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Intron polymorphism in small subunit rDNA of *Nectria galligena*

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PCR amplification of the small subunit (SSU) rDNA gene of 40 isolates of *Nectria galligena* revealed four length polymorphisms. PCR–RFLP analysis of the SSU rDNA gene divided the isolates into four categories similar, but not identical, to categories identified by Southern–RFLP analysis. Nucleotide sequence analysis revealed that isolates in three of the four SSU rDNA (18S) categories possess an intron of 363 bp, 1185 bp or 1423 bp at the NS 7 priming site. Isolates in the fourth category do not possess an intron. The nucleotide sequences of these introns did not contain the core elements characteristic of typical group I introns, nor did they exhibit a group I intron secondary structure. Homology between the introns indicates a common lineage, all three possibly having come from a larger intron and having been formed by subsequent deletions. PCR primers upstream of the SSU rDNA intron region and from within the internal transcribed spacer 1 region amplify a product specific to *N. galligena*, which will confirm the identity of the pathogen and reveal its 18S category in a single reaction.

Keywords: *Nectria galligena*, small subunit rDNA, introns

INTRODUCTION

Nectria galligena Bres [anamorph: *Cylindrocarpon heteronema* (Berk and Br.) Wollenw.] causes European canker of apple. The disease, which can be particularly damaging to young orchards and rots fruit in storage, is present in virtually all apple-producing regions of the world (Swinburne, 1975). It has been generally accepted that wood cankers arise from the infection of leaf scars and wounds by splash-dispersed conidia or airborne ascospores (Swinburne *et al*., 1975). Recently, however, investigations have indicated that a proportion of cankers may develop from systemic infection of apple trees and that infection appeared to originate from the nurseries in which the trees were produced, rather than from the orchard in which they were grown (Brown *et al*., 1994). Confirming the source of infection is a priority in developing more effective control strategies for the disease; to facilitate this, molecular markers are being sought which could be used to trace the origin of *N. galligena* isolated from infected apple trees. A previous study revealed RFLPs in the rDNA of *N. galligena* (Brown *et al*., 1994); however, there is a need for faster, non-isotopic means of detecting variation in the fungus. No variation was observed in the internal transcribed spacer (ITS) 1 or 2, nor in the 5·8S gene, of more than 70 isolates tested. This paper describes the presence of introns, giving rise to size polymorphisms in the small subunit (SSU) rDNA of *N. galligena*.

Abbreviations: ITS, internal transcribed spacer; SSU, small subunit.

The EMBL accession numbers for the sequences reported in this paper are Y12315 for the ITS region, Y16422 for the 363 bp SSU rDNA intron, Y16423 for the 1185 bp SSU rDNA intron and Y16424 for the 1423 bp SSU rDNA intron.

METHODS

Fungal growth conditions and DNA extraction. Forty isolates of *N. galligena* were used in this study (see Table 2); the 23 'A' isolates from orchards in England (all collected in 1991) and NI84 and NI89 isolates from Northern Ireland have been used in a previous study and bear the same reference number (Brown *et al*., 1994). The remaining 15 'NI' isolates were obtained from cankered Bramley's seedling apple wood from orchards in Northern Ireland in 1995. All isolates were maintained on 2% malt agar at 22 °C and were confirmed to be able to cause lesions in apple fruits.

Mycelium for DNA extraction was grown in liquid Czapek-Dox medium for 5 d at 22 °C in an orbital shaker (120 r.p.m.), harvested, lyophilized and total DNA extracted using a phenol/chloroform extraction method based on that of Raeder & Broda (1985), as described by Brown *et al*. (1994).

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Table 1. Nucleotide sequences of primers not published elsewhere

Primer name	Sequence (5′–3′)
NI 3/6 F(1)	CCA TGG GAC AAC CAT GCT
NI 3/6 F(2)	CTC CTC CTC CCT CAC CTC
NI 3/6 F(3)	GGT AAG GGC TTC GGT TTG
NI 3/6 F(4)	AGC GCA AGT CAT CAG CTT
NI 3/6 R(1)	CCA GAA CAT CTA AGG GCA
NI 3/6 R(2)	GGC ACC CCG AAG AGT CTC
NI 3/6 R(3)	ACC GCG AAA CAG CTT ACG
NI 3/6 R(4)	GCG CCC ACG TGA AGC TGA
NI 100/109	GTC GGC GTC GTC GTC GGG
NI 100/109 F4+	TTG AGT CCA TCG CGA CTG
NS 7 UP	GGC CGC TGG CTT CTT AGG GG
*Ch*Int*rev*	TCC TCT GGC GGG CCG CCG GAG CGG

