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Low genetic diversity among isolates of the nematode-trapping fungus *Duddingtonia flagrans*: evidence for recent worldwide dispersion from a single common ancestor

Dag AHREN[1], Margaret FAEDO[2]†, Balaji RAJASHEKAR[1] and Anders TUNLID[1]*

[1] *Department of Microbial Ecology, Lund University, Ecology Building, S-223 62 Lund, Sweden.*
[2] *Danish Centre for Experimental Parasitology, The Royal Veterinary and Agricultural University, 100 Dyrlaegevej, DK-1870, Frederiksberg C, Denmark.*
E-mail: *anders.tunlid@mbioekol.lu.se*

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The genetic variation of *Duddingtonia flagrans*, which has become a promising biocontrol agent of animal parasitic nematodes, was investigated in a worldwide collection of 22 isolates. We analysed the sequence variation in four nuclear genes, *tubA* (β-tubulin), *CMD1* (calmodulin), *EF1*α (translation elongation factor 1α), and PII (extracellular serine protease). 1428 aligned base pairs (bp) were analysed from the four genes, including 709 bp of introns. In addition, the variations in three anonymous genomic regions comprising 1155 bp were examined. Three single nucleotide polymorphisms (SNPs) were detected in the seven loci, none of them in the protein encoding genes. The genetic variation was significantly higher in the nematode-trapping fungus *Arthrobotrys oligospora*, the closest evolutionary relative to *D. flagrans*. Analysis of 12 isolates of *A. oligospora* revealed 30 SNPs in *tubA*, *CMD1*, *EF1*α and *PII*. The genetic variation in the isolates of *D. flagrans* was further examined using AFLP analysis. Five primer combinations were used to detect 159 bands, of which 94 (59.1 %) were polymorphic. A neighbour-joining tree based on the AFLP data showed no clear association between genotype and geographical origin. Furthermore, the AFLP data suggest that *D. flagrans* is mainly clonal and no recombination could be detected, not even within the same country. The low genetic variation in *D. flagrans* suggests that this fungus has recently diverged from a single progenitor. Based on estimations of mutation rates, it was calculated that this most recent common ancestor lived about 16 000–23 000 years ago.

INTRODUCTION

The nematophagous fungi comprise a large group of soil-living fungi that are parasites on nematodes. So far, more than 200 species of nematophagous fungi have been described. Among them, ascomycetes contain the largest group of nematode-trapping fungi. The nematode-trapping fungi can grow either as saprophytes using a vegetative mycelium or as parasites using specific hyphal structures. During the parasitic stages the fungi develop specialized morphological structures, traps, that are used to infect nematodes. The morphology of traps varies from three-dimensional nets, adhesive knobs, hyphal branches to constricting rings (Barron 1977).

The interest in the infection biology of nematode-trapping fungi is based partly on several of these fungi having been used as biocontrol agents of plant and animal parasitic nematodes. One of the most promising candidates for study was the net-forming nematode-trapping *Duddingtonia flagrans*. In 1992, a strain of *D. flagrans* was isolated in Denmark, whose spores (chlamydospores) survived passage through the gastrointestinal tract of cattle (Larsen *et al.* 1992). Subsequently, trials all over the world have demonstrated that the feeding of *D. flagrans* spores to grazing animals results in successful suppression of the numbers of parasitic nematodes on pasture, and hence diminishes the parasitic burden on various hosts (Faedo *et al.* 1998, Larsen 1999, Knox, Josh & Anderson 2002, Waller 2003).

We examined the genetic diversity of a worldwide collection of *D. flagrans* strains. Information was obtained on the population structure and mode of reproduction of *D. flagrans*. Knowledge of such patterns and processes are important for evaluating the risks of an unwanted spread and possible recombination following a mass application of a specific biocontrol strain. The sequence diversity of *D. flagrans* isolates was very low, and no geographic separation of the genotypes could be

* Corresponding author.
† Current address: Virology Division, Department of Microbiology, SEALS, The Prince of Wales Hospital, Randwick, NSW 2031, Australia.

Table 1. Examined strains and identified genotypes of *Duddingtonia flagrans*[a].

No.	Location	Strain[e]	Substratum	*tubA*	*EF1α*	*CMD1*	*PII*	D6	D7	D9
	Denmark									
1	Ringsted	1351	Horse f.[b]	*a*[c]	*b*	*f*	*h*	*j*	–	*n*
2	Fredensborg	1482	Horse f.	*a*	*b*	*f*	*h*	*j*	*l*	*o*
3	Hundested	1498	Horse f.	–	*b*	–	–	*j*	*l*	–
4	Gram	1725	Horse f.	–	*b*	*f*	–	*j*	*k*	*o*
5	Farum	1768	Horse f.	*a*	–	*f*	*h*	*j*	*l*	*o*
6	Sønderborg	1882	Horse f.	*a*	*b*	*f*	*h*	*j*	*k*	*n*
7	Tureby	1887	Horse f.	*a*	*b*	*f*	*h*	*j*	*k*	*n*
8	Tisvildeleje	1888	Horse f.	–	–	*f*	–	*j*	*k*	*n*
9	Greve	1889	Horse f.	*a*	–	*f*	*h*	*j*	*k*	*n*
10	–[d]	C I_3	–	*a*	*b*	*f*	*h*	*j*	*l*	*o*
11	–	HK II	–	*a*	*b*	*f*	*h*	*j*	*l*	*n*
12	–	Troll A	–	*a*	*b*	*f*	*h*	*j*	*k*	*n*
	United Kingdom									
13	Rothamsted	R4	Soil	*a*	*b*	*f*	*h*	*j*	*k*	*o*
14	London	CBS 565.50	Compost	*a*	*b*	*f*	*h*	*j*	*k*	*o*
15	Yorkshire	CBS 143.83	Leaf litter	*a*	–	–	*h*	*j*	*k*	*o*
	France									
16	–	MUCL	Meadow	*a*	*b*	*f*	–	*j*	*k*	*n*
	Germany									
17	Berlin-Dahlem	CBS 583.91	Soil	*a*	–	*f*	*h*	*j*	*k*	*n*
	USA									
18	California	ATCC 207101	Compost	–	*b*	*f*	*h*	–	*k*	*n*
19	–	CBS 561.92	–	*a*	*b*	*f*	*h*	*j*	*k*	*o*
	India									
20	–	DF-S-A	Sheep f.	–	–	*f*	*h*	*j*	*k*	*n*
21	–	DF-B-J	Bullock f.	*a*	–	*f*	–	*j*	*k*	*n*
	Malaysia									
22	Ipoh	2207	Sheep f.	–	–	*f*	*h*	*j*	*k*	*n*

