

**Molecular Phylogeny, Detection and Epidemiology of *Nectria
galligena* Bres. the incitant of *Nectria* Canker on Apple**

By

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Abstract

Nectria canker, incited by *Nectria galligena* (anamorph *Cylindrocarpon heteronema*), is prevalent in apple and pear orchards in all temperate growing areas of the world where it causes loss of yield by direct damage to trees, and rotting in stored fruit. Interpretation of the conventional epidemiology, from which current control measures are designed, is often inconsistent with the distribution of infections, particularly in young orchards, and may account for poor control in some areas, suggesting many original assumptions concerning pathogen biology and spread require revision. Earlier work has implicated nurseries as a source of infection. This thesis describes experiments designed to test this hypothesis and the development and application of molecular tools to examine intra-specific variation in *N. galligena* and its detection in woody tissue.

Two experimental trials based on randomised block designs were undertaken. In the first, trees comprising *cv.* Queen Cox on M9 rootstocks from five UK and five continental commercial nurseries were planted at a single site in East Kent. The incidence of *Nectria* canker over the ensuing five years was monitored. Significant differences in percentage of trees with canker between nurseries were observed, indicating a source effect. Analysis of data from a second experiment, comprising M9 rootstocks from three nurseries, budded with *cv.* Spartan material from a single source, showed significant differences in the frequency of disease incidence over the ensuing seasons. This latter trial provided strong evidence that rootstocks may be the source of the initial infection court, implying that symptomless infection can be 'transmitted' via the union graft to the developing scion. Results from both trials further imply that the period of latency associated with such infections may be longer than hitherto envisaged.

Two PCR-based methods, PCR-RFLP analysis of the rRNA inter-genic spacer region and arbitrary primed PCR techniques, were assessed for characterising intra-specific variation in *N. galligena*. Results of both approaches were largely congruent with the level of strain discrimination suitable for molecular ecological applications. Statistical analysis of the distribution of IGS PCR-RFLP haplotypes of isolates recovered from the

former trial indicated a relationship with the nursery origin of the tree. Although this was due exclusively to Nursery III consisting entirely of one particular haplotype, analysis of both trials provide additional confirmation that at least some part of initial infection observed in young plantations may originate in the nursery.

Two oligonucleotide primers, Ch 1 and Ch 2, were designed specifically for *N. galligena* from ITS sequence comparisons with closely related species of the *Nectria Coccinea* group, including *N. radicicola*, *N. cinnabarina* and species commonly associated with apple. Under optimal PCR conditons, a 412 bp fragment, specific to *N. galligena*, was amplified from total DNA extracts from infected woody tissue. Due to high levels of PCR inhibitors frequently encountered in these extracts, a magnetic capture-hybridization procedure was developed. Incorporation of this procedure into the conventional PCR assay resulted in increased sensitivity and the detection of the presence of the pathogen from symptomless material.

rRNA sequence analysis supported the retention of *N. galligena* as a single homogenous species, irrespective of derived host. Molecular characteristaion of Coccinea group species and strains of soil borne *N. radicicola*, were studied using rRNA spacer PCR-RFLP, arbitrarily primed PCR and ITS sequence analysis. Each set of molecular data was largely consistent. Coccinea species formed a monophyletic group, distinct from *N. radicicola*, exhibiting lower levels of inter-specific variation than observed intra-specifically between the strains of *N. radicicola* examined. ITS sequence phylogeny revealed an evolutionary association with *N. radicicola* which may infer a degree of ancestral biological function, in particular the ability to reside in, and infect from soil. In view of the challenges of the conventional epidemiological model as presented in this thesis, such an ability may help explain how propagated rootstocks, in particular rootstocks, as implicated in this research, may become initially infected at source.

The significance of infection originating from nurseries to the development of orchard epidemics and implications for control and disease management are discussed.

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**This Thesis is dedicated to the Memory of
Kathleen May Langrell**

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List of Abbreviations

m	metre(s)
cm	centimetre(s)
mm	millimetre(s)
g	gram(s)
mg	milligram(s)
μ g	microgram(s)
ng	nanogram(s)
pg	picogram(s)
fg	fentogram(s)
l	litre(s)
ml	millilitre(s)
μ l	microlitre(s)
ml ⁻¹	per millilitre
l ⁻¹	per litre
μ l ⁻¹	per microlitre
M	Molar
mM	milliMolar
nM	nanoMolar
μ M	microMolar
SDW	Sterile Distilled Water
EDTA	Ethylenediaminetetraacetic acid
CTAB	Hexadecyltrimethylammonium bromide
SE	Standard Error
ppm	parts per million
cv.	cultivar
cvs.	cultivars
var.	variety
ai	active ingredient
ED ₅₀	Effective Dose
DNA	Deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
ITS	Internal Transcribed Spacer
IGS	Inter-genic Spacer
LSU	Large Sub-Unit
SSU	Small Sub-Unit
mtSSU	mitochondrial Small Sub-Unit
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
PCR-RFLP	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
RAPD	Rapidly Amplified Polymorphic DNA
Kb	Kilobase

Kbp	Kilobase pair
bp	base pair
S	Swedberg, unit of sedimentation
v/v	volume per volume
V/cm	Volts per centimetre
UV	Ultra Violet
x g	times gravity
≅	approximately
min	minute(s)
°C	degree <i>Celsius</i>

Chapter 1. General Introduction

1.1 General

1.1.1 *Nectria* canker

Nectria canker, also known as apple canker, or European apple canker as it is commonly referred to in North America, is incited by the phytopathogenic fungus *Nectria galligena* (anamorph *Cylindrocarpon heteronema*). The disease is prevalent in apple and pear orchard plantations in virtually all temperate growing regions of the world, including all European countries east to the Ural mountain range, Iran, Iraq, Syria, Japan, Taiwan, Indonesia, New Zealand, Canada, USA, Mexico, Argentina and Chile (CMI Distribution Map No. 38, Edition 4), except Australasia, where a forty year eradication program at two locations in northern Tasmania (Stubbs, 1971; Sampson and Walker, 1982) has reputedly lead to its elimination (Ransom, 1997). The earliest recordings of the disease from some major apple and pear growing regions (appears to) coincides approximately with the introduction of rootstocks propagated vegetatively as clones instead of seedlings for the establishment of commercial plantations in North America (Nova Scotia and New York) (Paddock, 1900); South Africa (Zoutpansberg and Kimberley) (Doidge, 1924); New Zealand (Kokako, Whangarei) (Cunningham, 1925) and Chile (Tartakowsky, 1934). Although believed to have been a serious problem in Europe for centuries, the earliest documentation of *Nectria* canker in Britain dates from 1710 (Swales, 1921).

1.1.2 Economic importance

Nectria canker has long been considered one of the most destructive diseases of apple in Europe (Kennel, 1976; Swinburne, 1975) and humid productive regions of North America (Wilson and Nichols, 1964; Braun, 1997; Plante and Bernier, 1997). However, economic losses, in terms of reduction in crop yields, have never been accurately estimated, largely because they are difficult to quantify. As modern apple production systems increasingly require young trees with sufficiently developed, strong scaffold branch frameworks to bare a crop within two to three years of planting (Howard *et al.*,

1974), reduction in vigour, through establishment of infection, can lead to considerable losses through the development of cankers and the girdling of productive shoots, branches, scaffold limbs and even trunks. Tree mortality is possible in severe situations, and total losses of 5-10 % of trees from young plantations have been recorded, particularly in areas where the disease is prevalent (Lovelidge, 1995). Epidemics can make whole orchards uneconomic and in some regions have resulted in the removal of entire plantations (Jones and Aldwinckle, 1990). Trees infected in their later years of production are usually not as severely affected as young trees, although localised loss of productive wood is still possible. Pears are usually considered less affected than apple, although evidence supporting this claim is largely anecdotal (A. Berrie, Horticulture Research International, East Malling, personal communication, 1998). Reduced yields through direct loss of productive wood is further compounded by fruit rotting, both in orchard and post harvest storage, first recognised by Ferdinandsen (1922). Losses of 10-60% of the crop have been recorded from various parts of the world (Kavanagh and Glynn, 1966; Bondoux, 1967; McCartney, 1967; Agarwala and Sharma, 1968; Swinburne, 1970a; Burchill and Edney, 1972), with reported losses more severe in apples held in refrigerated controlled atmosphere (CA) stores than in barns (Swinburne, 1970a, Swinburne, 1970b, Swinburne, 1974). Reports on the reduction in yields tend to be predominantly anecdotal but losses of up to £5000 per 100 tons have been recorded from the south east of England (A. Berrie, Horticulture Research International, East Malling, personal communication, 1997). In the Netherlands annual losses have been estimated at ten million guilders (1975 prices) (Rijnders, 1975). Economic damage has also been reported for a range of forest hardwood species, particularly in North America. The species involved include *Betula lenta* and *B. alleghaniensis*, (Lortie, 1969; Plante and Bernier, 1997; S. Anagnostakis, Connecticut Agricultural Experimental Station, New Haven, Connecticut, personal communication, 1996), *Acer saccharum*, *A. rubrum*, *Populus* species (Lortie, 1969; G. Braun, Agriculture Canada, Kentville, Nova Scotia, personal communication) and *Juglans nigra* (Sinclair *et al.*, 1987). It has been identified as a component of the increasingly important beech bark disease complex affecting *Fagus sylvatica* and *F. grandifolia* (Houston, 1994). The presence of cankers on the

stem of infected trees, and associated pathogen invasion, can cause severe damage leading to significant reductions in log quality, value and inevitable loss in merchantable timber volume (Sinclair *et al.*, 1987; Houston, 1994; Plante and Bernier, 1997).

1.1.3 Early Nomenclature

Confusion regarding the early nomenclature of the true identity and taxonomic status of the causal organism of *Nectria* canker was in part due to overlapping host range and similar symptom development of two closely related species; *N. ditissima* Tul. and *N. coccinea* (Pers. ex Fr.) Fries, currently accepted as distinct taxa (Booth, 1959). Early research by both Hartig (1878) and Goethe (1880) attributed canker development to *N. ditissima* through their work on *Fagus*, *Quercus* and *Corylus*, and apple, respectively. Appel and Wollenwber (1910) supported this determination in inoculation experiments involving isolates of the same species taken from beech and apple cankers, which they identified as *Fusarium wilkommii*, the then accepted conidial state of *N. ditissima*. However, Weese (1911) concluded that *N. ditissima* was a synonym of *N. coccinea* and that this species did not cause canker, regarding it as a saprophyte. He defined the apple canker causing organism as the morphologically distinct species *N. galligena*, earlier proposed by Bresadola (Strasser, 1901). Wollenweber (1913) followed Weese's (1911) diagnosis for *N. galligena* and proposed the form genus *Cylindrocarpon* for the conidial state, naming it *C. mali* (Allesch.) Wollenweber. Voges (1914) rejected Weese's (1911) treatment of *N. ditissima*, however, Weese (1919) later re-examined Goethe's (1880) and Voges' (1914) original specimen material and confirmed that the apple pathogen was *N. galligena* and not *N. ditissima*, as they had each previously assumed. Agreeing with this finding, Ashcroft (1934) concluded that *N. ditissima* was distinct from *N. galligena* but synonymous with *N. coccinea*. In his view the canker causing organism on apple was *N. galligena*. *N. ditissima* was later shown to be identical to the specimens of the beech-bark disease and dieback pathogen collected by Ehrlich (1934) from Nova Scotia (Booth, 1959). Wollenweber (1924) re-established the identity of each of the three closely related species by conidial morphology, separating *N. ditissima* from both *N. galligena* and *N. coccinea* on macro and micro-conidial dimensions. In addition, Booth's

(1959) treatment accepts this separation based on perithecial characters, and from *N. coccinea* on the structure of the ascus.

1.1.4 Classification

The genus *Nectria*, the name originally proposed by Fries (1825) and later erected as a distinct taxa (Fries, 1849), is a large, economically important genus within the Ascomycotina, order Hypocreales, characterised by the production of colourless, non-apiculate, two to many celled ascospores within perithecia that are pigmented in shades of red and yellow (Samuels and Brayford, 1994). The modern concept of the taxonomy of the genus has primarily focused on perithecial wall structure for the separation of sub-generic groupings. Booth's (1959) treatment assigned each known British species of *Nectria* to one of nine proposed separate groups based chiefly on perithecial wall structure; *Cinnabarina*, *Coccinea*, *Aquifolii* and *Ochroleuca*, each exhibiting a normally well developed stroma, and *Mammoidea*, *Episphaeria*, *Peziza*, *Arenula* and *Lasionectria*, each characterised by a reduced or absent stroma, or replaced by a partial bysus. This classification was later shown to be broadly complimentary to the groupings assigned to the descriptions of the conidial states (*Cylindrocarpon*) (Booth, 1959; 1966) as originally proposed by Wollenweber (1913; 1917). The *Coccinea* group (equating with Wollenweber's original anamorphic *Willkommioetes* section (Booth, 1959), and later, *Cylindrocarpon* Group 1, as proposed by Booth (1966), in which *galligena* is placed, is separated from each of the other eight groups in having thick-walled globose cells in the outer layer of typically red and semi-translucent perithecial cells merging into thinner-walled, elongated inner layers (Booth, 1959). Morphologically, *N. galligena* was assigned to the *Coccinea* group, comprising *N. coccinea*, *N. coccinea* var. *faginata* Lohman, Watson and Ayers, *N. ditissima*, *N. punicea* (Schmidt ex Fr.) Fr. ex Rabenh., *N. punicea* (Schmidt ex Fr.) Fr. var. *ilicis* Booth, *N. fuckeliana* Booth, and *N. hederæ* Booth, (Booth, 1959). *N. fuckeliana* is an exception within the group, exhibiting atypical *Coccinea* perithecial wall features, but producing abundant microconidia in young cultures, consistent with *Coccinea* cultural characteristics. Taken together, the *Coccinea* group forms an important collection of tree saprobes and pathogens infecting the aerial

parts of woody plants, causing cankers or associated dieback symptoms of stressed hosts (Booth, 1959; Brayford, 1992). However, despite its relative economic importance only few of its over six hundred named *Nectria* species have been included in keys (Samuels and Brayford, 1994). Further, the original concept of the genus as outlined above is now considered to be artificial and attempts have been made in recent years to re-evaluate criteria of taxonomic significance in redefining genera and segregating sub-generic groupings (Samuels and Brayford, 1994).