Southern analysis. Genomic DNA (1–3 μg) was digested to completion at 37 °C with *Eco*RI according to the manufacturer's instructions (Promega). The restriction products were separated by electrophoresis on 0·8% agarose gels, transferred to nylon membranes (Hybond-N; Amersham) and probed with plasmid pMY60 containing a complete rDNA unit from *Saccharomyces carlsbergensis* Hansen (Verbeet *et al*., 1983) as described by Brown *et al*. (1994). The membranes were exposed to Hyperfilm *β*-max (Amersham) at −70 °C using intensifying screens.

PCR amplification of SSU rDNA. The SSU rDNA (18S) was amplified on a Perkin Elmer 480 thermal cycler using the following programme: 1 min at 94 °C, 1 min 30 s at 53 °C, 2 min at 72 °C, for 30 cycles. The reaction mixtures (100 μl) comprised 50 ng template DNA, 1·5 mM $MgCl_2$, 1 unit Red Hot *Taq* polymerase (Advanced Biotechnologies), 200 μM dNTPs (Sigma), 1 × PCR buffer IV (75 mM Tris/HCl, pH 9·0, 20 mM $(NH_4)_2SO_4$, 0·1%, v/v, Tween 20) and 0·6 μM primers NS 1, NS 3, NS 5 or NS 7, and NS 6 or NS 8 (White *et al*., 1990). PCR products were separated in 1 × TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8·3) on 1·0% agarose gels containing ethidium bromide (0·4 μg ml^{-1}) using *Hin*dIII-digested *λ* DNA (Promega) as the molecular size marker.

Restriction analysis of SSU rDNA. PCR products amplified with primers NS 1 and NS 8 were digested to completion with *Alu*I, *Cfo*I, *Msp*I and *Rsa*I (Promega) according to the manufacturer's instructions. Restriction fragments (500 ng) were separated by electrophoresis in 1 × TAE buffer on 2% Metaphor agarose gels containing ethidium bromide (0·4 μg ml^{-1}) with Marker VI (Boehringer Mannheim) as the molecular size marker. For data analysis, each fragment generated with the four restriction enzymes was treated as a separate character. DNA fragments of the same size were assumed to represent the same genetic locus and were scored as present or absent. The cluster analysis of the data was undertaken on a similarity matrix derived from Jaccard's similarity coefficient. The dendrogram was generated by the unweighted pair group method with arithmetic mean (UPGMA), on the GENSTAT5 program (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Bootstrap analysis of the PCR–RFLP data was carried out using the CONSENSE program (PHYLIP computer package; Felsenstein, 1993).

Sequencing of SSU rDNA inserts. PCR products amplified with primers NS 7 UP (50 bp upstream from NS 7; Table 1) and NS 8 were purified using the Wizard DNA Clean Up Kit (Promega). PCR products were sequenced using double-stranded template (400 ng) and initially 1 μM of the NS 7 UP (forward) and NS 8 (reverse) primers, following the protocol supplied with the Prism Ready Reaction Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Internal primers (Table 1) were designed from the resulting sequence data to complete the sequencing in both directions. Products were resolved on an Applied Biosystems model 373A DNA sequencer and the sequence data were compiled and edited using Sequence Navigator software (Applied Biosystems).

Amplification of products specific to *N. galligena*. PCR primers NS 7 UP (from the 5′ end of the *N. galligena* intron region) and ChInt*rev* (a reverse primer from the region of the *N. galligena*-specific primer, *Ch*Int, in the ITS1 region of the rDNA; Brown *et al*., 1993), were used to amplify *N. galligena*-specific products which included the SSU rDNA intron region. PCR amplification conditions were: 1 min at 94 °C, 1 min 30 s at 55 °C, 3 min at 72 °C, for 30 cycles (Perkin Elmer 480 thermal cycler). The PCR products were separated on 1 × TAE, 1·0% agarose gels, with digested pGEM (Sigma) as the molecular size marker and visualized as described above. To check the specificity of the primers, DNA from apple wood and other fungi that are potentially pathogenic or saprophytic on apple wood or fruit (*Alternaria alternata*, *Botrytis cinerea*, *Cladosporium herbarum*, *Epicoccum purpurascens*, *Fusarium lateritium*, *Monilinia fructigena*, *Monilinia laxa*, *Nectria cinnabarina Pestalotiopsis* spp., *Penicillium expansum*, *Rhizopus stolonifer*, *Trichoderma harzianum*) were also analysed. Primers ITS1 and ITS4 (White *et al*., 1990) were used to verify the suitability of the DNA preparations for PCR.