[a] *a-n* indicate different alleles of the following genes: *tubA* (β-tubulin), accession nos. AY444726–AY444739. *EF1α* (translation elongation factor alpha), accession nos. AY444695–AY444708. *CMD1* (calmodulin), accession nos. AY444623–AY444642. *PII* (serine protease), accession nos. AY444709–AY444725. Anonymous genome region D6, accession nos. AY444643–AY444652. Anonymous genome region D7, accession nos. AY444653–AY444673, $l=k$ but $8T \Rightarrow C$. Anonymous genome region D9, accession nos. AY444674–AY444694. $o=n$ but $242C \Rightarrow T$.

[b] f, Faeces.

[c] Underlining indicates that the data are from SSCP analysis.

[d] –, Not known.

[e] Strains are permanently preserved at the Danish Centre for Experimental Parasitology (The Royal Veterinary and Agricultural University, Frederiksberg).

detected. To investigate whether other species of nematode-trapping fungi have a similarly low level of genetic diversity, we also examined the genetic variation of a set of *Arthrobotrys oligospora* isolates, a species closely related to *D. flagrans* (Ahrén *et al.* 1998, Hagedorn & Scholler 1999). These two species are very similar in morphology, but they have been identified as separate species based on the conidiogenesis and conspicuous production of chlamydospores in *D. flagrans* (Rubner 1996).

MATERIALS AND METHODS

Fungal cultures and DNA extractions

22 isolates of *Duddingtonia flagrans* from different geographic regions were analysed in this investigation (Table 1). Additionally, 12 *A. oligospora* isolates were analysed (Table 2). All fungal isolates were maintained on corn meal agar (CMA 1:10). For DNA extractions, each isolate was grown in malt extract broth, and DNA was extracted as described in Ahrén *et al.* (1998). After the final ethanol precipitation, the DNA pellet was dissolved in TE buffer containing RNAse (20 μg μl^{-1}). Absorbance at 260 nm was used to determine the quantity of DNA.

DNA sequencing

The genetic variation of *Duddingtonia flagrans* was examined in four nuclear genes *tubA* (β-tubulin), *CMD1* (calmodulin), *EF1α* (translation elongation factor 1α), *PII* (an extracellular serine protease) and three anonymous genomic regions which we called D6, D7 and D9. In *A. oligospora* the genetic variation in *tubA*, *CMD1*, *EF1α* and *PII* were examined. The primers used for amplifying these genes are given in Table 3.

Table 2. Strains examined and identified genotypes of *Arthrobotrys oligospora*[a].

No.	Location	Strain	Code	*tubA*	*EF1α*	*CMD1*	*PII*
	Sweden						
1	Alnarp	ATCC 24927, CBS115.81	Lu7[c]	*a*	*g*	*l*	*r*
2	Lönnstorp	L.P.9002	Lu53	*a*	*g*	*l*	*r*
3	Bjäred	L.P.9009	Lu60	*a*	*g*	*l*	*r*
4	Mossagården	L.P.9036	Lu82	*e*	*g*	*l*	*r*
5	Mossagården	L.P.9202	Lu88	*a*	*g*	*p*	*r*
	Germany						
6	Berlin-Dahlem	A.R.9113, CBS337.94	Lu102	*e*	*g*	*p*	*r*
7	Kiel	Lu8	Lu8	*a*	*g*	*l*	*r*
	Netherlands						
8	–[b]	CBS106.49	Lu6	*e*	*g*	*p*	*r*
	USA						
9	–	CBS 111.37 Drechsler	Lu5	*b*	*h*	*n*	*s*
	South America						
10	Ecuador	A.R.936, CBS338.94	Lu136	*c*	*i*	*m*	*r*
11	Mexico	Mankau4	Lu146	*d*	–	*o*	*t*
12	Falkland Island	Mankau263b	Lu165	*f*	*g*	*q*	*r*