1.1.5 Taxonomy and Spore Production

1.1.5.1 Asexual stage

The anamorph of *N. galligena*, *Cylindrocarpon heteronema* (Berk. and Br.) Wollenw., is generally considered to be haploid throughout the vegetative phase of its life cycle including when grown on a variety of media. On Malt Extract agar (MEA) or Potato Dextrose agar (PDA), growth is predominantly mycelial, being normally of a white, floccose appearance under normal conditions on 2-3 week old plates. Variably septate macroconidia develop as sporodochia from short cylindrical phialides, measuring 12-16 X 2-2.5 μm , borne on multi-branched conidiophores (Booth, 1966). Cylindrical shaped macroconidia are generally either straight or curved with rounded ends, with a mean size (varying according to the degree of septation), ranging from 10-28 X 4-5 μm for spores with one septum to 45-65 X 4-7 μm for those with four or more septa. The dimensions quoted are for material directly derived from host tissues or from artificial media (Booth, 1966). Hyaline, aseptate microconidia reflect the general cylindrical spore morphology, but are significantly smaller than macroconidia, measuring 4-8 X 2-3 μm . On susceptible hosts, conidia are produced in white to cream coloured, cushion-like sporodochia on, or near, the surface of an infected area, or more commonly along the folds of callus tissue of a young developing canker lesion.

1.1.5.2 Sexual cycle

The teleomorph of *N. galligena* is typical for a member of the ascomycetes. Thin walled clavate asci, measuring 75-95 X 12-15 μm , arise from the penultimate or terminal cell of ascogenous hyphae within the developing bright red, ovate perithecia, which are typically 250-350 μm in diameter (Booth, 1959). Perithecia are commonly found scattered, or in clusters, along the margins and within the cracks and callus folds of mature canker lesions. Typically, each ascus contains eight colourless, bicellular, oval, ellipsoid, or spindle shaped, hyaline ascospores measuring 14-22 X 6-9 μm , slightly constricted at the central septum (Booth, 1959).

Lortie (1964) reported the production of perithecial fruiting bodies on cultures derived from single ascospore isolates inoculated onto whole grain and powdered birch bark media. El-Gholl *et al.* (1986) also confirmed the homothallic nature of *N. galligena*, with isolates derived from *Swietenia mahagoni*. They found that perithecia developed in *in vitro* cultures initiated by transferring uninucleate cells taken from the distal ends of germinating macroconidia onto carnation leaf pieces resting on water agar. In contrast, the earlier work of Kruger (1974), to which El-Gholl *et al.* (1986) make no reference, concluded *N. galligena* to be heterothallic from the results of crosses between single-spore cultures of ascospore sets isolated from different localities, reporting the existence of two intrasterile, but interfertile mating groups, capable of producing perithecia. Mature ascospores of *N. galligena* are bicellular, each comprising two uninucleate cells, the homo- or hetero-thallic nature of which has not been fully resolved, although it may be possible that the sexual status is variable between strains in this species. Despite this, the general opinion, through further observations and genetic experimentation, accepts *N. galligena* as homothallic (Bernier and Hamelin, 1993; Plante and Bernier, 1995; L. Bernier, Universite Laval, personal communication, 1997), recognising some degree of possible out crossing under some conditions (L. Bernier, Universite Laval, personal communication, 1997).

1.1.6 Infection and colonisation

External windborne or rainsplashed inoculum from neighbouring sporulating lesions are generally considered the main source of infective inoculum in the infection process of lignified woody tissue as well as developing fruits. Entry of the host, and subsequent infection occurs primarily through wounds and natural openings, in particular leaf-scars (Wiltshire, 1921; Zeller, 1926). Penetration of intact bark has not been observed (Wiltshire, 1921; Zeller, 1926), although suggested for pear (Harris, 1925) and willow (Zalasky, 1968). In order to establish active infection, viable inoculum must reach the cambium where colonisation of parenchymatous cells and penetration of xylem tissues, such as xylem parenchyma, vessels, fibres, tracheids and medullary rays, is possible (Crowdy, 1949). Entry to xylem tissue by infective hyphae is either through pit cells (Crowdy, 1949) or medullary rays (Goethe, 1880). Further internal spread of infection is widely considered to be responsible for the extension of the lesions and ultimately in the severity of symptom development (Crowdy, 1949). Invasion and colonisation of the cortex and phloem tissues is mainly intercellular during the early stages of infection, becoming intracellular in the later stages (Crowdy, 1949). Infections confined to these peripheral tissues are subsequently checked to some extent by successive layers of wound phellogens (Crowdy, 1949, 1952). Colonisation of the deeper xylem and parenchymatous tissues is restricted by tyloses and gum barriers, and phellogen deposition, respectively (Crowdy, 1949, 1952). Callus wound tissue deposition is subsequently laid down defining the area of infection (Crowdy, 1952). Swelling around active canker lesions is a characteristic symptom and continues as the infection spreads in the lumen of xylem fibre tissues (Crowdy, 1952). Such swellings have been associated with changes in indolyl-3-acetic acid (IAA) and cytokinin concentrations within infected tissues (Beltra *et al.*, 1969; Taris and Clemencet, 1970). The observation that *N. galligena* can effectively kill host tissue cells, such as bark, cambium and the outermost sapwood, in advance of penetration and colonisation has implicated the role of a fungal toxin in the infection process (Zeller, 1926; Mooi, 1948; Crowdy, 1949). In addition, death of distal leaves prior to stem girdling may also indicate production of a diffusable toxin from within active lesions (Wormald, 1935; and personal observation).

1.1.7 Disease Symptoms

The first observable symptom of *Nectria* canker is the discolouration of the bark, which develops reddish colours then becomes blackened and sunken, followed by the successive deposition of callus layers around the area of necrosis. Typical 'open' canker lesions may develop on a range of host tissue of varying ages once infection has become established through an appropriate infection court (Crowdy, 1949). Lesion development is usually centered on leaf-scars, twig stubs, spurs, buds or other natural wound openings on younger fruit producing wood and extension shoots, in limb crotches or on the much older main stem (trunk) or rootstock. Tip die-back of young shoots, exhibiting no obvious infection focus, are also encountered, particularly on heavily infected trees where overt lesions are already evident on older, lower parts of the tree (T. Swinburne, Wye College, personal communication, 1995; and personal observation). Colonisation by invading inoculum within developing young cankers can be relatively rapid. Girdling of young 2, 3 or 4 year old productive wood can quickly follow, leading to death of the distal portions of shoot tissue. Crotch cankers may be particularly damaging, especially in relation to important branches and overall scaffold limb structure and physical strength of the tree. Invariably, canker infection leads to a loss of tree vigor and ultimately reduction in fruit production. On older wood, including main stems and rootstocks, expansion of lesions may continue over successive seasons resulting in a series of concentric ridges in the bark tissue giving the canker lesion a typical 'target' appearance (Crowdy, 1949). After infection has been established, the cortical and phloem tissues below the bark dry out, separate from the wood, crack, and later break away exposing the xylem (Crowdy, 1949). Perithecia may develop in the cracks at this stage. Complete girdling of main stems and rootstock is not uncommon, and under severe conditions heavily infected trees can exhibit all symptoms in a range of combinations, leading to reduced productivity and potentially limb or entire tree death. In addition to the usual range of 'open' canker symptoms, 'closed' lesions may form, typically on younger wood, which appear as shoot or branch swellings. Usually the bark above the swollen, invaded area is roughened and cracked, but often remains intact for several years (Crowdy, 1949). Sporodochial development on bark is characteristic of this form

of infection and may extend beyond the immediate area of swelling. All gradations between both 'open' and 'closed' symptom types, including tip die-back, may be encountered on trees in varying combinations depending on cultivar and level of infection.

Rotting by *N. galligena*, of apple fruits, and to a lesser extent pear, can occur on unripe and ripened fruit both pre and post-harvest. Eyerot, first recorded by Salmon and Wormald (1915), is typically considered to occur around the open calyx, and is characterised by circularly expanding slightly depressed, sunken, brown necrotic areas on the fruit surface accompanied by internal cortical degradation (Burchill and Edney, 1972; Swinburne, 1964). During the latter stages of fruit infection, white or yellow coloured pustules or mycelial mats may appear over the rot from which numerous conidia may be produced (Swinburne, 1964).

1.1.8 Host Range and Specialisation

Apart from apple and pear, *N. galligena* has been recorded on more than 60 tree and shrub species from over 20 genera, representing a wide spectrum in family diversity, including *Acer*, *Betula*, *Carpinus*, *Crataegus*, *Cydonia*, *Fagus*, *Fraxinus*, *Juglans*, *Magnolia*, *Populus*, *Prunus*, *Quercus*, *Salix* and *Sorbus* (Ashcroft, 1934; Lohman and Watson, 1943; Manion and Fench, 1967, Lortie, 1969), which was extensively reviewed by Flack and Swinburne (1977).

Little evidence of host specificity among isolates ascribed to *N. galligena* in cross-inoculation experiments is apparent from the literature. Ashcroft (1934), Lortie (1969), Ng and Roberts (1974), Barnard *et al.* (1988) and Plante and Bernier (1997) each reported isolates of *N. galligena* capable of infecting a wide range of hardwood hosts upon cross-inoculation, confirming the polyphagous nature of the pathogen (Plante and Bernier, 1997). Despite this, some notable exceptions do exist. Manion and French (1967) showed that although isolates derived from red maple (*Acer rubrum*) could cause canker on paper birch (*Betula papyrifera*) and balsam poplar (*Populus tacamahaca*)

using wound inoculations, attempts with a paper birch isolate failed to infect balsam poplar. Similarly, Dubin and English (1975) indicated failure of isolates derived from three North American hardwood species, viz. *Betula papyrifera* (paper birch), *B. alleghaniensis* (yellow birch) and *Populus grandidentata* (bigtooth aspen), to infect two year old apple trees of cultivars Gravenstein and Golden Delicious when inoculated at leaf scars. Lortie (1969) suggested that wounding was a prerequisite to infection by *N. galligena* and discrepancy between results may be due to the method of inoculation employed. Richter (1928) reported the occurrence of variability in the pathogenicity and specificity of *N. galligena* by showing that isolates from ash (*Fraxinus* spp.) were unable to infect *Malus*, *Fagus*, *Populus* and *Acer*. Wollenweber (1924) had earlier proposed that isolates derived from ash represented a distinct variety from those on apple, based exclusively on spore dimensions, and named those on ash as *N. galligena* var. *major*. This was rejected by Ashcroft (1934), and later by Booth (1959) who could not justify the separation morphologically, but recognised a possible degree of physiological specialisation. An account of two 'varieties' of the pathogen from Chile, based on conidial dimensions and pathogenicity towards apple, has never received any serious attention (Vergara Castillo, 1953), and most probably represented intraspecific variation based on these characters. Spaulding *et al.* (1936) reported that white ash (*Fraxinus americana*) was resistant to infection by isolates recovered from other deciduous species, including beech, maple, apple, aspen, elm, birch, oak and hickory, re-confirmed more recently by Plante and Bernier (1997). Flack and Swinburne (1977) indicated differences in host range and symptomology between isolates derived from either apple or ash, supporting the earlier findings of Richter (1928), and proposed the designation of two distinct formae speciales; *N. galligena* f.sp. *fraxini* on common ash (*Fraxinus excelsior*) and *N. galligena* f.sp. *mali* on apple, as previously suggested by Booth (1959).

In apple, complete resistance is not known to exist (van de Weg, 1989) and all recognised cultivars are considered susceptible to varying degrees, suggesting resistance is under polygenic control. Preliminary studies on the genetics of virulence of *N. galligena* towards apple (cv. Golden Delicious) and yellow birch (*Betula alleghaniensis*)

supports this proposition (Plante and Bernier, 1995; Plante and Bernier, 1997) which may partially account for the extensive host range exhibited by this species, although much further research is required in this area.

The centre of origin of this species remains unclear, obscured in part, by its limited host specificity and non-exclusive association with any one given host or narrow group of host species.

1.1.9 Epidemiology

The aerial discharge and rainsplash dispersal of both ascospores and conidia are features central to the interpretation of disease spread. Wiltshire (1921) was the first to investigate the significance of air-borne inoculum to the spread and subsequent establishment of the disease in apple and outlined stages in the development of leaf scar infection shortly after the taxonomic confusion regarding the true identity of the causal organism had been elucidated (Goethe, 1904), even though the association of infection with buds and frost damage was earlier recorded by Goethe (1880). Wiltshire's (1921) initial assumptions, made at Long Ashton, rest wholly on the incidence and development of canker lesions from regions of buds of some seedling trees of the cider cross, Kingston Black X Medaille D'Or (Wiltshire, 1921). Wiltshire (1921) also recorded a dramatic increase in incidence of cankers on shoots of these trees over five consecutive years (1915-1919). As these trees were never pruned, Wiltshire took the residual count of cankers between years as an indication of the number of individual aerial infections of buds during the previous growing cycle/year (Wiltshire, 1921). Wiltshire also made observations of other cultivars at Long Ashton, on adjacent plots of Cox's Orange Pippin, King of the Pippins, Worcester Pearmain, Devonshire Quarenden and Beauty of Bath (each considered susceptible to canker). Individual trees exhibited wide discrepancies in the number of bud infections which developed during the winter 1919-20, ranging from 0 to 149 lesions, a result Wiltshire was unable to explain (Wiltshire, 1921).