RESULTS

*Eco*RI RFLP patterns

Digestion of the rDNA with *Eco*RI gave the same four banding patterns as observed in a previous study (Brown *et al*., 1994; data not shown) and placed the 40 *N. galligena* isolates into one of the four *Eco*RI RFLP categories (Table 2). The majority of 'A' isolates were in categories 1 and 2, as previously reported (Brown *et al*., 1994). The majority of the 'NI' isolates were in category 1; category 2 was not found amongst the 'NI' isolates analysed and no category 3 was found amongst

Table 2. *Eco*RI rDNA and 18S categorization of *N. galligena* isolates obtained from English and Northern Irish orchards

Isolate no.	Isolate code	*Eco*RI RFLP category	18S PCR–RFLP category
1	A3	1	1
2	A4	1	1
3	A5	1	1
4	A6	1	1
5	A7	2	2
6	A9	2	2
7	A10	2	2
8	A11	1	1
9	A12	1	1
10	A13	1	1
11	A17	2	2
12	A18	2	2
13	A20	2	2
14	A21	2	2
15	A22	2	2
16	A23	4	2
17	A24	2	2
18	A25	2	2
19	A26	4	2
20	A27	1	1
21	A29	1	1
22	A30	1	1
23	A31	2	2
24	NI84	3	3
25	NI89	1	1
26	NI2	1	1
27	NI3	3	3
28	NI4	1	1
29	NI5	3	3
30	NI6	3	3
31	NI7	1	1
32	NI8	1	4
33	NI47	1	4
34	NI55	1	4
35	NI70	1	4
36	NI93	1	1
37	NI100	1	1
38	NI109	1	1
39	NI119	1	1
40	NI123	1	4

the 'A' isolates (Table 2). Isolates A23 and A26 were the only isolates in category 4.

PCR–RFLP analysis of the 18S gene

PCR products generated using primers NS 1 and NS 8 varied in size from approximately 1·8 kb to approximately 3·5 kb; four distinct fragments were visible (data not shown). Each of the four endonucleases used digested the NS 1/NS 8 PCR products into four visually

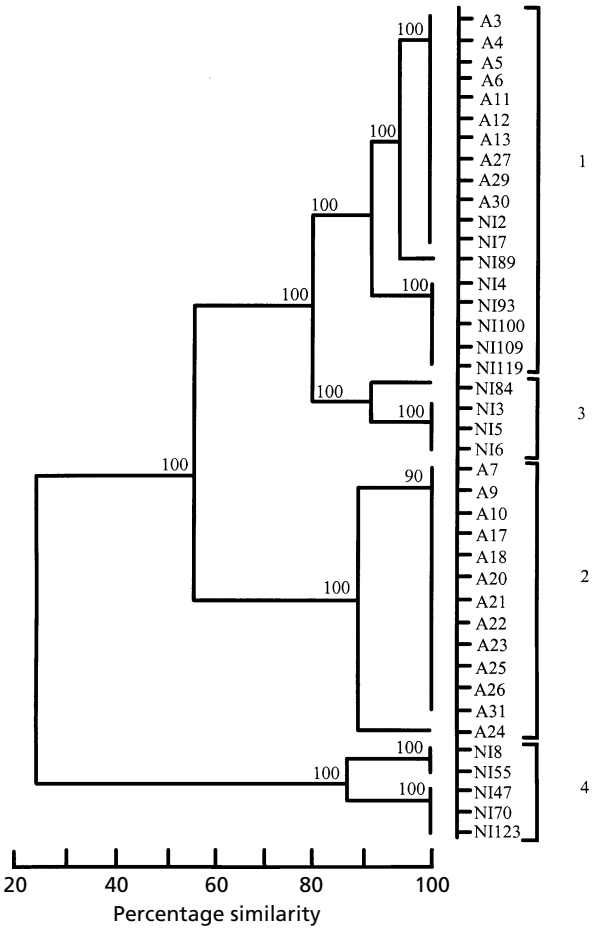

Fig. 1. Clustering of *N. galligena* isolates from pairwise comparison of SSU rDNA restriction fragment banding patterns by the 'group average' method. The values at the branch points are statistical values representing the strength of the relationship between branches using bootstrap analysis.

distinct fragment patterns. A dendrogram (Fig. 1) produced from cluster analysis of 36 polymorphic characters divided the 40 *N. galligena* isolates studied, allowing for minor variation among some of the isolates, into four main groups corresponding to the four length-polymorphic NS 1/NS 8 PCR products. The isolates in 18S category 3 were the same as those in *Eco*RI RFLP category 3 and most of the isolates in 18S category 2 were the same as those in *Eco*RI RFLP category 2, except that isolates A23 and A26 (*Eco*RI RFLP category 4) also fell into this category. All but five of the isolates in 18S category 1 corresponded to the isolates in *Eco*RI RFLP category 1; the five constituted a fourth 18S group (Fig. 1, Table 2).