[a] *a-r* indicate different alleles of the following genes: *tubA* (β-tubulin), accession nos. AY444609–AY444620, *b* = *a* but 17T ⇒ C, 93A ⇒ G, 318A ⇒ C; *c* = *a* but 17T ⇒ C, 58A ⇒ G, 81T ⇒ C, 318A ⇒ C; *d* = *a* but 17T ⇒ C, 170C ⇒ T, 186C ⇒ T, 193T ⇒ C, 259C ⇒ T, 318A ⇒ C; *e* = *a* but 318A ⇒ C; *f* = *a* but 17T ⇒ C, 318A ⇒ C. *EF1α* (translation elongation factor alpha), accession nos. AY444586–AY444597, *h* = *g* but 111 G ⇒ A, 122 A ⇒ G; *i* = *g* but 88T ⇒ C; *f* = *g* but 25C ⇒ T. *CMD1* (calmodulin), accession nos. AY444574–AY444585, *m* = *l* but 98C ⇒ T, 119A ⇒ G, 156G ⇒ A, 204 Del. 330G ⇒ T, 356A ⇒ C; *n* = *l* but 22C ⇒ T, 31T ⇒ C, 40A ⇒ G, 62T ⇒ C, 98C ⇒ T, 215C ⇒ T; *o* = *l* but 98C ⇒ T, 129A ⇒ C; *p* = *l* but 22T ⇒ C, 31T ⇒ C, 40A ⇒ G, 62T ⇒ C, 98C ⇒ T, 170C ⇒ T; *q* = *l* but 22C ⇒ T, 31T ⇒ C, 40A ⇒ G, 62T ⇒ C, 98C ⇒ T. *PII* (serine protease), accession nos. AY444598–AY444608; *s* = *r* but 186C ⇒ T, 197T ⇒ C, 212C ⇒ T; *t* = *r* but 57C ⇒ T, 186C ⇒ T.

[b] –, Not known.

[c] Code used in the culture collection at the Department of Microbial Ecology (Lund University) where the strains are permanently preserved.

Table 3. Primers used to detect DNA sequence variation in genes *tubA*, *EF1α*, *CMD1, PII, D6, D7 and D9*.

Primer	Gene	Direction	Sequence
P112[a]	*tubA*	F[d]	5′-GGTAACCAAATCGGTGCTGCTTTC
P113[a]	*tubA*	R	5′-ACCCTCAGTGTAGTGACCCTTGGC
P128[b]	*EF1α*	F	5′-TATCAACGTCGTCGTCATCG
P129[b]	*EF1α*	R	5′-ATGAAATCACGATGTCCGGG
P190[b]	*EF1α*	F	5′-GGGCAAGGGTTCCTTCAAGT
P191[b]	*EF1α*	R	5′-TAACCCCGCACAAACAACCC
P186[c]	*CMD1*	F	5′-GAGTTCAAGGAGGCCTTCTCCC
P187[c]	*CMD1*	R	5′-CATCTTTCTGGCCATCATGG
P120[b]	*PII*	F	5′-TTTGACAAGGCAACTCTCCAGG
P121[b]	*PII*	R	5′-CCACCAGCGCTAAGAACCTTAAC
P138[b]	D6	–	5′-CGAGAGTCTAGAAGAACGGGTCG
P139[b]	D6	–	5′-CTGTGCCTTGGGCTTAGAGGA
P140[b]	D7	–	5′-AGGTAGCCCAACTTTTGCCTGC
P141[b]	D7	–	5′-TGTTCGAAATCGGGGATGCC
P148[b]	D9	–	5′-GCTGAGCGGTGGCCGTATAT
P149[b]	D9	–	5′-AAGGTCGAGGTCTCGGAGGA

[a] Glass & Donaldsson (1995).

[b] This study.

[c] Carbone & Kohn (1999).

[d] F and R, forward and reverse direction respectively; –, direction not known.

Anonymous clones were obtained by making a small plasmid library of *D. flagrans* (CBS 565.50). DNA was digested with *Bam*HI and *Pst*I and fragments of sizes between 500–800 bp were isolated from an agarose gel and cloned into the compatible sites in pUC19. Ten clones (D1–D10) were randomly picked and sequenced using the M13 forward and reverse primers. Based on the sequences obtained, primer pairs were designed to amplify a region of approximately 500–600 bp in length in three of the clones (Table 3). By using the program Getorf and Showorf from the EMBOSS sequence analysis package (Rice, Longden & Bleasby 2000), the anonymous clones were analysed for possible open reading frames. After translating sequences using Transeq from EMBOSS, a BLASTP search was performed against the non-redundant database and a TBLASTN search of the nucleotide sequences against the dbEST database (Boguski, Lowe & Tolstoshev 1993).

Sequencing was performed according to the manufacturer's description using ABI 377 automated DNA sequencer and Big Dye terminator mix (Applied Biosystems, Foster City, CA).

SSCP analysis

To limit sequencing of a large number of alleles with identical DNA sequences, some of the *tubA* and D6 alleles of *Duddingtonia flagrans* were analysed using Single Strand Conformation Polymorphism (SSCP). The PCR gene fragments of *tubA* and D6 were cut with restriction enzymes to obtain fragments of sizes between 100–300 bp: *tubA* – *Hin*dIII (513 bp→1342 bp and 171 bp); clone D6 – *Eco*RI (466 bp→1182 bp and 284 bp). The samples were denatured and then placed

on ice and the resulting fragments were run on pre-made SSCP gels (ExcelGel DNA Analysis Kit, Pharmacia Biotech, Uppsala). Silver staining was used to visualize the bands on the gels (DNA Silver Staining Kit, Pharmacia Biotech).