Based on his observations, Wiltshire (1921) proposed two predominant periods of leaf scar infection, namely early autumn and spring, coinciding with leaf fall and bud swell respectively (Wiltshire, 1921). Scab wounds (caused by *Venturia inaequalis*) were also considered to act as infection courts (Wiltshire, 1922). These periods correspond closely with the observations of Cayley (1921), one of the first to characterise the life cycle of *N. galligena* associated with apple infection, who observed macroconidia release during the months of September and October and perithecial discharge in spring, mostly in April. Although ascospores and conidia can be found on cankers throughout the year, their production and release have been shown to be dependent on rainfall. Cayley (1921), and later Wiltshire (1922), defined the periods of maximum aerial dispersal for both ascospores and conidia in Southern England with the objective of timing fungicidal spraying programmes with maximum effect. Interestingly, subsequent research from various apple producing regions of the world have timed aerial dispersal to different periods of maximum spore release, even over relatively short geographical distances, implicating environmental factors, particularly rainfall, in the epidemiology of the disease. Munson (1939), surveyed spore release at Long Ashton through the use of vasaline slide spore traps positioned adjacent to perithecial fruitifications on orchard trees. He reported high levels of ascospore discharge in all rainfall periods from November to April or May, and maximum conidial release in the autumn, supporting earlier observations (Wiltshire, 1921; Cayley, 1921; Wiltshire, 1922), but in general defining the continual presence of inoculum throughout the year (Munson, 1939). Employing a similar approach, Marsh (1940), also working at Long Ashton, reported the importance of rainfall in conidial release from spring to autumn, as well as a close correlation with ascospore discharge, but not temperature, humidity, wind or sunlight.

At East Malling, Moore and Bennett (1960) attempted to establish the period(s) of greatest natural infection from autumn to spring by affixing mature cankers exhibiting sporodochia or perithecia to singly planted 'bushes' of M8 rootstocks in a row and recording the incidence of natural infection over the following three growing seasons (1947-50). Each of six sections of the row received a one month exposure period to the

inoculum source from November to March (later extended from October to April) and the incidence of canker was recorded at the end of each season. From their results, the incidence of infection was found to peak in November and December, with a secondary increase in March, which they associated with leaf fall and bud burst, respectively (Moore and Bennett, 1960).

On the continent, Saure (1962), reported a close correlation between the amount of rainfall in summer and autumn with the inception of perithecial development in the apple producing regions of Germany. The commencement of ascospore discharge was shown to differ over two consecutive years (November 1959 to August 1960) and was related to the timing of perithecial formation (Saure, 1962). These findings were later supported by the work of Kennel (1963) who reported a rapid development of the perithecial stage in abundant humidity, being most rapid during the summer months (July-September). Additionally, Saure (1962) observed rapid perithecial development from three to four month old artificial infections. This was considered unusual, as perithecial development was normally not encountered until the following year after infection or on older mature cankers (Saure, 1962). In Ireland, Loughnane and McKay (1959) noticed a similar early emergence of perithecia on current seasons growth of the cultivar Laxton's Superb following the wet summer and autumn of 1958. Working at Versailles, Bulit (1957) examined the conditions necessary for the emission of spores in France through a similar spore trapping approach to that of Munson (1939). Based on the observations of a given canker, Bulit (1957) reported ascospore discharge continued for over a year, particularly during rainy periods, with maximum emission recorded for autumn. Working with four French cultivars; Calville blanc, Reinette blanche de Canada, Reinette grise de Canada and Belle fille rose, (during 1953-55) he also confirmed macroconidial production is, in general, restricted to winter and the beginning of spring (Bulit, 1957). In both cases he directly correlated ascospore and conidial liberation to the level of rainfall, quoting abandonment of spore production during periods of fine weather (Bulit, 1957).

In Northern Ireland, Swinburne (1971), employing a spore trapping procedure based on the same technique as Munson (1939), surveyed spore release over a three year period (1968-1971) from fruiting cankers of indeterminate age on trees of *cv.* Bramley's Seedling. Results from this region revealed a maximum release of conidia from early summer to late autumn and spring to early summer (with a second minor period of discharge in autumn) for ascospore discharge (Swinburne, 1971) in slight contrast to the lower proportion of the annual total ascospore release during the same yearly period recorded in south-west England by Munson (1939) and the earlier observations of Wiltshire (1921, 1922), and Cayley (1921). In addition, Swinburne (1971), showed spore discharge in Northern Ireland was more closely related to the number of hours of leaf wetness than to the volume of precipitation, suggesting differences in seasonal spore discharge between localities with similar levels of rainfall might be due to this factor.

In Oregon, Zeller (1926), who conducted the first major study of the disease in North America, timed the commencement of ascospore discharge to two months after the onset of autumn rains continuing until the the dry summer period, observing more severe damage on pear trees than on apple (Zeller, 1926). In California, Dubin and English (1975), surveyed conidial dispersal on three cultivars (Red Delicious, Golden Delicious and Rome Beauty) on a weekly basis employing a plastic funnel and receptacle positioned on the under side of cankers to collect rainwashed conidia over the winter period 1970-71 (Red Delicious only) and 1971-72. Relatively high concentrations of conidia were recorded over the period November to the end of April, when winter rains decreased greatly (Dubin and English, 1975). Peak release was found during November and December, correlating well with the much earlier observations of Zeller (1926) in Oregon. In contrast, Lortie and Kuntz (1963) recorded conidial release from May to August on cankers occurring on *Betula alleghaniensis* near Quebec. Using vaseline spore traps they recorded peak conidial release in May and June over the period August 1959 to September 1960, but similar to the experience of Bulit (1957) in France, they recorded a continual discharge of ascospore throughout the year, peaking in August and September. In South America, Vergara Castillo (1953), and later Lolas and Latorre

(1996), employing similar techniques to that of Dubin and English (1975), reported maximum ascospore discharge and conidial release during the periods of spring and autumn, respectively, primarily on cultivars of the Delicious type from the Central Valley region of Chile.

1.1.10 Control

Current strategies for the prevention of *Nectria* canker are based on the application of protectant fungicides during those times of the year in which both inoculum and infection sites are available, namely autumn and spring. Initially, the recommended programmes were based on fungicides containing copper or mercury (Wiltshire, 1921; Byrde *et al.* 1952, 1965) which soon became established as the standard procedure in canker control in the United Kingdom and in most other apple producing regions of the world (Vergara Castillo, 1953; Brook and Bailey, 1965; Mulder, 1966; Wilson, 1968; Bennet, 1971a; Corbin, 1971). Classical Bordeaux preparations eventually gave way to those containing mercury, such as phenyl mercury chloride (PMC), which was found more effective in control (Byrde *et al.*, 1952, 1965; Moore and Bennett, 1960; Bennett, 1971a, 1971b). Later, phenyl mercury nitrate (PMN) fungicides replaced PMC as the most effective organo-mercury compound against canker (Mulder, 1966; Bennett, 1971; Swinburne *et al.* 1975).

However, increasing environmental concerns regarding the use of organo-mercury compounds as pesticides, particularly as applied sprays, led to them being superseded by less toxic materials. Evaluation of new compounds and the re-evaluation of copper-based formulations for canker control was investigated in detail during the 1980's (Berrie, 1991; Cooke *et al.*, 1993; Cooke and Watters, 1994). Typically, sprays are applied at 5% and 50% leaf fall and again in spring for continuous leaf-scar protection. Applications of the benzimidazole Carbendazim during summer months to the control of canker, particularly in orchards where significant levels of infection has already been established, are particularly common in Northern Ireland (Cooke *et al.*, 1993; Cooke and Waters, 1994). Today, use of recommended fungicides for the control of apple scab

(*Venturia inaequalis*) and mildew (*Podosphaera leucotricha*) in summer simultaneously reduce canker (Cooke and Watters, 1994).

In addition, appropriate orchard management and sanitary measures, used as part of an integrated programme, compliment chemical treatments, and may lead to satisfactory control of the disease. Excised cankers should be removed and burned including, under severe outbreaks, entire trees. In addition, good orchard practice, such as maintaining orchard floor hygiene is recommended as cankered wood left on the orchard floor can continue to sporulate for up to a period of 2 years (Saure, 1962), as well as infected fruits, which can become mummified while still on the tree, becoming a further source of infection (Dillon-Weston, 1927).

1.2 Molecular Taxonomy of Fungi

1.2.1 Sources of Genetic Variation in Filamentous Fungi

The study of the extent, frequency and nature of intra-specific genetic variation in filamentous fungi is central to the elucidation of evolutionary processes in population structures, dynamics and the emergence of new specific or intra-specific forms such as varieties, *formae speciales*, races, and under certain agricultural regimes, fungicide resistant strains. Host selection and chemical control practices can each induce directional constraints that may lead to population genetic shifts, or other micro-evolutionary events under modern crop production systems, in phytopathogenic fungi (Hansen, 1987). Traditionally, population structures of fungal plant pathogens were investigated almost exclusively by testing for conventional genetic characteristics such as virulence traits, pathogenicity and anastomatic or vegetative compatibility.

Despite recent advances, the mechanisms responsible in generating variation are poorly understood. Apart from established processes identified in sexually reproducing fungi, recombination frequencies may be low or non-existent in nature (Michelmore and Hulbert, 1987), resulting in limited or infrequent allelic assortments exhibiting low levels of variation. Further, the potential for intra-specific gene flow is still also difficult

to determine in natural conditions (Michelmore and Hulbert, 1987). Asexual fungi (*Fungi Imperfecti*) lack a known sexual phase to their life cycle, but in many cases are capable of rapid evolution over relatively short periods of time, e.g. the emergence of pathogenic *formae speciales* and physiological races such as in *Fusarium oxysporum* (Gordon and Martyn, 1997; Daboussi, 1997). Continuous evolution and the emergence and establishment of genetic changes continues to cause considerable controversy in the development of biological species concepts (Mayr, 1988). Although the ability to interbreed sexually has been accepted by most mycologists as the main criterion in species definition (Seifert *et al.*, 1995), population parameters, as adopted by Brasier and Hansen (1992) in their treatment of species in the genus *Phytophthora*, help define the concept of biological species as operational units of evolution comprising populations that share a common lineage and have maintained genetic similarity in morphology, physiology and ecological behaviour (Love, 1964). Geographic and genetic isolation, predominately through the regulation of heterokaryon formation via vegetative incompatibility processes can serve to sub-divide fungal populations and lead to changes attributable to standard population genetic forces such as mutation, selection, migration and drift (Hartl and Clark, 1989), or a suite of DNA turnover mechanisms (Dover, 1982). Such events may lead to the emergence of new forms or physiologically specialised races depending on prevailing environmental parameters. Individuals within given Vegetative compatibility groups (VCG) are potentially capable of exchanging genetic information via a parasexual cycle (Leslie, 1993; Pontecorvo, 1956), although this has not been established in natural populations (Glass and Kulda, 1992).

1.2.2 Possible sources of selection on *Malus* derived *N. galligena*

Resistance to *N. galligena* has not been observed in any of the known cultivars of *Malus X domestica*, although some appear to be more prone to *Nectria* canker than others. The apple cultivars used for commercial production have either arisen from chance seedlings, often of unknown parentage, or through successive introgressive breeding programmes from a particularly narrow genetic base (Watkins, 1995). Any differential resistance would be incomplete, or horizontal and expressed as differing rates of disease

progression and severity of the symptoms induced. Such resistance is understood to be controlled polygenically and is quantitative in its inheritance, mechanism(s) and epidemiological effects (Robinson, 1976). Consequently, no host specificity has been reported from the *Malus domestica*/*N. galligena* pathosystem which would ultimately act as a selective pressure on a given pathogen population. However, the tendency to use a very small number of scion cultivars with a limited range of clonal rootstocks has probably influenced the structure and variability of extant populations of the pathogen in commercial plantations (although a differential varietal set and definitive inoculation assay which could reveal finer details of host pathogen interactions in this pathosystem is lacking). Evidence from cross-inoculation experiments exists for varying levels of host specificity from natural hardwood populations, although in most cases this is not complete (Plante and Bernier, 1997; Flack and Swinburne, 1977; see Chapter 3, point 3.4).

1.2.3 Molecular characterisation of Fungi

Traditionally, fungal classification and identification has relied almost exclusively on phenotypic characteristics such as the morphology of spores, hyphae and organised structure of fruiting bodies, and to a lesser extent colony morphology. Later, simple biochemical and physiological characters such as the presence or absence of chitin, growth and colony characteristics on defined media and vegetative compatibility gained taxonomic credence. However, morphological features/phenetic characters by which species are defined are often insufficiently distinct to resolve closely related species. Many important taxonomic characters have ranges that overlap, in addition, intra-specific plasticity of phenetic characters is often observed in filamentous fungi where identification based on morphometric assessments can often differ considerably from the type species. Further, morphological characters are often not applicable in distinguishing varietal or formae speciales status of plant pathogens that exhibit physiological specialisation with a given host or narrow range of hosts. The difficulty to resolve at or below the species level continues to confound and frustrate research and hinder progress in the area of conventional fungal systematics, especially in problematic taxa such as

Phoma, *Colletotrichum*, including the *Fusaria*, and in population and epidemiological applications.

The advent of molecular biology and the continued development and diversification of nucleic acid based technologies such as conventional hybridisation genetic markers, PCR based methodologies and advances in gene sequencing technology, have found direct application in fungal systematics (including evolutionary relationships), identification, species delineation, population studies, diagnostics and detailed molecular genetic analysis, offering a level of specific and sub-specific resolution previously unattainable. A growing range of techniques are now available for addressing taxonomic problems in fungal systematics over a wide range of evolutionary divergence, in particular at the specific and sub-specific level where resolution by conventional means has been problematic or impossible. Molecular approaches are set to continue to be widely exploited to characterise relationships at varying taxonomic levels across the fungal kingdom.