Location of size variation in SSU rDNA

Using the conserved series of primers, the region of variation in the 18S gene of the *N. galligena* isolates was found to be between the NS 5 and NS 8 primer sites. All of the isolates, except those in 18S category 4, failed to amplify when primer NS 7 was used with

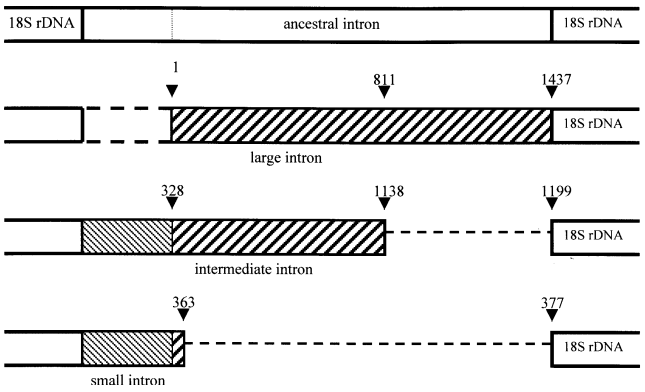

Fig. 2. Diagrammatic representation of the position of the introns within the SSU rDNA of *N. galligena* and possible evolution from an ancestral form

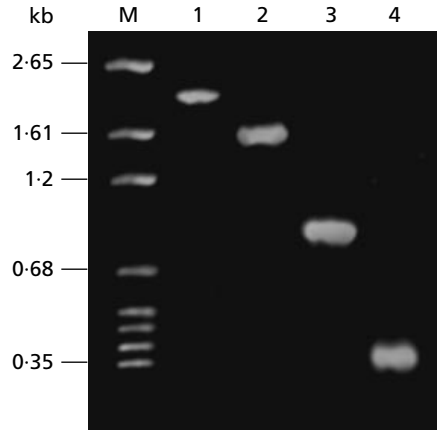

Fig. 3. Amplification of *N. galligena*-specific fragments from total DNA using target primers NS 7 UP and *Ch*Int*rev*. Lanes 1 to 4 are *N. galligena* isolates A11, NI3, A23 and NI70 respectively; lane M, digested pGEM used as molecular size marker.

NS 8. Nucleotide sequence analysis of PCR products generated with primers NS 5 and NS 8 from all but the 18S category 4 isolates revealed insertions of varying size which spliced the NS 7 priming site (GGCAATAACAGGT↓CTGTGATGC). When used with primer NS 1, the reverse primer of NS 7 (NS 6) gave the same size product with all 40 *N. galligena* isolates (data not shown). Primer NS 7 UP, used with NS 8, gave products of 333 bp (18S PCR–RFLP category 4), 696 bp (18S PCR–RFLP category 2), 1558 bp (18S PCR–RFLP category 3) and 1763 bp (18S PCR–RFLP category 1) (Table 2). The 333 bp fragment was homologous with conserved nucleotide sequence data for the 18S gene.

Sequence analysis of SSU rDNA inserts

The nucleotide sequence data of the SSU rDNA inserts from *N. galligena* isolates revealed the presence of 363 bp, 1185 bp and 1423 bp inserts. Comparison of these nucleotide sequences using the BESTFIT program (Genetics Computer Group, Wisconsin Package, version 8.1) revealed that the large- and intermediate-sized introns showed more than 99 % homology over 1088 bp, whilst the small- and intermediate-sized introns showed more than 88 % homology over approximately the first 300 bp. The positions of the alignments are shown in Fig. 2.

Amplification of *N. galligena*-specific fragments

Primers NS 7 UP and *Ch*Int*rev* amplified fragments, including the intron region, which were specific to *N. galligena* (Fig. 3). These primers did not cross-react with apple-wood DNA nor with the other fungi tested.