AFLP analysis

The protocol used for the AFLP analysis was a modification of that of Vos *et al.* (1995). Briefly, genomic DNA of *Duddingtonia flagrans* was digested with two restriction enzymes, *Eco*RI and *Mse*I. The adapter sequences were ligated to the sticky ends of the fragments using T4 DNA ligase. The *Mse*I adaptor sequences were: 5′-GACGATGAGTCCTGAG and 5′-TACTCAGGACTCAT. *Eco*RI adaptor sequences were: 5′-CTCGTAGACTGCGTACC and 5′-AATTGGTACGCAGTCTAC. The ligated DNA was pre-amplified by adding a reaction mixture containing the primers, *Eco*RI (sequence above) with a T extending from the 3′end, and *Mse*I (sequence above) with a C extending from the 3′-end. Selective amplifications were done using five primer combinations labeled with a fluorescent dye (TAMRA; Applied Biosystems) (Table 5). The PCR products were mixed with loading buffer and run on an ABI 377 sequencer. The peak patterns extracted from the gelfile were manually checked using the GeneScan software (Applied Biosystems) and subsequently imported into the Genographer software (version 1.4.0, Montana State University). In order to check the reproducibility of the data, the DNA fragments were ligated, amplified and analysed at least twice. All bands between 35 and 350 bp that could unambiguously be scored in both samples were selected and used for further analysis.

The index of association (I_A) was calculated using the Multilocus software with 1000 randomizations (Agapow & Burt 2001). The software tests whether the observed dataset is significantly different from the null hypothesis of complete panmixia. The genetic differentiation calculated by theta (θ) was used as an estimate of the gene flow between different geographical areas, on a global as well as the regional scale. θ is Weir's formulation of Wright's F_{ST} (Weir 1996). The null hypothesis is that there is no genetic differentiation between the populations. The statistical significance of the null hypothesis was calculated as the difference in variance between 1000 randomized datasets and the observed data.

Phylogenetic analysis

For phylogenetic analysis, nucleotide alignments of *tubA* and *CMD1* were constructed separately from both species. The nematode-trapping *Monacrosporium haptotylum*, which captures nematodes using adhesive knobs, was used as an outgroup (Ahrén *et al.* 1998, Hagedorn & Scholler 1999). The accession nos. for *tubA* and *CMD1* of *M. haptotylum* are AY444622 and AY444621, respectively. Fragments of *tubA* and *CMD1* were amplified from genomic DNA of *Duddingtonia flagrans* and the products were sequenced using the primers P112/P113 and P186/P187 (Table 3). Phylogenetic trees were constructed using the neighbour-joining (NJ) method (with the Jukes–Cantor model for nucleotide substitution) from the MEGA2 software (Kumar *et al.* 2001). Support values were obtained by bootstrapping with 1000 replicates. For calculating the polymorphism of the analysed loci, the DNA sequences from *D. flagrans* and *Arthrobotrys oligospora* were aligned separately using the ClustalX program (Thompson *et al.* 1997).

To estimate the divergence time between *D. flagrans* and *A. oligospora*, a phylogenetic tree of 16 different species of nematode-trapping fungi and 3 other ascomycetes was constructed based on 18S ribosomal DNA (18S rDNA) sequences. The sequences were aligned using the AliBee program (Brodsky *et al.* 1992) and the ambiguous sites and non-overlapping ends in the multiple alignments were removed manually, leaving 1674 nucleotide sites for the analysis. The tree was inferred using the NJ method with the Jukes–Cantor model for nucleotide substitution (Kumar *et al.* 2001). The topology of this tree was identical to previously published phylogeny of nematode-trapping fungi (Ahrén *et al.* 1998). Subsequently a linearized phylogenetic tree was constructed and the divergence time was calculated using the MEGA2 software (Kumar *et al.* 2001). For calculating the divergence time between *D. flagrans* and *A. oligospora*, we used the estimated divergence time (375 Myr) between *Neurospora crassa* and *Saccharomyces cerevisiae* as the calibration point (Berbee & Taylor 2001).

A neighbour-joining (NJ) tree of the *D. flagrans* isolates was reconstructed from the AFLP bands with no gaps (i.e. missing data) or ambiguities using the PAUP* software (version 4.0.64) (Swofford 2000) and 1000 bootstraps to reconstruct the tree.

Nucleotide diversity

The nucleotide diversity (π) was estimated from AFLP data using the method by Innan *et al.* (1999). The indices were estimated separately for each primer pair. Nucleotide diversity of DNA sequences, defined as the average number of pairwise nucleotide changes per site (Nei 1987), was calculated using the DnaSP software (Rozas *et al.* 2003).

RESULTS

Variation in DNA sequences

We analysed the sequence variation in four protein-encoding genes, namely, *tubA* (β-tubulin), *EF1*α (translation elongation factor 1α), *CMD1* (calmodulin) and the extracellular serine protease *PII* from a worldwide collection of *Duddingtonia flagrans* (Table 1). PII is a

cuticle-degrading protease belonging to the subtilisin family of serine proteases and has been shown to be involved in the killing and digestion of captured nematodes by *Arthrobotrys oligospora* (Åhman *et al.* 1996, 2002). Primers for the protein-encoding genes were constructed to cover intron sequences, which are the most variable parts of protein-encoding genes together with 4-fold degenerated sites (Li 1997). In total 1428 aligned bp were analysed from the four genes, including 709 bp of introns. In addition, the variations in three anonymous genomic clones (D6, D7 and D9) comprising 1155 bp were also examined. The anonymous clone D9 displayed a weak homology (E-value 0.003) with the *Homo sapiens* cDNA clone NIH_MGC_14 in the dbEST database. The sequences from D6 and D7 did not show any significant hit against sequences found in public databases (nr and dbEST).

All seven loci of *D. flagrans* were analysed and three single nucleotide polymorphisms (SNPs) with no indels (insertions/deletions) were observed (Table 1). In the protein-encoding genes no SNPs were detected. D9 had a SNP dividing the isolates into two groups of eight and 13, with no correlation to geographic regions whereas two SNPs were observed in the D7 genomic clone.