1.2.4 Molecular markers

The development and application of molecular genetic markers is well established in the molecular characterisation of fungi for a wide range of research and identification purposes. Central to applying molecular approaches to assessing inter and intra-specific variation is a consideration of procedures which reveal levels of discrimination between genotypes appropriate to the objective of the intended study. The range and variety of molecular marker systems applied to all the major phytopathogenic fungal taxa has increased considerably since the review of Michelmore and Hulbert (1987). Weising *et al.* (1994) identified the most important properties of a molecular marker as those that exhibit both a highly polymorphic and co-dominant behaviour (permitting homo- and heterozygotic discrimination in diploid organisms), and are evenly, as well as frequently, distributed throughout the genome for maximum genetic informativeness. Bruns *et al.* (1991) have reviewed a range of molecular methods and their usefulness in fungal systematics at varying taxonomic levels.

Conventional restriction fragment length polymorphisms (RFLP) analysis on total genomic or maternally inherited fungal mitochondrial genomes, using a range of single to multi-copy homologous or heterologous gene probes, have been identified that discriminate well at the population level in fungi. Multilocus DNA fingerprinting, distinguishable from conventional RFLP analysis by the use of anonymous repetitive DNA probes to repetitive sequences distributed as interspersed or tandemly repeated sequences throughout the genome have also been widely exploited.

Both approaches, although proven in the study of genetic variation, especially at the population level, are relatively expensive, technically demanding, labour intensive, and, in the case of radioactive labelling, potentially hazardous in their application. Alternative methods that combine ease of operation, cost and rapidity with similar or improved levels of polymorphic resolution are now becoming established as methods of choice in studies of genetic variation and molecular characterisation of fungi (reviewed by Bruns *et al.*, 1991).

1.2.5 The Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR), a method whereby DNA is enzymatically amplified exponentially *in vitro* with the use of thermostable DNA polymerase in the presence of short oligonucleotide primers sequences (and a number of reaction components), has revolutionised the study of fungal biology, proving indispensable as a research tool in modern molecular mycology (Foster *et al.*, 1993). Since its first introduction, PCR has found a wide and effective range of diverse applications in fungal systematics, detection, population genetics and gene cloning in areas such as medical and industrial mycology, soil and environmental microbiology and plant pathology.

Arbitrary primed PCR, either randomly amplified polymorphic DNA (RAPDs) (Williams *et al.*, 1990) or mini or micro-satellite based primers (single primed arbitrary reactions), are useful techniques for discriminating fungi at the population level. RAPDs have been extensively used in a range of applications including the assessment of genetic

diversity (Hamelin *et al.*, 1993), genetic mapping, linkage analysis (Paran and Michelmore, 1993) and for differentiating races and pathotypes within *formae speciales* of *Fusarium* species (Crowhurst *et al.*, 1991; Grajal-Martin *et al.*, 1993; Kelly *et al.*, 1994). The more technically robust random amplified microsatellite technique (Zietkiewicz *et al.*, 1994) is becoming an established complementary technique in population studies (Hamelin *et al.*, 1993; Hantula and Muller, 1996; Stenlid *et al.*, 1994) and, increasingly, in epidemiological applications. More recently, the development of Amplified Fragment Length Polymorphisms (AFLPs) (Vos *et al.*, 1995) has been found useful in resolving variation between fungal genomes (Majer *et al.*, 1996). In addition, PCR access to the variable non-coding nuclear ribosomal RNA (rRNA) gene spacer regions, including both the internal transcribed (ITS) and the intergenic (IGS) spacers (White *et al.*, 1990), and mitochondrial rRNA (White *et al.*, 1990) has facilitated direct restriction fragment length polymorphism (RFLP) and sequence analysis in a growing number of applications including species delineation (Edel *et al.*, 1996; O'Donnell, 1992), species complex and sub-generic aggregate resolution (Bryan *et al.*, 1995; O'Donnell and Gray, 1995) and phylogenetics (Cooke and Duncan, 1997; Morales *et al.*, 1995).

1.2.6 Phylogenetics

Two fundamental evolutionary patterns are the source of sequence divergence exploited in molecular phylogeny: the first that DNA evolves over time, acquiring substitutions, insertions and deletions, and the second, that separate lineages evolve independently once they diverge from each other and for the most part, acquire different mutations, and at different rates, from each other (Bowman, 1995). Fungal ribosomal RNA (rRNA), comprising functionally conserved genes coding for the 5.8S, small sub-unit (SSU) and large sub-unit (LSU) rRNA and associated non-coding spacer regions (Moss *et al.*, 1985), have attracted considerable interest in fungal molecular systematics for their versatility in inferring phylogenetic relationships at varying taxonomic levels as the various genes, and their spacer regions, evolve at different rates (Hillis and Dixon, 1991). Each successive ribosomal operon, organised in multicopy tandem arrays, is

separated by a region of DNA comprising an external transcribed spacer region (ETS), containing ribosomal transcription signals upstream of the SSU rRNA gene (Moss *et al.*, 1985), and a non-transcribed spacer region (NTS), collectively known as the intergenic spacer region (IGS). Sizes of entire rRNA repeat units vary in size and copy number.

The non-functional internal transcribed spacer (ITS) regions (ITS 1 and ITS 2), flanked by the SSU and the LSU rRNA respectively and separated by the 5.8S, lack a functional role (Nues *et al.*, 1994), which is thought to explain the high levels of sequence variation observed within them. Universal primers designed against conserved flanking SSU, 5.8S and LSU rRNA sequences has facilitated direct access to these regions by PCR amplification (White *et al.*, 1990), allowing direct characterisation by sequencing or direct restriction enzyme digestion analysis (e.g. *Phytophthora* (Cooke and Duncan, 1997), *Fusarium* (Waalwijk *et al.*, 1996).

The NTS is considered the most rapidly evolving spacer region (Hoshikawa *et al.*, 1983) comprised predominantly of tandem arrays of subrepeating elements which are widely believed to serve as enhancers for the control of pre-ribosomal transcription (Moss *et al.*, 1985; Reeder, 1984). PCR-RFLP of the IGS appears useful for assessing and characterising variation at the population level in a growing range of fungi including *Fusarium oxysporum* (Apples and Gordon, 1995; Edel *et al.*, 1995), *Chondrostereum purpureum* (Ramsfield *et al.*, 1996), *Armillaria* spp. (Harrington and Wingfield, 1995), *Tuber* spp. (Henrion *et al.*, 1994) and *Saccharomyces* spp. (Molina *et al.*, 1993). However, the utility of sequence comparisons in this region depend on mechanisms by which the region evolves, secondary structures and the presence of pre-transcriptional enhancer sequences (Baldrige *et al.*, 1992; Appel and Gordon, 1996).

Both the LSU (Guadet *et al.*, 1989) and the SSU (Harrington *et al.*, 1999), perhaps the slowest evolving regions of the rRNA operon, and good candidates for exploring ancient evolutionary events, have both been exploited in inferring relationships at higher taxonomic levels.

The process of concerted evolution (Arnheim *et al.*, 1980) has been proposed to account for the high degree of homogeneity observed between the ribosomal multicopy arrays and their associated spacer regions, particularly the IGS region, within species allowing their effective treatment as single copy genes. Both unequal crossing over and gene conversion have been proposed as the operative mechanisms responsible for this process. Although the relative contribution of both these mechanisms to rRNA homogenisation remains debatable (Dover, 1982), the process(es) continue to maintain the usefulness of rRNA multicopy operons in phylogenetic analysis at varying levels of systematic relatedness.

Advances in sequencing technology coupled with the further development of 'universal' primers to mitochondrial RNA (White *et al.*, 1990) and other unlinked genes (O'Donnell and Cigelnik, 1997; Glass and Donaldson, 1995) continues to facilitate direct access to DNA sequences of phylogentic informativeness. The impact of molecular characters and DNA sequences to fungal systematic, phylogenetics and evolutionary history has been extensively reviewed by Bruns *et al.* (1991) and Samuels and Seifert (1995).

1.2.7 Molecular detection

Accurate, fast and reliable plant disease diagnosis is essential to effective disease management and in preventing crop losses, especially at an early stage of infection (Seong, 1988). Adaptation of existing molecular biological technologies for the nonconventional diagnosis of plant diseases at early stages of development using nucleic acid based techniques are increasingly being developed, promising to revolutionise the approach to practical plant disease management, optimizing fungicide application and control measures in the field. Earlier molecular approaches exploited species specific single copy anonymous sequence probes for dot-blot hybridization detection from total tissue preparations. However, with the advent of PCR, molecular detection has largely given way to the amplification of target DNA through the exploitation of species specific primers, sometimes designed to probes used in earlier diagnostic applications, or to

intronic sequence divergence in readily accessible genes such as rRNA ITS spacers (reviewed by Miller and Martin, 1988; Miller, 1996; Henson and Fench, 1993; Annamalai *et al.*, 1995).

PCR detection systems can be made as broad ranging or specific in their detection of species as is appropriate for the particular diagnosis in question, depending on the design of primers to regions of sequence divergence between the organism(s) of interest and those to be excluded from detection. Overall, diagnostic primer design depends on the same fundamental evolutionary patterns responsible for sequence divergence exploited in inferring molecular phylogenies (see Chapter 1, point 1.2.6). The essence of diagnostic PCR design strategy is that where sequences unite the species of interest and differ from those of non-target organisms, these differences are exploited for primer and, to a lesser extent, probe design, allowing the exploitation of such evolutionary patterns for the development of PCR detection systems at different taxonomic levels. These principles have been widely used in the design of diagnostic primers for a wide range of plant pathogenic fungi from across the fungal kingdom (see Chapter 4, point 4.1).

1.3 Origin of *N. galligena* responsible for epidemics in young orchards

Whilst the control measures described in point 1.1.10 are effective under experimental conditions, serious epidemics continue to occur, particularly in young plantations where the origin of the inoculum responsible remains unclear. The conventional epidemiology of the disease, since Wiltshire's pioneering observations (Wiltshire, 1921), has long since implicated rain splashed ascospores and air-borne conidia, both considered functional inocula, to the spread, transmission and incidence of the disease. Consequently, independent infection events of aerial origin at susceptible infection courts such as leaf scars, scab wounds, (Wiltshire 1921, 1922), points of attachment of fruits, spurs (Kennel, 1963), pruning cuts, bud fissures, frost cracks, growth cracks and damage wounds (Swinburne, 1971), became firmly established as the central component in the epidemiology of this disease (Wiltshire, 1921; Zeller, 1926; Marsh, 1939; Bulit, 1957; Kennel, 1963; Saure, 1962; Wilson, 1966; Swinburne, 1971; Dubin and English,

1974, 1975). If this were so, then the most likely sources would either be alternative hosts in hedgerows or neighbouring infected orchards. Four facts relating to such epidemics in young orchards make such sources less probable;

(1) They can occur in the absence of susceptible hosts in the hedgerow, now predominantly alder, and in locations remote from any obvious source of infection including infected orchards,

(2) The distribution of infections within such orchards often appear random, not relating to any obvious disease gradient, moreover, individual trees can become severely infected, whilst neighbouring trees remain canker free, as was originally noted by Wiltshire (1921) and many workers since (McKay, 1947; Brown *et al.*, 1994; T. R. Swinburne, Wye College, personal communication, 1994; D. Rosenberger, Cornell Hudson Valley Laboratory, personal communication, 1996; personal observation),

(3) The surface area of typical infection courts (in particular leaf scars) implicated in the conventional epidemiological model are small in relation to the total surface area of the tree and are only receptive to infective propagules for short periods, becoming less susceptible to infection shortly after exposure (Crowdy, 1952; Dubin and English, 1974). Under the conventional model, such infection site resistance should reduce their significance in the spread of the disease. However, it is difficult to reconcile this phenomena with the simultaneous appearance of many cankers on individual trees in young orchards where there is no obvious local source of inoculum. For such events to result from air or splash dispersed inocula, it would have to be assumed that the normal distribution of propagules from a prime source was subverted in some way.

(4) Air-borne conidia are seldomly dispersed by wind but are often washed off by rain, appearing in the water running down the tree (Swinburne, 1975). Ascospores, considered better adapted for long distance dispersal as they are forcibly discharged from mature swollen perithecia, are produced in comparatively low numbers, ejected in groups of

eight within asci and, being relatively heavy, generally require high winds to be disseminated over large distances. In addition, the ascus ejection mechanism frequently fails in wet weather, when asci and ascospores emerge as an ooze which is often washed off by rain (Swinburne, 1975). Although both Munson (1939) and Mulder (1966) reported maximum spore dispersal of 10m and 125m, respectively (relatively small distances in relation to modern orchard dimensions), they had no effective way of discriminating infection types or linking inoculum sources within their studies.

Thus, whilst tree to tree transmission is clearly possible, it seems doubtful if aerial-borne or rain splashed inocula could be responsible for the incidence of severe infections occurring individually, deep within the centres of otherwise healthy, canker free orchards, within a few seasons of planting. For these reasons the classical interpretation of the epidemiology of the disease, as described in previous sections, was challenged by an Apple and Pear Research Council (APRC) funded research project initiated in 1991. Li (1995) examined a range of isolates obtained from overt canker infections in young orchards and noted variation in sizes of conidia between isolations. From detailed morphometric analysis it was shown that variation in conidial size was not related to cultivar, or locality of the orchard from which the samples were taken, but was related to the nursery source in which the trees were raised (Li, 1995). A further study of these isolates employing conventional RFLP techniques (Brown *et al.*, 1994) revealed a general association with the nursery source. If, as these studies suggested, infections originate in the nursery and are later expressed in the orchard, then it must be assumed that infections can remain unexpressed for longer periods than has been suspected. Using monoclonal antibody techniques Dewey *et al.*, (1995) showed that hyphae could be detected within xylem tissue at sites remote from active lesions. Systemic invasion was also confirmed by direct isolation from symptomless young shoots of the susceptible cultivars such as Discovery (Li, 1995). The implication of these findings suggest that at least some of the observed infections in young orchards are caused not by external inoculum arriving at susceptible infection court(s), but arise from cryptic infections already present within the tissues. However, as there is no information on the mechanism

of infection within nurseries, and no data which can reveal the frequency of such infection or it's relative significance as opposed to direct tree to tree transmission, this current study was undertaken to address the research objectives as outlined in section 1.4.

