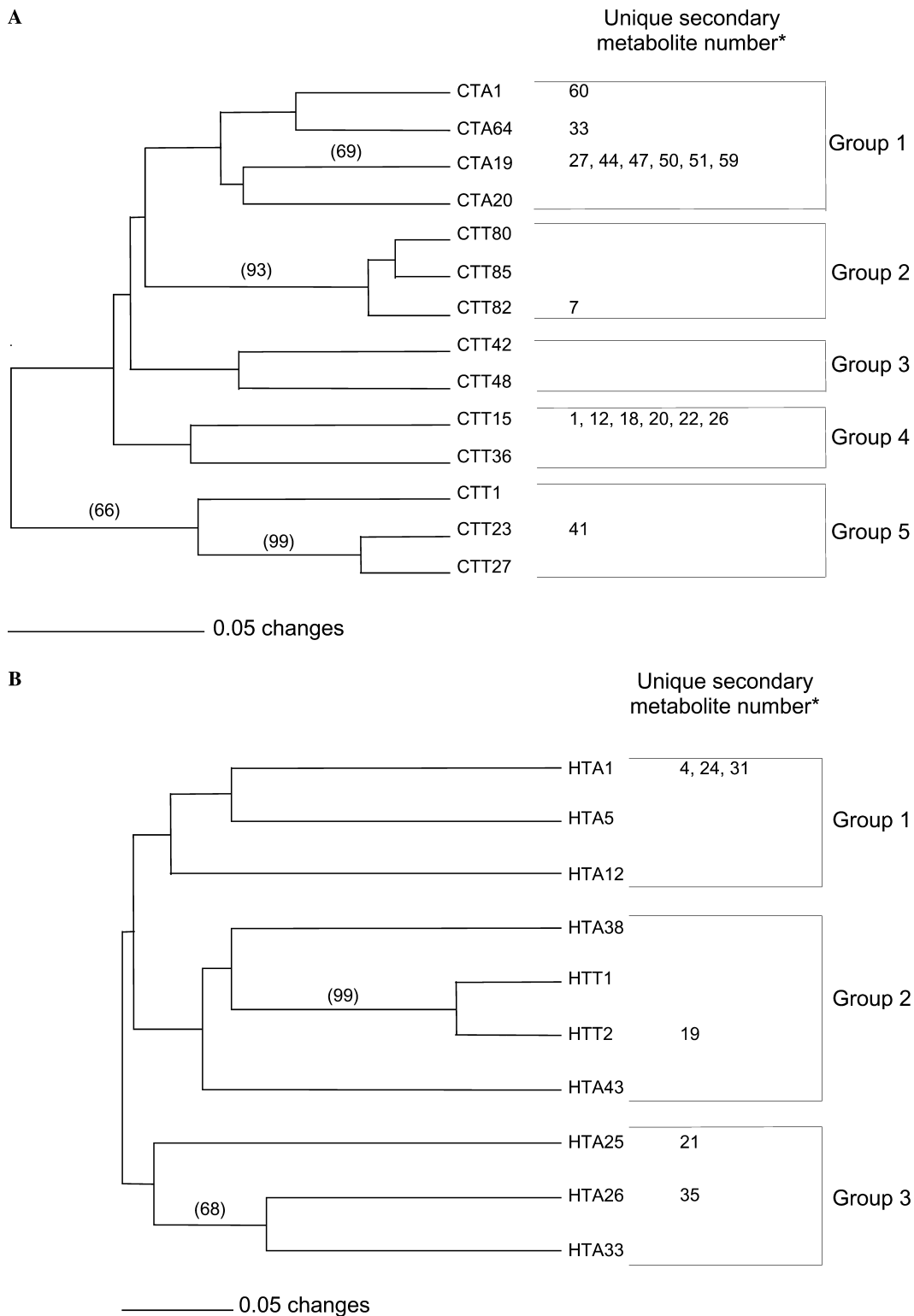


Fig. 7. Dendrograms derived from RAPD analysis of (A) *C. destructans* and (B) *H. lugdunensis* isolates collected from roots of a single Common Alder tree (*A. glutinosa*) at site 2. These represented the most diverse sub-set of isolates based on RAPD profiles. For each isolate metabolite profiling was carried out using HPLC of ethyl acetate extracts. The presence of a unique metabolite peak, defined as one produced by a single fungal isolate, is shown (numbered from 1 to 60).

sufficiently confident in the correlation between genotypic classification and metabolite profile, however, to test the relationship directly. For this we picked a sec-

ond site and a much larger collection of fungi. These were subjected to selection for metabolite profiling based solely on genotype analysis using 2 RAPD

primers. Ten of the selected fungal isolates from this second population produced unique metabolites in comparison to our original indiscriminate selection in which only 2 isolates produced completely unique metabolite peaks.

There are two main conclusions that have resulted from this study. The first is that assessing the potential metabolic diversity of a fungal species cannot be adequately performed by investigating a single 'representative' isolate; significant metabolic diversity is present among isolates of a single species collected from a single site. This emphasises the enormous capacity for secondary metabolic variation that appears to exist in fungal genomes (Galagan et al., 2003), and the importance of environmental and developmental factors on secondary metabolite expression. The second conclusion to this study is that a simple form of genotypic analysis may be used as a pre-selection procedure in fungal screens. The best form of genotype testing would be a simple multi-locus method as used here, or another PCR-based method such as single nucleotide polymorphism (SNP) analysis at numerous loci, or microsatellite analysis, which might be more robust and scalable than using RAPDs. Utilising a genotype screen to pre-select the most diverse fungal isolates in an environment may therefore provide a means of reducing the genetic and chemical redundancy that is inherent in natural product selection programmes, thus providing more efficient generation of novel lead structures for the development of new drugs.

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