To compare the variation in the DNA sequence of *D. flagrans*, isolates with another closely related species of nematode-trapping fungus, 12 worldwide isolates of *A. oligospora* were analysed for genetic variation (Table 2). 1574 aligned bp, including 846 intron sites, were analysed from *tubA*, *EF1α*, *CMD1* and *PII*. We observed nine SNPs (seven in introns) in *tubA*, 4 (3) in *EF1α*, 13 (13) in *CMD1* and 4 (3) in *PII* making 30 SNPs, of which 26 were from introns.

A phylogenetic tree for *tubA* genes was constructed using the nematode-trapping fungus *Monacrosporium haptotylum* as an outgroup (Fig. 1). The strains from *D. flagrans* and *A. oligospora* formed two well supported clades. As expected, there was no resolution in the branch of the strains of *D. flagrans*. By contrast, the isolates of *A. oligospora* were at least partly separated according to their geographical origin. A tree with similar topology was constructed using the *CMD1* sequences (data not shown).

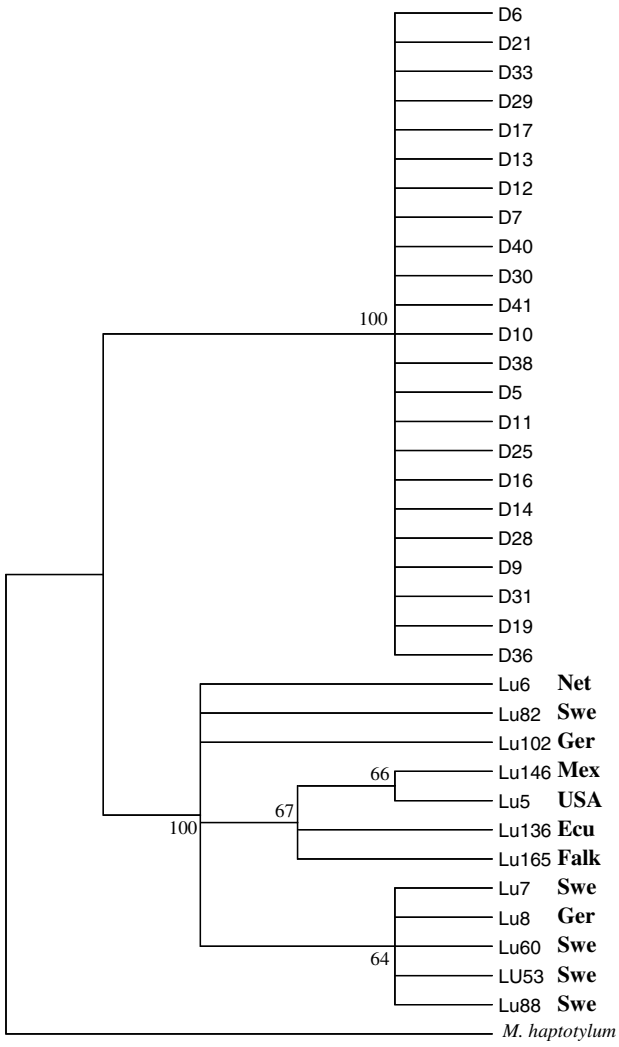

Fig. 1. Neighbour-joining tree of *tubA* with 1000 bootstrap replicates reconstructing the phylogenetic relationship between strains of *Duddingtonia flagrans* (designated with D5-, Table 1) and *Arthrobotrys oligospora* (Lu7-, Table 2). *Monacrosporium haptotylum* was used as an outgroup. Net, The Netherlands; Swe, Sweden; Ger, Germany; Mex, Mexico; Ecu, Ecuador; and Falk, The land Islands.

Estimation of divergence times

The limited variation in DNA sequence in *Duddingtonia flagrans* indicates that this fungus diverged recently from a single progenitor. This divergence time (t) can be estimated by using the following equation (based on Rich *et al.* 1998):

$$t = S/(\mu_a \Sigma n_i l_i + \mu_b \Sigma n_i m_i + \mu_c \Sigma n_i I_i + \mu_d \Sigma n_i N_i) \quad (1)$$

where, S is the observed number of single nucleotide polymorphisms (SNPs); n_i is the number of analysed sequences at the ith locus; μ_a is the mutation rate of 4-fold degenerate sites (including 3- and 6-fold sites); μ_b is the mutation rate of 2-fold degenerate sites; μ_c is the mutation rate of intron regions; μ_d is the mutation rates of non-degenerate sites; l_i and m_i are the average numbers of potentially 4-fold synonymous sites (including 3- and 6-fold sites) and 2-fold sites (at the ith locus), respectively; I_i is the number of sites in introns (at the ith locus); N_i is the number of potentially non-synonymous sites (at the ith locus).

The mutation rates of the various sites (μ_a–μ_d) were estimated by comparing the sequences of *tubA*, *EF1α*, *CMD1* and *PII* from *D. flagrans* (strain D7) with its homologue from *Arthrobotrys oligospora* (strain Lu7). The homologous genes were aligned and the number of substitutions at 4-fold, 2-fold synonymous and non-synonymous sites, and introns were examined (Table 4). The divergence time of *D. flagrans* and *A. oligospora* was estimated to be 25 MYA (Fig. 2). Based on the values of substitutions and the divergence time between *D. flagrans* and *A. oligospora*, the average mutation rates were estimated using the Jukes–Cantor Poisson

Table 4. Estimate of substitution rates in four nuclear genes of *Duddingtonia flagrans*[a].