1.4 Research objectives

The objectives of this research were to;

1. To appraise the significance of nursery sources to the incidence of orchard infections.
2. To identify and develop informative PCR based molecular genetic markers for the molecular characterisation of *N. galligena* and their ability to distinguish intra-specific variation for use as research tools for molecular epidemiological applications.
3. To develop a robust, sensitive, species specific PCR based diagnostic assay based on phylogenetic divergence characterised in objectives 2 and 4 to detect and quantify *N. galligena* from infected apple propagating material as a research tool in support of objective 1.
4. To appraise the molecular phylogenetic relationship of *N. galligena* with members of the *Coccinea* group and the aggressive ubiquitous root rot pathogen *N. radicicola* through direct ITS sequence analysis and the application of molecular markers development in objective 2.

This research initially formed part of an Apple and Pear Research Council funded project and later a much larger strategic research programme sponsored jointly by the Apple and Pear Research Council and the Ministry of Agriculture Fisheries and Food (OC9518). The molecular tools developed and results of this research formed an integral part of this strategic research programme further evaluating the implications of the results of this thesis.



Chapter 2. Variation in the incidence of *Nectria* canker in apple trees obtained from different nurseries

2.1 Introduction

Wiltshire (1921) first demonstrated that infection could be established by placing conidia on freshly exposed leaf scars. This and subsequent studies (Zeller, 1926; Crowdy, 1952; Bennett, 1971; Swinburne, 1971) led to the general assumption that all infections took place through such natural openings and pruning wounds and that each lesion was the result of a separate event. The first spores to be produced on new canker lesions are conidia. These are predominantly dispersed by rain-splash with a range estimated to be 10m (Munson, 1939; Taylor and Byrde, 1954). Perithecia do not normally develop in the first year following infection but appear in the second and subsequent years (Saure, 1962). Ascospores are violently discharged from perithecia and it has been assumed that they are disseminated by wind and consequently constitute the primary means of long distance dispersal (Wilson, 1966). However, as ascospores are discharged in groups of eight within the epiplasm (Swinburne, 1971) the unit of dispersal has a high mass relative to other air-borne propagules. Moreover, in periods of prolonged leaf wetness rapid ascospore discharge results in the blocking of the ostioles, with the consequence that further dissemination relies on rain-splash (Swinburne, 1971).

The source of inoculum giving rise to infections in new plantations is assumed to be either neighbouring orchards or alternative hosts within the hedge-row (Flack and Swinburne, 1977). If this was correct then it would be expected that infections in a new plantation would be randomly distributed among the available infection sites (predominantly leaf scars) with the probable frequency of infection inversely proportional to the distance of each tree from the source of inoculum. Often the distribution of infections observed in young orchards appears random with some trees exhibiting a disproportionately large number of cankers relative to their neighbours. However, in certain instances it is not. Thus Byrde and Clarke (1973), analysing earlier data, found that the distribution of cankers in one orchard followed a negative binomial.

Such distributions could arise if the primary infection within individual trees led to secondary infections confined within a limited range. This tends to suggest that tree to tree transmission within and between orchards is infrequent and could only account for a slow development of epidemics, consistent with the probable low mobility of both spore types.

Severe *Nectria* canker is sometimes found in young (< 7 years old) plantations of apple in the UK, particularly in susceptible cultivars such as Gala, Discovery, and Red Pippin (syn. Fiesta) (unpublished data). A characteristic feature of these epidemics was the simultaneous appearance of several lesions on each tree. This was difficult to explain in the absence of primary infections within the orchard and, in several instances, the absence of inoculum in surrounding areas. In an attempt to identify the source of inoculum, the pathogen was isolated from samples obtained from such orchards. It was noted that there was a variation in the size of conidia, particularly in length, between isolates obtained from different orchards. Morphometric analysis of the conidia from these isolates indicated that this variation was not associated with either the cultivar or the geographical location of the orchard. However, distinct and statistically significant size groupings were apparent when the data was subjected to discriminant analysis with respect to the nursery in which the trees had been propagated (Li, 1995). Subsequently, these isolates were also examined for DNA polymorphism. Two ribosomal-DNA random fragment length polymorphism (RFLP) types were found, both of which were further divided into two mitochondrial DNA categories (Brown *et al.*, 1994). The majority of isolates originating from trees propagated in the same nursery, irrespective of where they were finally planted, belonged to the same category, suggesting that they may have become infected during propagation.

If infections within the nursery can lead to *Nectria* canker development in the orchard at a later date, it must also be assumed that such infections can remain symptomless for long periods. Crowdy (1949) noted that infections could remain latent for several months. Evidence that the pathogen can remain quiescent for even longer periods has

been obtained by direct isolation (Li, 1995) and by immunofluorescent microscopy (Dewey *et al.*, 1995).

This chapter describes two experiments undertaken to provide further evidence that nurseries can be a source of infection leading to canker epidemics in the orchard. Both were based on the assumption that if trees or rootstocks of the same clones, but obtained from different nursery sources, were planted together in an orchard, any differential development of *Nectria* canker could be explained by infections which occurred within nurseries.

2.2 Materials and Methods

2.2.1 The Aylesham Trial

Twenty-five maiden trees of the cultivar Queen Cox on M9 rootstocks were obtained from 10 nurseries during the winter of 1991/92. Of the collaborating nurseries five were located in the UK and five in continental Europe. Upon receipt at Wye College the trees were heeled into peat compost to prevent drying until planting on 2nd February, 1992 at Rattling Court Farm, Aylesham, Kent. The experimental plot was part of a much larger commercial orchard planted concurrently with the same cultivar, and set out as 'three row beds'. This dictated the design of the trial, which was planted in five adjacent beds in the south eastern corner of the orchard. Two sides of the trial site were bordered by hedgerow, predominantly grey alder (*Alnus incarna*), and the other sides by the commercial plantation. Within the beds, trees were planted in staggered rows one metre apart. Pollinator trees (*Malus cv. Katy*), supplied by the farm, were planted in the outside rows of each bed alternately to provide one pollinator for every five trees of the *cv. Queen Cox*. Grass alleyways, 2 metres across, separated the beds which were clean cultivated. Five trees from each nursery plus one pollinator were planted as a single plot, randomly assigned to a position within each of the five beds. Guard plots were planted at the ends of each bed. These contained five trees of the *cv. Queen Cox* from the same source as the commercial plantation and one pollinator. Plots within the beds were not separated in any way.

The trees within the experimental plot received the same treatments with respect to pesticides as the surrounding orchard. The selection of materials and the timing of their application was at the discretion of the management. Control of apple scab (*Venturia inaequalis*) was achieved by the application of dithianon at approximately 10 day intervals from bud-burst until mid April, followed by Captan at the same interval until July. Mildew (*Podosphaera leucotricha*) was controlled by the application of bupirimate at 14 day intervals during June and July. All pesticide applications were made at levels recommended by the manufacturers.

In each of the next four seasons, trees were examined for the presence of cankers attributable to *N. galligena* on the basis of their macroscopic appearance. These were not deliberately removed, but many were cut out during the course of normal pruning operations. Likewise some main stem cankers were treated with canker paint as part of the routine farm operations. Results were recorded as the number of trees per plot which had visible cankers, cumulatively at each time of inspection.

2.2.2 The Budding Trial

Two hundred rootstocks of M9 were obtained from each of two UK and one continental nursery in the winter of 1992/93. Upon receipt the rootstocks were individually planted in polybags (25cm diameter, 15 l capacity) containing compost (Pan Britannica, P-base) prepared from 75% peat 25% sand (v/v). The polybags were then placed in a plunge bed remote from any known sources of *N. galligena*. Two plots of 20 rootstocks from each nursery were randomly assigned to positions within each of 5 blocks. Within each plot the pots were arranged in four rows of five. The spacing between the plots and blocks was 0.5m but within the plots the pots were touching. In the absence of any fungicidal treatment powdery mildew was quite severe on the M9 rootstocks in the season prior to budding.

In July 1993 budwood of the cv. Spartan was obtained from a group of mother trees growing on a farm at Linton, Kent. *Nectria* cankers were present in each of these trees

but the budwood was taken from branches free from obvious symptoms. After removal of the leaves half of the budsticks were immersed for 30 mins. in a suspension of 10 g l^{-1} of a commercial formulation of benomyl (50% ai, Benlate, Dupont). The remaining budsticks were left untreated. Buds were grafted to rootstocks using the T-graft method (Garner, 1988), so that each block contained one plot from each nursery source budded with either Benlate-treated or untreated buds. The rootstocks above the graft were removed in the early spring of the following year allowing the scion shoots to develop. The trees were examined monthly and those with visible lesions attributable to *Nectria* were removed to minimise distribution of inoculum to neighbouring trees.

Benomyl-sensitivity of four sample isolates was assessed by transferring mycelial discs cut from the leading edge of colonies growing on 20 g l^{-1} Malt Extract agar to fresh plates of the same medium containing 0, 1.0, 1.5 or 2.0 mg l^{-1} benomyl. The diameter of four replicate colonies of each isolate on each concentration was measured after 10 days incubation at 22°C .

2.2.3 Isolation of *N. galligena* from infected wood

At the conclusion of the fifth season of the Aylesham Trial all overt canker lesions were excised, and wood samples stored in labelled polythene bags at -20°C pending isolation in the laboratory. Samples of internal wood taken from the margin of diseased areas were aseptically excised and transferred to Malt Extract Agar as described in Chapter 3, point 3.2.1. Amongst the fungal colonies that emerged, those of *N. galligena* were identified by colony and conidial characteristics (Booth, 1966) and species-specific PCR (Chapter 4).

2.2.4 Data evaluation

Cumulative inspection data from the Aylesham and Budding trials were subjected to angular transformation to achieve homogeneity of variance and analysed using analysis of variance, with differences between individual nurseries in the Aylesham trial being tested using the Tukey's LSD (SAS Inc., 1990). For the Budding trial, the number of

trees which had developed *Nectria* canker by mid-summer of the following two years was transformed to arcsine to achieve homogeneity of variance and subjected to analysis of variance.

To assess the benomyl-sensitivity of sample *N. galligena* isolates, the concentration of benomyl that reduced growth by 50% (ED_{50}) was calculated from the regression of colony diameter against the log of the concentration of benomyl. Data were subjected to analysis of variance with respect to the nursery of origin and benomyl treatments in the Budding trial.

2.3 Results

2.3.1 The Aylesham Trial

There was no evidence of *Nectria* canker lesions on any of the trees at the time of their receipt from the ten collaborating nurseries, nor when they were planted in their final field positions in the Aylesham orchard.

The number of trees from each source that developed *Nectria* cankers at each time of inspection during the following four years is shown in **Fig. 2.1**. At the first inspection in mid-summer 1992, trees from five nurseries had developed cankers, and approximately a quarter of trees from three nurseries (VII, VIII and X) had at least one lesion. Most of these lesions took the form of die back of the youngest shoots, particularly the leader. Analysis of variance of this data indicated that the differences between nurseries was significant ($p = 0.032$) but Tukey's test failed to reveal differences in paired comparisons.

Despite the removal of almost all these infected shoots during pruning operations in the winter of 1992/93, additional cankers developed on the new shoots which grew out in the summer of 1993, particularly in trees from Nursery VII. Some trees from this source had developed lesions on the main stem which had seemingly arisen as the result of die-back of side shoots. The number of trees which had developed *Nectria* cankers was

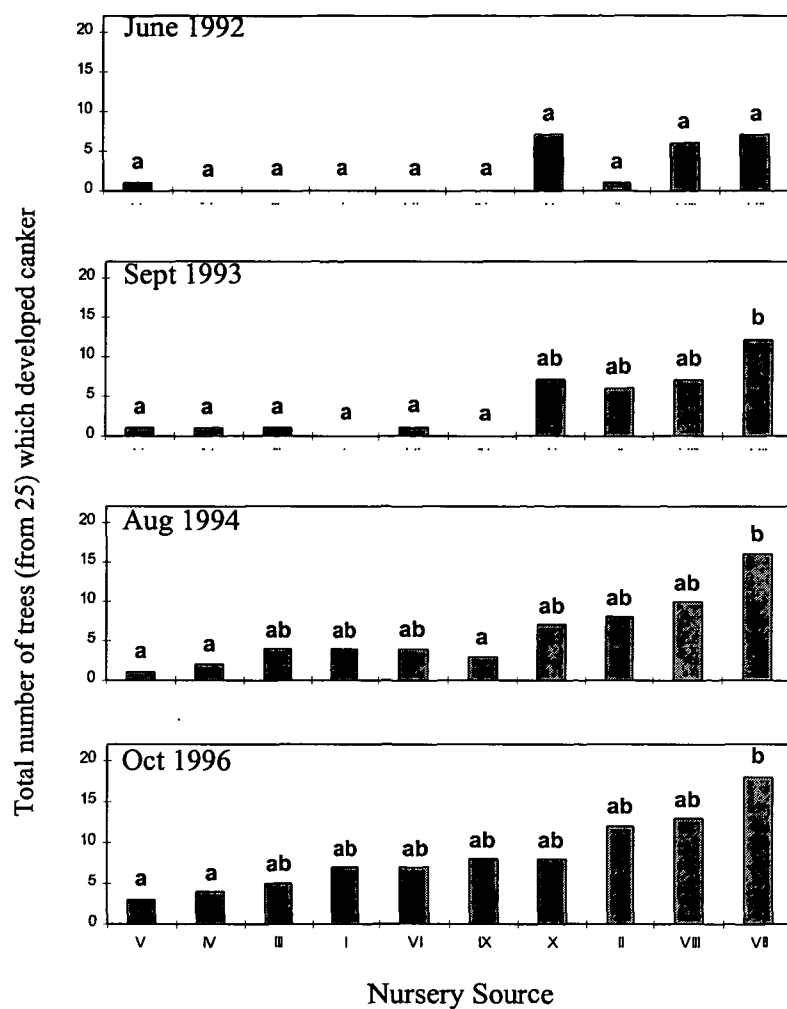


Fig. 2.1. Number of apple trees (Queen Cox on M9) obtained from different nurseries which developed canker following planting in February 1992 at a single site. Values sharing the same letter do not differ significantly using Tukey's test.

significantly related to the nursery of origin ($p = 0.003$) and Tukey's tests showed that significantly more trees from Nursery VII were infected than those from Nurseries I, III, IV, V, VI and IX.