DISCUSSION

The rRNA gene block of *N. galligena*, previously shown by RFLP analysis to be polymorphic (Brown *et al*., 1994) has no variation in the ITS 1 or 2 regions, which vary considerably in some fungal species (Lee & Taylor, 1992; Muthumeenakshi *et al*., 1994; Sreenivasaprasad *et al*., 1996). Amplification of the 18S gene, however, revealed four length polymorphisms. Restriction analysis of NS 1/NS 8 PCR products grouped the *N. galligena* isolates in a similar, although not identical, manner to the *Eco*RI RFLP data. This would suggest that differences in the makeup of the 18S gene are, to a large extent, responsible for the reported polymorphisms in rDNA of this fungus (Brown *et al*., 1994). PCR amplification of the SSU rDNA showed no variation among the isolates upstream of the NS 5 priming site or downstream of the NS 8 priming site. This, together with the failure to amplify all but the smallest 18S gene with the NS 7 primer, suggested that the different length insertions probably occurred at the same site and at the NS 7 priming site; this was confirmed by nucleotide sequence analysis.

To sequence the introns, a primer was designed upstream of the NS 7 priming site (NS 7 UP) and the sequence data from the NS 7 UP/NS 8 PCR products revealed the relationship between the introns within the SSU rDNA of *N. galligena*. The presence of group I introns is quite common in many fungal species (Fulton & Brown, 1997, Nishida *et al*., 1993, DePriest & Been, 1992) and they are characterized by conserved core elements and secondary structure (Cech, 1988). They also show a high degree of variability in terms of size, number of insertions and location of insertion (Gargas *et al*., 1995, Oliveira *et al*., 1994; Nishida *et al*., 1993). The nucleotide sequences of the *N. galligena* introns however, do not contain these core elements and preliminary analysis of the secondary structure, using the MFOLD program (Zuker, 1989), showed no correlation with the typical group I intron structure. BLASTX (GCG Computer Package) analysis of the intron sequences showed no significant homology with any peptide sequence in the SWISS-PROT database, nor were any putative open reading frames identified using the FRAMES program (GCG Computer Package). How-

ever, the intron insertion site in *N. galligena*, disrupting the NS 7 priming region, has been shown in other studies to be a region 'targeted' by group I introns (position 1199), notably, in other Ascomycotina (DePriest & Been, 1992; Gargas *et al*., 1995). The conservation of the insertion site may simply reflect the exposed position of this location within the 18S tertiary structure, rather than a specific targeting by group I introns. Since there is no homology between the *N. galligena* introns and group I introns in other fungi it is impossible to predict the mode of action of insertion of these introns into the 18S gene. It is possible that these introns are an as yet unclassified type of group I intron or the vestiges of a larger one (Grube *et al*., 1996). From the sequence alignments it would appear that the intermediate intron may have arisen from the large intron as the result of a deletion. The homology of the small and intermediate introns also suggests a common lineage, possibly all three arising from deletions of an ancestral intron (Fig. 2). The large intron, which is considerably longer than most fungal group I introns (Gargas *et al*., 1995), appears to have been formed as a result of the loss of the 328 bp fragment common to both the small and intermediate introns, whilst the intermediate intron could have been formed from a single deletion of 565 bp from the ancestral intron. The small intron appears to be a remnant left after the loss of most of the large intron. Those *N. galligena* isolates without an intron may never have possessed one, but it is more likely that they arose as a result of the loss of the entire intron.

The distribution of group I introns within the genomes of many organisms has often been attributed to lateral transfer mediated through transposition (Sogin & Edman, 1989). In *N. galligena* there is no evidence to suggest that the introns have arisen from horizontal transfer from another genome, due to the lack of homology with any published group I sequence data. The conservation of the intron insertion site and the homology of the primary sequence strongly suggests that intron variation within *N. galligena* is due to deletions from an ancestral intron which does not appear to be a typical group I intron.

It was not the intention of this study to attempt to link DNA categorization with provenance of the *N. galligena* isolates, especially as this has already been alluded to in the case of the 'A' isolates (Brown *et al*., 1994), but rather to identify regions of molecular variation in this fungus. It is, however, interesting to note that none of the 'NI' isolates were in 18S category 2 (those with the smallest intron), and of the isolates tested, those without an intron and those with the intermediate-sized intron were collected only in Northern Ireland. Nothing about the origin or spread of *N. galligena* in Northern Irish orchards could be deduced from the small number of isolates used in the present study; this will require a much larger sample of isolates of known provenance. In such a study, however, amplification of an NS 7 UP/*Ch*Int*rev* product specific to *N. galligena* will provide rapid confirmation of the identity of the fungus and reveal its 18S category in a single PCR reaction.

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