		Exon								
		Synonymous				Non-synonymous				
		4-fold		2-fold		0-fold			Intron	
Loci	Length (bp)	$D_a = s_a/n_a$	$\mu_a \times 10^{-9}$	$D_b = s_b/n_b$	$\mu_b \times 10^{-9}$	$D_d = s_d/n_d$	$\mu_d \times 10^{-9}$	Length (bp)	$D_c = s_c/n_c$	$\mu_c \times 10^{-9}$
TubA	198	0/37	–	0/30	–	8/125	1.3	225	30/225	2.9
EF1α	75	0/16	–	0/8	–	3/46	1.3	91	13/91	3.1
CMD1	141	2/18	2.4	1/28	0.7	0/95	–	316	69/316	5.1
PII	300	15/53	7	10/44	5.3	13/201	1.3	54	15/54	6.8
Average			4.7		3.0		1.3			4.5

[a] Rates of nucleotide substitution were calculated by the Jukes–Cantor correction model between the D7 strain of *D. flagrans* and the Lu7 strain of *Arthrobotrys oligospora*. D_x indicates the proportion of observed differences between the sequence D7 and Lu7 for x sites (x = a, b, c, d stands for 4-fold, 2-fold, intron and non-synonymous sites, respectively); s_x is the number of substitutions in x sites; n_x is the number of x sites; μ_x is the mutation rate for each site.

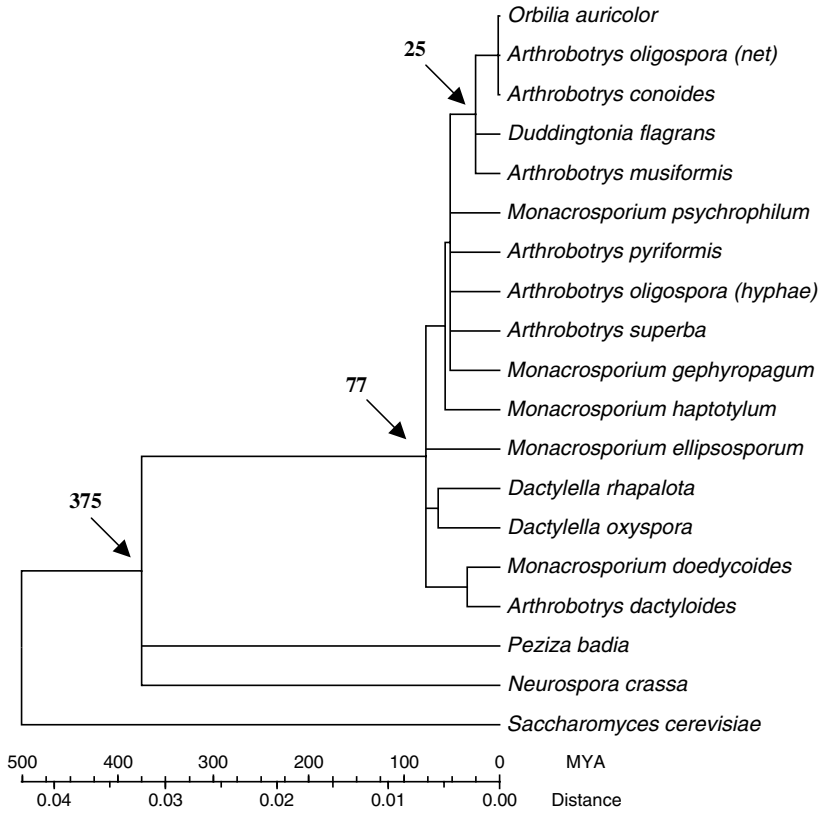

Fig. 2. A linearised phylogenetic tree of nematode-trapping fungi based on the 18S rDNA sequences. For calculating the divergence time between *Duddingtonia flagrans* and *Arthrobotrys oligospora*, we used the estimated divergence time (375 Myr ago) between *Neurospora crassa* and *Saccharomyces cerevisiae* as the calibration point (Berbee & Taylor 2001). The GenBank accession number of the sequences used to estimate the divergence time are: U72598 (*Orbilia auricolor*), J001996 (*Monacrosporium gephyropagum*), AJ001995 (*M. ellipsosporum*), AJ001990 (*M. haptotylum*), AJ001986 *Arthrobotrys oligospora* (net), AJ001987 *A. oligospora* (hyphae), AJ001988 *A. pyriformis*, AJ001985 *A. musiformis*, AJ001983 *A. conoides*, AJ001895 *Duddingtonia flagrans*, AJ001998 *Monacrosporium psychrophilum*, AJ001989 *A. superba*, AJ001994 *M. doedycoides*, AJ001997 *A. dactyloides*, AJ001992 *Dactylella rhopalota*, AJ001993 *Dactylella oxyspora*; J01353 *Saccharomyces cerevisiae*; X04971 *Neurospora crassa*; and L37539 *Peziza badia*.

correction model (Li 1997) to be: $\mu_a = 4.7 \times 10^{-9}$, $\mu_b = 3.0 \times 10^{-9}$, $\mu_c = 4.5 \times 10^{-9}$ and $\mu_d = 1.3 \times 10^{-9}$ (substitutions per nucleotide site per year). These values were similar to those given by Li: $\mu_a = 3.7 \times 10^{-9}$, $\mu_b = 2.2 \times 10^{-9}$, $\mu_c = 3.5 \times 10^{-9}$ and $\mu_d = 0.8 \times 10^{-9}$. The estimated mutation rates together with the values in Table 4 were substituted in equation 1 and the divergence of the *D. flagrans* isolates was estimated to have occurred approximately 16 000 yr ago. Based on the mutation rates given by Li (1997), the divergence of *D. flagrans* isolates was estimated to have occurred approximately 23 000 years ago.