In the third season after planting, canker lesions were found on trees from all nursery sources but the magnitude of the differences between nurseries was less than the previous season. However, the nursery of origin was the only significant factor contributing to the number of trees with *Nectria* canker ($p = 0.007$). Tukey's tests showed that Nurseries V, IV and IX had significantly fewer trees infected with *Nectria* canker than Nursery VII at this time.

At the final inspection, in the autumn of the fifth season, most of the cankers observed were located at the junctions between different year's growth and had the typical swollen shape and many had perithecia present. At this stage die-back symptoms on the current years growth was rare. Again there were significant differences between nursery sources ($p = 0.026$) and Turkey's test indicated that fewer trees from Nurseries IV and V had developed *Nectria* canker than those from Nursery VII. At this time, 18 of the 25 trees supplied by Nursery VII had several cankers and some trees had branches which were girdled and consequently dying. In contrast, only 3 of the 25 trees supplied by Nursery V had developed any symptoms of *Nectria* canker by the fifth season, and only one of these was found to have more than one lesion at the final inspection.

Cankers were found at each inspection in the trees of the main plantation, both in the row flanking the experimental site and those trees acting as guards at the end of the blocks. However, no significant block effect was found at any inspection. Three of the pollinator trees also developed cankers but this did not appear to be related to the frequency of infection in their neighbouring trees. Isolations were not achieved from sample lesions taken from these trees.

2.3.2 The Budding Trial

At the time of receipt and planting all of the rootstocks appeared to be healthy. However, typical *Nectria* cankers developed on a few rootstocks in the following spring and early summer. The total number of infected rootstocks was too small to detect significant differences between nursery sources and appeared to be randomly distributed between blocks. Seven of the 200 rootstocks from Nursery I, one from Nursery II and two from Nursery VI developed such cankers, all of which were located around excised side shoots. The infected rootstocks were retained in the experiment, but the majority rapidly developed additional cankers around the budding wound and were removed before the end of the first summer. Cankers also developed at the budding wounds on apparently healthy rootstocks. The majority of all cankers which developed during the first year following budding (**Fig. 2.2**) occurred at the budding wound, with the consequence that the scion buds failed to grow.

In the second year most of the new lesions which developed were on the scion shoots, but a few were found on suckers emerging from the rootstock. Lesions on the scion shoots were mostly at the apex, resulting in progressive die-back. These lesions had no obvious point of origin. A few lesions were found at the base of the scion shoot and appeared to originate at the union with the rootstock. Examples of some of these overt symptoms observed on rootstocks and Spartan budded maidens during the Budding Trial are given in **Fig. 2.3**.

Of the total of 118 *Nectria* cankers observed on the trees over both years, 45% occurred at the union between rootstock and scion, 40% were on the scion shoot remote from the union and 15% on rootstock suckers. No differences were detected in this distribution between either rootstocks from different sources or between benomyl treated and control bud sticks. The total number of trees which developed *Nectria* canker in each of the two years following budding is shown in **Fig. 2.2** and the statistical analysis of the accumulated data for the second year in **Table 2.1**.

The number of trees which developed *Nectria* canker on rootstocks from Nursery I was significantly less than in trees from Nurseries II and VI. This difference was observed in both years, but was particularly striking in the second year, when most of the cankers which occurred on trees on rootstocks from Nurseries II and VI appeared.

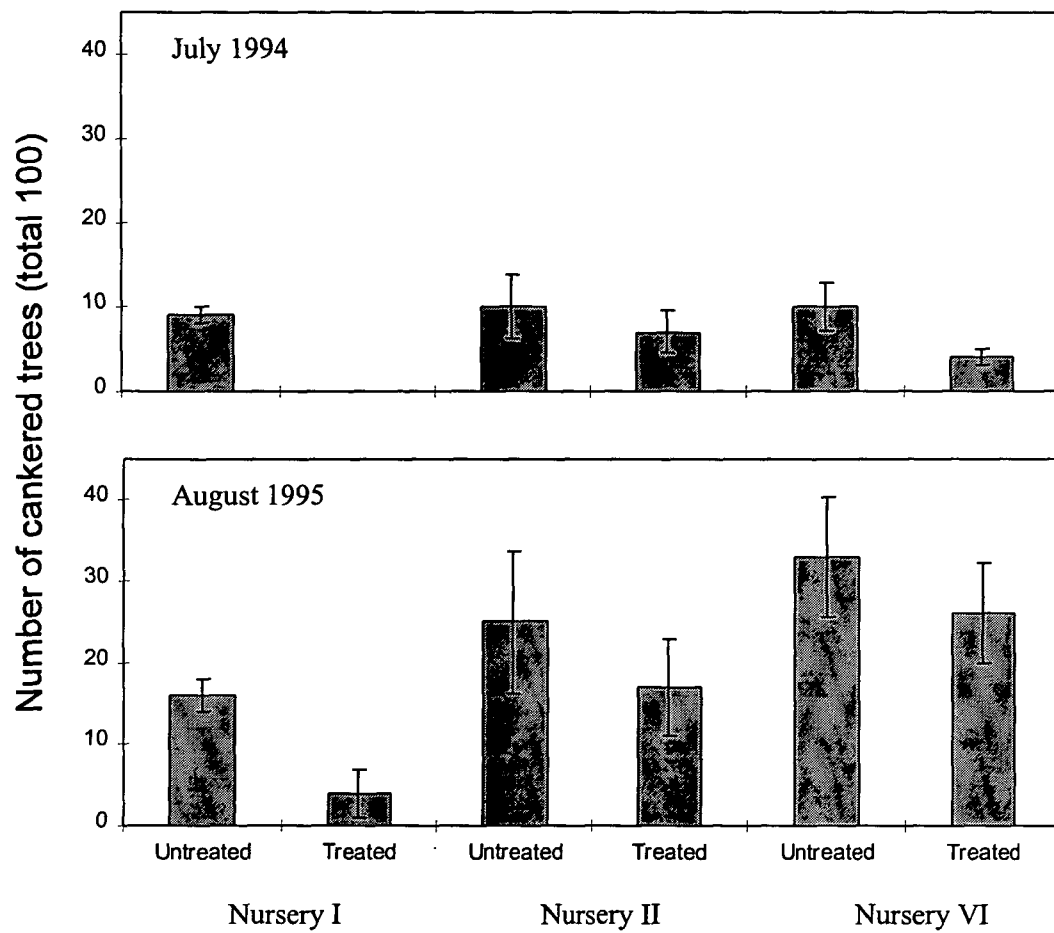


Fig. 2.2. Canker development in young trees, *cv.* Spartan, budded in July 1993 onto M9 Rootstocks obtained from three commercial nurseries, with and without benomyl treatment of the budsticks (untransformed data with S.E. bars).

Table 2.1. Analysis of variance of the data shown in Fig. 2.2 for 1995, following arcsine transformation.

Source of variation	d.f.	m.s.	v.r.	F.pr
Blocks	4	128.25	1.44	
Nurseries	2	743.71	8.37	0.002
Benlate Treatment	1	582.54	6.55	0.019
Nursery x Benlate	2	105.94	1.19	0.324
Residual	20	88.88		

Treatment of the bud-sticks with benomyl reduced the number of trees which developed *Nectria* cankers in each of the two years following budding of rootstocks from each nursery. The magnitude of the reduction obtained with rootstocks from Nursery I was much greater than that for Nurseries II and VI. This was particularly evident in the second year.

The ED₅₀ of benomyl was estimated for four isolates of *N. galligena* from cankers on trees grown on rootstocks from each source (Table 2.2). There was no significant difference in sensitivity between any of the isolates tested.

Table 2.2. Mean ED₅₀ (ppm) of benomyl for isolates of *N. galligena* obtained from trees (cv. Spartan) budded on rootstocks (M9) obtained from three commercial nurseries.

Nursery	ED ₅₀	S.E.
I	1.15	0.08
II	1.09	0.02
VI	1.18	0.10

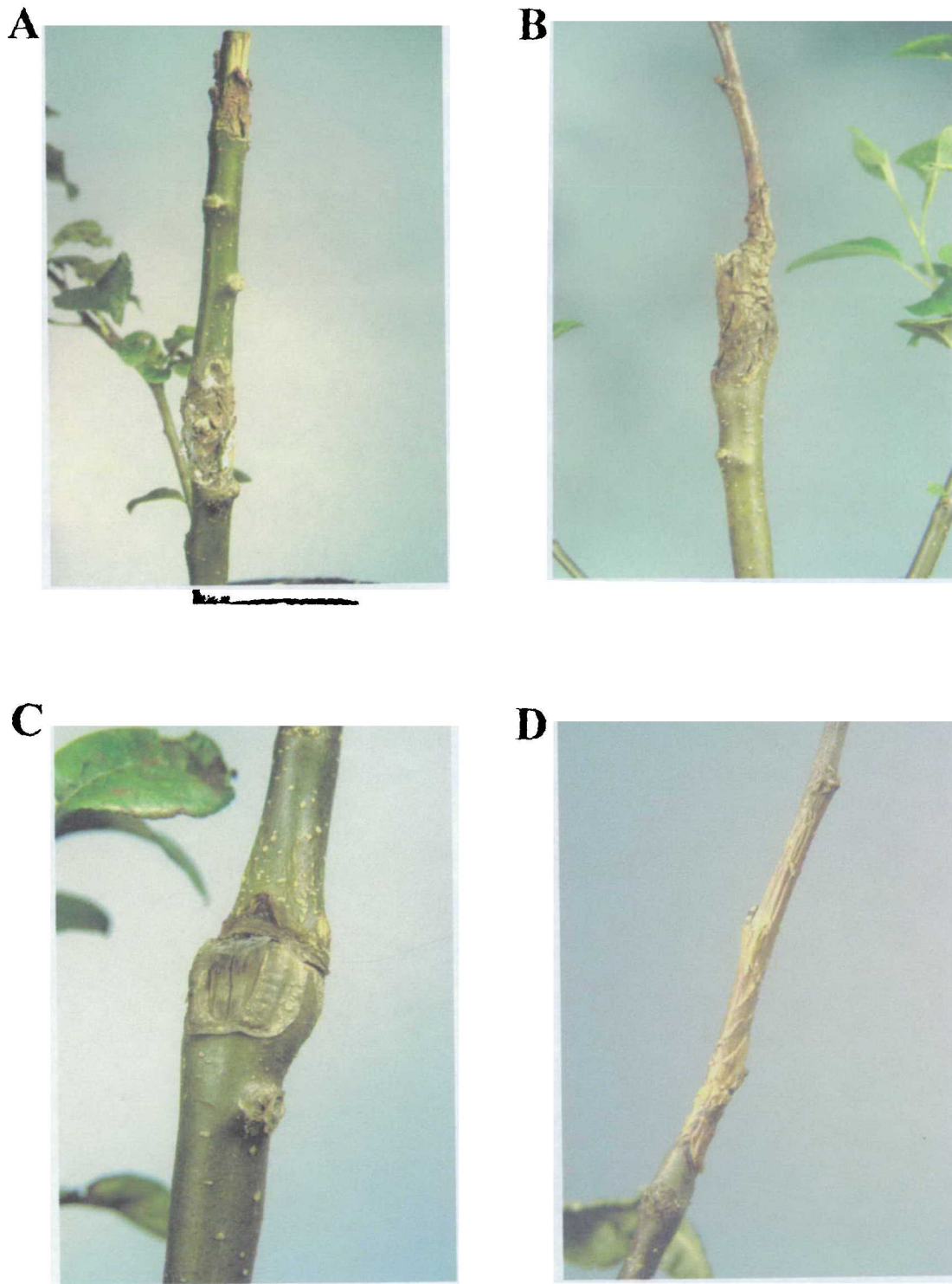


Fig. 2.3. Examples of some overt *Nectria* canker symptoms on rootstocks and Spartan budded maidens observed during the Budding Trial. A, *Nectria* canker development at the site of budding and on rootstock base, B, Union canker development after bud has grown out, C, Development of union canker 2 years after budding, D, Tip die-back of young whip, note no defined infection focus.

2.4 Discussion

The Aylesham trial demonstrated that apple trees from different commercial nurseries have differing potentials to develop *Nectria* canker in the first years following planting in orchards. A number of factors could have contributed to this outcome. Firstly, whilst the trial was conducted with one cultivar, Queen Cox on M9 rootstocks, these had been produced from different mother trees and stoolbeds where clonal variation could result in variable levels of susceptibility. Given that all the trees were raised under National Certification schemes, clonal differences would be expected to be minor. A second factor to be considered is that differing agronomic practices between the nurseries may have resulted in the production of trees of differing vigour. There is abundant anecdotal evidence that susceptibility to *Nectria* canker is increased by practices which encourage lush growth (reviewed by Swinburne, 1975). However, the trees in this trial were produced to industry standards and showed only minor differences in size at the time of receipt or in subsequent years in the orchard. Even so, a more complex experiment involving the exchange of rootstocks and budwood between nurseries would be required to eliminate concerns regarding both clonal variation and vigour as factors in this trial.