Table 5. AFLP analysis of *Duddingtonia flagrans*. The columns indicate the used primers combination, number of bands scored, number of polymorphic bands, the proportion of shared bands (*F*) and nucleotide diversity (π).

Primers	Amplified bands	Polymorphic bands	*F*	π
*Eco*RI-TGA/*Mse*I-CAT	44	20	0.87	0.0080
*Eco*RI-TGA/*Mse*I-CGA	31	20	0.82	0.0112
*Eco*RI-TGA/*Mse*I-CGG	29	25	0.84	0.0102
*Eco*RI-TGA/*Mse*I-CGC	26	11	0.87	0.0078
*Eco*RI-TGA/*Mse*I-CGT	29	18	0.85	0.0092

Estimation of dispersion and clonality using AFLP data

159 fragments were scored when the five AFLP primer combinations were pooled. In total 65 bands were monomorphic and 94 were polymorphic (Table 5). Fig. 3 shows the unrooted, Neighbour-joining tree constructed from the AFLP data using strict consensus of ten trees. The topology of the tree showed no clear support for divergence according to geographic origin.

The statistical value of θ (theta) was used to estimate gene flow between various subsets of *Duddingtonia flagrans* isolates. Gene flow among the European isolates was significantly different from the null hypothesis of no genetic differentiation ($P_\theta = 0.02$). Among the Danish isolates, the genetic differentiation was not significantly different from the randomized dataset ($P_\theta = 0.07$).

The index of association was calculated on several subsets of *D. flagrans* isolates to look for indications of recombination events on the global, regional and local scale. On the global scale 22 isolates (Table 1) were analysed and found to be significantly different from the randomized datasets ($P_{IA} < 0.01$). On a continental scale, the European population subset (17 isolates) showed significantly different P_{IA} from the corresponding randomized datasets ($P_{IA} < 0.01$). Even the Danish data subset was significantly different from the randomized datasets ($P_{IA} < 0.01$).

Nucleotide diversity

The estimated mean nucleotide diversity (π) of *Duddingtonia flagrans* based on the AFLP data was 0.0093 (range 0.0780 to 0.0112) (Table 5). The π values calculated from the DNA sequences of *tubA*, *EF1α*, *CMD1*, *PII*, D6, D7 and D9 were 0.0000, 0.0022, 0.0001, 0.0007, 0.0000, 0.0031 and 0.0016, respectively (mean 0.0011).

DISCUSSION

The global genetic diversity of the strains of the nematode-trapping fungus *Duddingtonia flagrans* was significantly lower than that of the closely related *Arthrobotrys oligospora*. Using the proportion of variable sites in the aligned nucleotide characters as a measure of global genetic diversity, *D. flagrans* had 0.3% variable sites (three SNPs in 2583 aligned nucleotide sites from seven loci), and *A. oligospora* had 1.9% variable sites (30 SNPs at 1574 alignable nucleotide sites from four loci). The level of genetic polymorphism in *A. oligospora* is within the reported range of several other parasitic fungi. For example, the proportion of variable sites in global populations of the wheat pathogenic *Fusarium graminearum* is 3.8% (six loci; 7120 nucleotides), the human pathogen *Coccidioides immitis* has 1.4% (five loci; 2384 aligned sites), and *Cryptococcus neoformans* 16.3% (four different nuclear and mitochondrial genes comprising 1945 aligned sites) (Koufopanou, Burt & Taylor 1997, O'Donnell *et al*. 2000, Xu, Vilgalys & Mitchell 2000).

The low level of nucleotide polymorphism in the protein-encoding genes of *D. flagrans* can be explained by a recent population bottleneck, and that the extant world populations of the parasitic fungus could have originated from a recent single ancestral strain or progenitor. Based on the estimated mutation rates, it was calculated that this most recent common ancestor lived about 16–23 000 yr ago. Following this bottleneck, the population has spread throughout the world. Other possible explanations, apart from a recent severe population bottleneck and a demographic sweep that could account for the low level of substitutions in *D. flagrans*, include: (1) persistent, low-effective population size; (2) low rates of spontaneous mutation; and (3) one or more selective sweeps, i.e. one genotype has been favoured by natural selection and has recently replaced all others (Kaplan *et al*. 1989, Rich *et al*. 1998).

The effective population size of *D. flagrans* is not known. However, given the worldwide distribution of this species, it is unlikely that the populations have been very small for many generations in recent time (hypothesis 1). Furthermore, considering the divergence in nucleotide sites of *tubA*, *EF1α*, *CMD1* and *PII* between *D. flagrans* and the closely related species *A. oligospora*, the mutational rates in *D. flagrans* did not appear to be exceptionally low (Li 1997) (hypothesis 2). It is known in other organisms that natural selection (hypothesis 3) can lead to a rapid spread of favoured genotypes, particularly when the population is large and/or the selection is strong (Rich *et al*. 1998). Since the analysis of the AFLP data indicated that the population structure of *D. flagrans* is mainly clonal, such a selective sweep could simultaneously have affected all loci in the genome. Thus it is at present not possible to reject the selective sweep hypothesis to explain the relative lack of SNPs in *D. flagrans*.