The third factor which could have resulted in the differing frequencies of *Nectria* canker in the Aylesham trial is that they developed from infections which occurred in the nursery during propagation. This would be consistent with the findings of Li (1995) and Brown *et al.* (1994) who found that isolates of *N. galligena* recovered from commercial orchards could be linked to the nursery in which the trees were raised, using morphometric and RFLP techniques, respectively.

Evidence that infection can and does occur in the commercial nurseries was provided by the early development of *Nectria* cankers on rootstocks obtained for the Budding trial within weeks of receipt. But, as was observed in the Aylesham trial, significant differences in the numbers of trees infected with *Nectria* canker became apparent only from the second season onward. No attempt was made to remove cankers during the Aylesham trial, and within the intensively planted three-row bed system employed, it is

possible that the cankers observed in the latter years of the trial were the result of inoculum produced on those lesions formed in the first two. In the Budding trial infected wood was removed before sporulation was initiated, and as the experimental trees were remote from any external source of inoculum it seems likely that they were infected before budding.

Crowdy (1949) observed that a period of several months may elapse between infection by *N. galligena* and the expression of symptoms of apple canker. Results from both trials reported here may imply that this latent period can extend to one or more seasons and that the site of lesion development may be remote from that of the original infection. Li (1995) isolated *N. galligena* from within the xylem tissue of young apple shoots distal to cankers on older branches. Using immuno-fluorescent microscopy, Dewey *et al.* (1995) detected hyphae within xylem vessels of symptomless shoots. The *Nectria* cankers which developed on Spartan scions in the second year following budding may have originated from inoculum moving with the transpiration stream from the rootstocks. Such movement was demonstrated in *in vitro* experiments by Li (1995). However, it is also possible that some of the cankers observed were the result of prior infection or contamination of the bud-sticks. Bennett (quoted in Howard *et al.*, 1974) concluded that this was an important source of infection within nurseries and demonstrated the efficacy of dipping bud-sticks in benomyl. In this trial benomyl treatment reduced the incidence of canker on rootstocks from two of the three nurseries in the first year after budding, but only one in the second year. Evidently, this differential response was not due to the presence of benomyl tolerant strains of the pathogen. Benomyl treatment appeared most effective and persistent with the rootstocks from Nursery I, which gave the lowest percentage of infected trees overall. Given that all of the buds came from a single source with equal probability of infection, it must be concluded that more rootstocks from Nurseries II and VI were infected prior to budding than those from Nursery I, but that contamination of buds made some contribution to cankers on trees on all rootstock sources.

Chapter 3. Intra-Specific Variation in *Nectria galligena* Characterised by Arbitrarily Primed PCR, Nuclear rRNA Gene Complex PCR-RFLP and Sequence Analysis

3.1 Introduction

The results of Chapter 2, challenging the conventional interpretation of the disease being spread through aerial external inoculum sources (Wiltshire, 1921; Zeller, 1926), has indicated that young trees can be infected symptomlessly during propagation. As control strategies are based on the earlier assumption, the ability to reliably discriminate between individual isolates would assist the evaluation of novel dissemination routes through molecular epidemiological appraisals of the structures of populations of known provenance, leading to a better understanding of disease spread and possible opportunities for more appropriate control measures.

Molecular approaches continue to be widely exploited to characterise relationships at varying taxonomic levels throughout the fungal kingdom. PCR-based access to the variable non-coding nuclear ribosomal RNA (rRNA) gene spacer regions (White *et al.*, 1990) (the internal transcribed (ITS) and the intergenic (IGS) spacers) has facilitated direct restriction fragment length polymorphism (RFLP) and sequence analysis in a growing number of applications including species complex and sub-generic aggregate resolution (Bryan *et al.*, 1995; O'Donnell and Gray, 1995), species identification and characterisation (Edel *et al.*, 1996; O'Donnell, 1992) and phylogenetics (Cooke and Duncan, 1997; Morales *et al.*, 1995). The higher variability observed in the IGS region makes this locus particularly attractive for intra-specific characterisation (Appel and Gordon, 1995, 1996; Edel *et al.*, 1995, Harrington and Wingfield, 1995; Hyun and Clark, 1998) especially for possible epidemiological application. Some rRNA gene coding regions have also been shown to be variable, but usually at or above the species level (Edel *et al.*, 1996; Guadet *et al.*, 1989; O'Donnell, 1993; Qu *et al.*, 1988; Sheriff *et al.*, 1994).

The use of arbitrarily primed PCR, either randomly amplified polymorphic DNA (RAPDs) (Williams *et al.*, 1990) or mini- or micro-satellite based primers, is also a useful strategy for discrimination at the population level. RAPDs have been extensively used in a range of applications including assessing genetic diversity (Hamelin *et al.*, 1993), genetic mapping, linkage analysis (Paran and Michelmore, 1993) and for assessing race and pathotype differentiation within *forma speciales* of *Fusarium* species (Crowhurst *et al.*, 1991; Grajal-Martin *et al.*, 1993; Kelly *et al.*, 1994). In addition, the use of the more technically robust random amplified microsatellite technique (Zietkiewicz *et al.*, 1994) is becoming an established complementary technique in population studies (Hamelin *et al.*, 1993; Hantula and Muller, 1996; Stenlid *et al.*, 1994) and, increasingly, in epidemiological applications.

This chapter describes the evaluation of two distinct PCR-based methods, PCR-RFLP analysis of rRNA ITS and IGS regions, and arbitrarily primed PCR, for strain discrimination at the population level within *N. galligena*. Some sequence analysis has also been used to independently assess relationships indicated by the others techniques. The potential usefulness of these techniques in molecular ecological applications against Aylesham and Budding Trial derived isolates, obtained from Chapter 2, are assessed.

3.2 Materials and Methods

3.2.1 Isolates and cultural conditions used in this study

Isolations of *N. galligena* were made from canker lesions on two to four year old shoots from *Malus* and *Pyrus* cultivars of various provenance's within the United Kingdom and Ireland. Typically, 3-5 wood discs (approximately 5 mm in length) were excised aseptically from the leading edge of bark stripped lesions, surface sterilised in 10 % sodium hypochlorite (BDH) for 75 seconds, rinsed twice in sterile distilled water and blotted dry on sterile Whatman Paper No. 1 (Whatman, UK). Surface sterilised wood discs were fragmented longitudinally with a fixed blade scalpel, placed on 12 g l⁻¹ Malt Extract Agar (MEA) (Oxoid, USA) supplemented with Augmentin™ (which contains amoxycillin sodium and potassium clavulanate) (SmithKline Beecham Pharmaceuticals,

UK) at 0.6 mg ml⁻¹ and incubated at 20°C under continuous fluorescent light (Philips TLD 50W/84HF) until fungal emergence. Anamorph species confirmation (as *C. heteronema*) was established by colony morphology, pigmentation, growth rate and conidial characteristics (Booth, 1959, 1966) on Spezieller Nahrstoffarmer Agar plus yeast extract (Nirenberg, 1976), MEA and Potato Dextrose Agar (PDA) (Oxoid, USA) media and by PCR with a diagnostic primer set (see Chapter 4). Isolates were confirmed as pathogenic to apple fruits, except the birch isolates CBS 100472, CBS 100473 and CBS 100477 which were not pathogenic to fruits (data not shown), and maintained on 12 g l⁻¹ MEA at 20°C.

Additional isolates, to a total of 50, of *N. galligena* (identification established by their suppliers and as above) were obtained from other collections and were selected to represent the broadest spectrum of geographic and host range available (see **Table 3.1**). Isolates not previously accessioned to a major international fungal collection were deposited with the express permission of suppliers. Samples of DNA isolated from a further six authenticated *N. galligena* isolates were supplied by Dr. K. O' Donnell, United States Department of Agriculture, Peoria, USA (MUCL 28137), Dr. J. Rahe, Simon Fraser University, British Columbia, Canada (Ng-FV-4, Ng-FV-5, Ng-FV-6) and Dr. D. Brayford, CABI Bioscience, Egham, UK (IMI 376385 and IMI 376387).

Table 3.1. Provenance of *N. galligena* isolates used in this study with their original host, year and place of isolation, the name of the original isolator and the approximate size (bp) of PCR product spanning IGS and adjacent rRNA gene coding sequences.

Number	Isolate	Host	Year	Origin	Isolator/Supplier	IGS ^d
1	CBS 100318 ^a	<i>Malus</i>	1995	N. Ireland	A. MacCracken	2850
2	CBS 100474	<i>Malus</i>	1995	N. Ireland	A. MacCracken	2850
3	CBS 100317 ^a	<i>Malus</i>	1995	N. Ireland	A. MacCracken	2950
4	CBS 100316	<i>Malus</i>	1995	N. Ireland	A. MacCracken	2850
5	IMI 378755 ^{ab}	<i>Populus</i>	1997	N. Ireland	S.R.H. Langrell	2700
6	MUCL 40722	<i>Malus</i>	1997	Great Britain	S.R.H. Langrell	2850
7	MUCL 40716 ^a	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2700
8	IMI 375724	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2750
9	MUCL 40723 ^c	<i>Malus</i>	1997	Great Britain	S.R.H. Langrell	2850
10	IMI 378754 ^{ab}	<i>Malus</i>	1991	Great Britain	T.R. Swinburne	2850
11	MUCL 40782 ^a	<i>Malus</i>	1991	Great Britain	T.R. Swinburne	2750
12	ICMP 13269 ^{ab}	<i>Malus</i>	1994	Great Britain	S.R.H. Langrell	2750
13	IMI 375721	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2700
14	IMI 375722	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2700
15	MUCL 40713	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2750
16	CBS 100326 ^a	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2750
17	CBS 100325 ^a	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2850
18	CBS 100327	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2750
19	CBS 100324	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2750
20	MUCL 40784 ^a	<i>Malus</i>	1995	Great Britain	S.R.H. Langrell	2750
21	ICMP 13275	<i>Malus</i>	1996	Great Britain	S.R.H. Langrell	2750
22	MUCL 40719	<i>Malus</i>	1997	Great Britain	S.R.H. Langrell	2750
23	MUCL 40786	<i>Malus</i>	1997	Great Britain	S.R.H. Langrell	2750
24	MUCL 40718	<i>Malus</i>	1997	Great Britain	S.R.H. Langrell	2750
25	MUCL 40717 ^a	<i>Malus</i>	1997	Great Britain	S.R.H. Langrell	2750
26	ICMP 13270	<i>Malus</i>	1995	Great Britain	S.R.H. Langrell	2750
27	ICMP 13271	<i>Malus</i>	1995	Great Britain	S.R.H. Langrell	2850
28	ICMP 13272	<i>Malus</i>	1995	Great Britain	S.R.H. Langrell	2750
29	MUCL 40720 ^{ab}	<i>Pyrus</i>	1997	Great Britain	S.R.H. Langrell	2850
30	MUCL 40721 ^c	<i>Pyrus</i>	1997	Great Britain	S.R.H. Langrell	2850
31	ATCC 11684 ^a	<i>Fraxinus</i>	1947	Great Britain	- ^e	2850
32	IMI 376406 ^a	<i>Malus</i>	1997	Eire	S.R.H. Langrell	2900
33	MUCL 40712 ^a	<i>Malus</i>	1995	France	S.R.H. Langrell	2900
34	MUCL 28137 ^{ab}	<i>Malus</i>	1983	Belgium	J. Marot	2950
35	IMI 376405 ^{ac}	<i>Malus</i>	1997	Norway	S.R.H. Langrell	2900
36	MAFF 410257 ^{abc}	<i>Fraxinus</i>	1984	Japan	K. Sasaki	3150
37	ICMP 5430-77 ^{ab}	<i>Malus</i>	1965	New Zealand	J.M. Dingley	2900
38	ICMP 9472-87 ^{ab}	<i>Malus</i>	1987	New Zealand	W.F.T. Hartill	2900
39	ICMP 13255 ^{ab}	<i>Malus</i>	1997	New Zealand	P.R. Johnston	2850
40	ICMP 13256 ^{ab}	<i>Malus</i>	1997	New Zealand	P.R. Johnston	2850
41	CBS 100472 ^{ab}	<i>Betula</i>	1996	USA	S. Anagnostakis	2450
42	CBS 100473	<i>Betula</i>	1996	USA	S. Anagnostakis	2450
43	CBS 100479 ^{ac}	<i>Fagus</i>	-	USA	D.R. Houston	2600
44	CBS 100477	<i>Betula</i>	1994	USA	F. Plante	2750
45	Ng-FV-4 ^a	<i>Malus</i>	1996	Canada	J. Rahe	2900
46	Ng-FV-5 ^a	<i>Malus</i>	1996	Canada	J. Rahe	2900
47	Ng-FV-6 ^a	<i>Malus</i>	1996	Canada	J. Rahe	2900
48	IMI 376407 ^a	<i>Malus</i>	1993	Canada	P.G. Braun	2900
49	CBS 100319	<i>Malus</i>	1997	Canada	P.G. Braun	2600
50	CBS 100320 ^{abc}	<i>Populus</i>	1997	Canada	P.G. Braun	2600
51	CBS 100321 ^{ab}	<i>Acer</i>	1997	Canada	P.G. Braun	2750
52	CBS 100478	<i>Malus</i>	1994	Canada	F. Plante	2900
53	IMI 378752 ^{abc}	<i>Malus</i>	1996	Chile	S.R.H. Langrell	2900
54	IMI 378753 ^a	<i>Malus</i>	1996	Chile	S.R.H. Langrell	2900
55	IMI 376385	<i>Malus</i>	1997	Chile	B.A. Latorre	2900
56	IMI 376387	<i>Malus</i>	1997	Chile	B.A. Latorre	2900

Continuation of **Table 3.1** from previous page.

^aIsolates which have had their entire ITS regions sequence characterised (**Fig. 3.2**).

^bIsolates which have had their 5'-terminal domain of the LSU rRNA gene sequence characterised (**Fig. 3.3**).

^cIsolates subjected to PCR-RFLP analysis of the β -tubulin gene intron and mtSSU with *AluI*, *HinfI*, *HpaII* and *RsaI* (no polymorphisms observed).