Since the nucleotide variation in the analysed loci was too low to infer the mode of reproduction and dispersion patterns of *D. flagrans*, the AFLP method was used (Majer *et al*. 1996). Indeed, the method revealed a genetic variation between the isolates of *D. flagrans*. With a mean value close to 0.01, the nuclear diversity (π) calculated from the AFLP data was approximately an order of magnitude higher than the

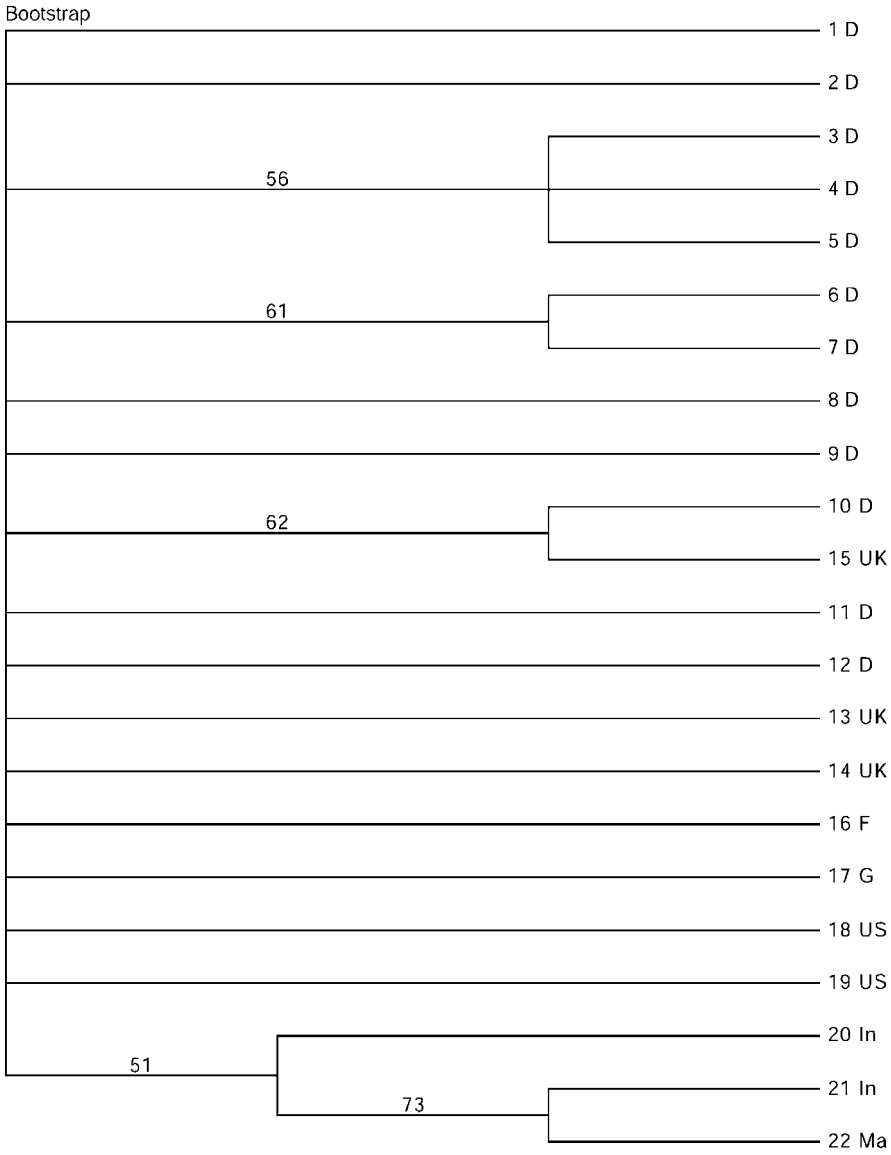

Fig. 3. Reconstructed phylogenetic tree of *Duddingtonia flagrans* isolates revealed by Neighbour-joining analysis with 1000 bootstrap replicates from AFLP data. Strains are listed in Table 1. D, Denmark; UK, United Kingdom; F, France; G, Germany; US, USA; In, India; and M, Malaysia.

π value calculated from the sequences of the nuclear genes. As pointed out by Innan *et al*. (1999), nucleotide diversity calculated from AFLP tends to be larger than nucleotide diversity estimated from DNA sequences (Innan *et al*. 1999). There are several reasons for this discrepancy. First, AFLPs represents the nucleotide diversity of the total genome, which contains regions of both low and high constraints. Secondly, the method by Innan *et al*. (1999) assumes that insertions and deletions are rare. If such events are common, the π values calculated from AFPLs might be an overestimate. Although the importance of genome rearrangement in *D. flagrans* is not known, it is well known that the genomes of fungi display a high degree of plasticity, and that genome rearrangement including deletions and insertions can occur during evolution (Zolan 1995, Dunham *et al*. 2002).

Teleomorphs have been identified for a number of nematode-trapping fungi, including *A. oligospora* (Pfister 1997). It is, however, not known to what extent sexual recombination occurs in natural populations of nematode-trapping fungi. It should be noted that an analysis of the AFLP data indicated that *D. flagrans* is mainly clonal and no recombination could be detected even between isolates from a local area. The facts that the AFLP analysis revealed a significant genetic differentiation among the strains, and that the genotypes identified by the AFLP data did not correlate with the geographical origins of the isolates, are consistent with a recent and ongoing (or multiple) dispersal of *D. flagrans*. One possibility is that the fungus has been dispersed during the transport of livestock, since *D. flagrans* are commonly isolated from the faeces of cattle (Table 1).

The data presented here have implications for the use of *D. flagrans* as a biocontrol agent of animal parasitic nematodes. To date, most of the successful field trials have been done used a single Danish 'super-isolate' selected for its ability to survive passage through the gastro-intestinal tract of cattle (Larsen *et al*. 1992). The low level of genetic variation and the lack of geographical differentiation should be considered in future screening programs aiming at isolating strains of *D. flagrans* with an improved capacity to control parasitic nematodes. For example, it is unlikely that strains will be found that are adapted to a specific geographical region. Furthermore, the AFLP data suggests that mass application of an isolate for biological control will not recombine with local isolates.

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