^d See point 3.2.3.1 and **Fig. 3.1** for position of primers.

^eNot known.

Culture collections where isolates available. CBS, Centralbureau voor Schimmelcultures, AG Baarn, The Netherlands; IMI, International Mycological Institute, Egham, United Kingdom (now CABI Bioscience); MUCL, Mycotheque de L'Universite Catholique de Louvain, Louvain-La-Neuve, Belgium; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; ATCC, American Type Culture Collection, Manassas, United States of America; MAFF, National Institute of Agrobiological Resources, Ibaraki, Japan.

3.2.2 High Molecular Weight DNA Preparation

High molecular weight DNA was extracted from fresh mycelium of *N. galligena* isolates using a procedure modified from that of Lecellier and Silar (1994). A mycelial plug, taken from the actively growing edge of a fresh culture, was used to inoculate the centre of a cellophane membrane (Bio-Rad Laboratories, USA) disc covering the surface of Augmentin™ supplemented MEA. The mycelial mat resulting from 7-9 days growth (approximately 50-100 mg fresh weight) was harvested from the surface of the cellophane membrane with the aid of a sterile disposable pipette tip (inverted) and placed in a sterile 1.5 ml microfuge tube to which was added 750 μ l extraction buffer (10 mM Tris-HCL (pH 8.0), 1 mM EDTA, 100 mM NaCl and 20 g l⁻¹ sodium dodecylsulphate (SDS)). The mycelium/buffer mix was vortexed, snap frozen for about 1 min in liquid nitrogen and thawed at 65°C. The vortexing and freeze/thaw procedure were repeated a further 3-4 times to ensure sufficient cell and nuclear disruption for DNA release. Following centrifugation (10 min, 11,600 x g), the supernatant was transferred to a fresh microfuge tube and extracted twice with an equal volume of Tris-washed phenol (Fisher Scientific):chloroform:isoamyl alcohol (25:24:1). Following centrifugation (10 min, 11,600 x g), the aqueous supernatant was transferred to another sterile microfuge tube and an equal volume of ice-cold propan-2-ol added and gently mixed by inverting several times. Tubes were refrigerated at -55°C for up to 1 hour and centrifuged (10 min, 11,600 x g), to pellet the DNA. The supernatant was carefully removed and the pellet washed with 1 ml 70 % ethanol. Following centrifugation (2 min, 11,600 x g), the supernatant was removed and discarded, the DNA pellets air-dried at room temperature and dissolved in sterile Milli-Q AR grade water. DNA concentrations were measured spectrophotometrically and by direct comparison (also to check quality) electrophoretically with a standard dilution series of bacteriophage λ DNA (Promega) and stored at -20°C.

3.2.3 PCR amplification conditions

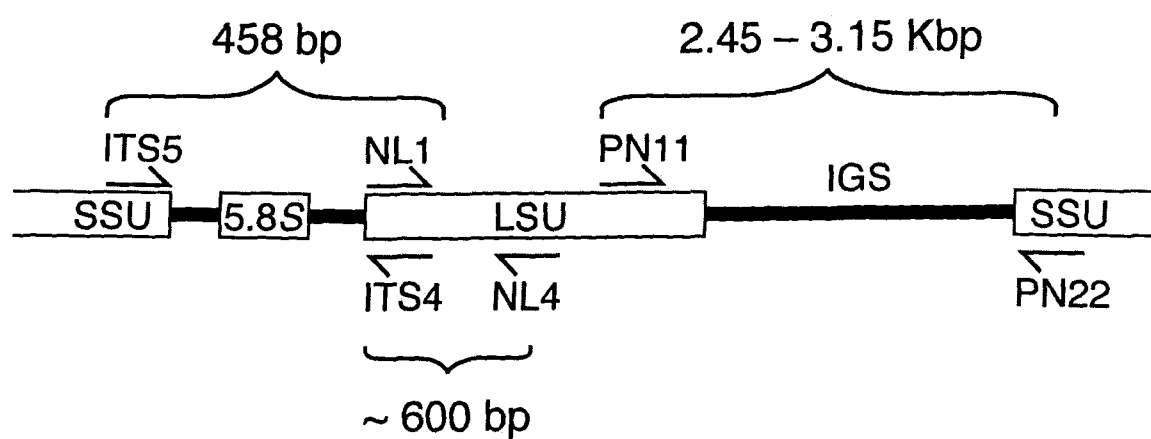
3.2.3.1 Nuclear Ribosomal RNA and unlinked loci

The phylogenetically informative nuclear rRNA internal transcribed spacer regions (ITS 1 and 2) and the hypervariable D1 and D2 domains at the 5' end of the LSU rRNA gene (Guadet *et al.*, 1989) were PCR amplified with primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), and NL1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NL4 (5'-CTT CCG TCA ATT CCT TTA AG-3'), respectively (White *et al.*, 1990). The unlinked mitochondrial small sub-unit (mtSSU) rRNA and the β -tubulin genes were amplified with primers MS1 (5'-CAG CAG TCA AGA ATA TTA GTC AAT G-3') and MS2 (5'-GCG GAT TAT CGA ATT AAA TAA C-3') (White *et al.*, 1990), and with T1 (5'-AAC ATG CGT GAG ATT GTA AGT-3') and T22 (5'-TCT GGA TGT TGT TGG GAA TCC-3') (O'Donnell and Cigelnik, 1997), respectively. PCR reaction conditions for the amplification of the ITS and 5' end of the LSU rRNA were as follows; 5 ng template DNA (from a standard concentration of 0.5 ng μl^{-1}), each primer to 0.1 mM, 100 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia, Uppsala, Sweden), 1.25 units Biotaq DNA polymerase, 10 μl 10 x PCR reaction buffer (160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris-HCL (pH 8.8 at 25° C), 1 ml l^{-1} Tween-20) and 1.5 mM MgCl_2 (all Bioline, UK). Total reaction volumes were made up to 100 μl with sterile Milli-Q AR grade water in 0.2 ml Apex thin walled polypropylene tubes (Alpha Laboratories, UK). Reaction mixtures were subject to the following amplification conditions on a Perkin-Elmer 9700 thermal cycler (Norwalk, USA); a preliminary 3 min incubation at 94°C (initial denaturation) followed by 30 cycles of 1 min/94°C (denaturation), 1 min/55°C (annealing) and 1 min/72°C (extension) with a final extension stage of 72°C/10 min. For the mtSSU rRNA and the β -tubulin gene amplification conditions were as above except that an annealing temperature of 50°C was used.

The entire IGS region of *N. galligena* isolates, including part of the 3' end of the LSU rRNA gene, was amplified using primers PN11 (5'-GCT GGG TTT AGA CCG TCG

TG-3') and PN22 (5'-CAA GCA TAT GAC TAC TGG C-3') (Mouyna and Brygoo, 1992). The forward primer, PN11, anneals at a position approximately 490 bp upstream from the 3' end of the LSU rRNA gene. The reverse primer, PN22, is derived from a conserved region approximately 50 bp downstream from the 5' end of the *Saccharomyces cerevisiae* SSU rRNA gene. The location and orientation of all primers used to assess the rRNA operon in this study are given in **Fig. 3.1**. PCR reaction conditions were essentially as for the other loci detailed above except 20 ng template DNA (from a standard concentration of 5 ng μl^{-1}) and 1.5 units Biotaq DNA polymerase were used. IGS reaction mixtures were thermocycled as follows; a preliminary 3 min/95°C was followed by 30 cycles comprising of 90 sec/95°C, 1 min/59°C and 90 sec/72°C with a final extension of 10 min/72°C.

Fig. 3.1. Schematic showing the approximate positions of the primers used within the rRNA gene repeats with the sizes of the amplicons.



3.2.3.2 Arbitrarily Primed PCR

Sixty-eight random decamer oligonucleotide primers from Kits E, I, R and X (Operon Technologies Inc., Alameda, CA, USA) were evaluated for their ability to reveal highly reproducible polymorphisms between three *N. galligena* isolates (IMI 378755, MUCL 40720, CBS 100478). From these, one, X11 (5'-GGA GCC TCA G-3'), which consistently revealed unambiguous polymorphisms was selected. Three micro-satellite based primers, (CA)₈, (CAT)₅ and (GACA)₄ (Meyer *et al.*, 1993; Weising *et al.*, 1995) originally designed for DNA fingerprinting (Meyer *et al.*, 1992) and the core sequence of mini-satellite M13 bacteriophage DNA (5'-GAG GGT GGC GGT TCT-3') (Stenlid *et al.*, 1994; Vassart *et al.*, 1987) were assessed as above. Of these four, only the M13 primer gave clear, consistent results in the preliminary tests. Using empirically optimised reaction and cycling conditions, primers X11 and M13 were used in all subsequent RAPD-PCR and PCR-fingerprint amplifications as follows: arbitrary primer X11, 25 ng target DNA, 200 μ M each of dATP, dCTP, dGTP and dTTP, 0.25 U *Biotaq* DNA polymerase, 10X PCR reaction buffer, 1.5 mM MgCl₂, 5 pmoles primer, to a final reaction volume of 35 μ l with sterile Milli-Q AR grade water; thermocycling on a Perkin-Elmer 9700 thermal cycler was for an initial 94°C/5 min followed by 45 cycles of 94°C/1 min, 33°C/1 min and 72°C/2 min with a final extension at 72°C/10 min; M13 derived primer, 2.5 ng target DNA was mixed with 200 μ M each of dATP, dCTP, dGTP and dTTP, 1.25 U *Biotaq* DNA polymerase, 10 x reaction buffer, 3.5 mM MgCl₂, and 200 nM primer in a final reaction volume of 50 μ l with sterile Milli-Q AR grade water in 0.5 ml polypropylene tubes (Fisher Scientific) overlaid with white mineral oil (Sigma, UK); thermocycling on a Techne PHC-3 thermal cycler (Techne, Cambridge, UK) was as follows: an initial 95°C/3 min followed by 45 cycles of 94°C/20 sec, 50°C/1 min and 72°C/20 sec with a final extension of 72°C/10 min. Both RAPD-PCR and PCR fingerprint profiles were assessed for reproducibility by performing duplicate independent reactions for each isolate/primer combination. Reactions containing no template DNA were included with every experiment as controls.

3.2.4 Restriction endonuclease digestion conditions

Typically, 10-15 μl of PCR product was digested, according to the manufacturer's instructions, in a total volume of 20 μl with 10 units of the following restriction endonucleases (Sigma, UK); *AluI*, *DpnI*, *HaeIII*, *HinfI*, *MboI*, *MseI*, *MspI* and *TaqI* for the ITS regions, and *AluI*, *HaeIII*, *HhaI*, *HinfI*, *MboI*, *MspI*, *RsaI* and *TaqI* for the IGS region. Reactions were incubated at 37°C overnight to ensure complete digestion and terminated by addition of 3-4 μl of loading buffer and incubating at 65°C for 10 minutes.

3.2.5 Agarose gel electrophoresis, staining and documentation

Individual PCR reactions were assessed by electrophoresing 5-10 μl of post-amplification reaction mix on a 10 g l⁻¹ agarose gel (Biogene, UK) in 1 X TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) using a 1 Kb ladder as a size marker (Life Technologies) and quantified by direct comparison with known concentrations of bacteriophage λ DNA (Promega). ITS and IGS restriction fragments were separated on a 20 g l⁻¹ Metaphor agarose (FMC Bioproducts) gel in 1 x TBE following the manufacturer's preparation instructions. Electrophoresis was at 4 V/cm for 3.5 hours along with 4 μl of $\phi\text{X174}/HinfI$ fragments (0.1 $\mu\text{g } \mu\text{l}^{-1}$) as molecular mass markers (Promega). For arbitrarily primed PCR, amplification products were separated in 10 g l⁻¹ agarose gels at 4.5 V/cm for 2 hours and 15 g l⁻¹ agarose gels at 5.5 V/cm for 4 hours, using a 1 Kb ladder as a size marker, for RAPD and the M13 core sequence primer, respectively. Gels were stained in a solution of ethidium bromide (1 mg ml⁻¹) for up to 45 minutes and destained in filtered water for up to 15 minutes. Restriction fragment and amplification profiles were recorded on Polaroid 665 film using a Polaroid MP-4 Land Camera using UV trans-illumination.

3.2.6 DNA Sequencing

PCR amplification products for sequencing were ethanol precipitated, resuspended in sterile Milli-Q AR grade water and further purified through Sepharose® CL-6B (Pharmacia). Purified PCR products were directly sequenced on both strands, employing

the primers used to generate the product (Rao, 1995; White *et al.*, 1990), with the Applied Biosystems ABI Prism™ Dye terminator Cycle Sequencing Ready Reaction Kit (Foster City, CA) on a Perkin-Elmer 9700 thermal cycler using the fastest ramp times available for 25 cycles of 96°C/10 sec, 50°C/5 sec and 60°C/2 min. Purified products were analysed on an Applied Biosystems 310 DNA sequencer. Sequences were edited using the Editview, EditSeq and SeqMan modules in the Lasergene sequence software package (DNASTar, Inc., Madison, USA). All sequences generated in this study were submitted to the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk>; see **Figs 3.2** and **3.3** for accession numbers).

3.2.7 Data evaluation

Sequence data were aligned using the computer program CLUSTAL V (Higgins and Sharp, 1989). Amplification products or restriction fragment sizes were estimated by direct comparison of migration distances to known sized markers and converted into base pairs using the program DNA-FIT (Oerter *et al.*, 1990). Pairwise profile comparisons of fragments were scored on the basis of the presence or absence of bands of the same electrophoretic mobility (considered as identical gene loci) to produce binary matrices using Jaccard's similarity coefficient, *i.e.* the number of bands shared by the pair/number of the shared plus non-shared bands (Sneath and Sokal, 1973). Phenograms were constructed using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA). All calculations were produced using Genstat 5 (Release 3.2, Lawes Agricultural Trust, Rothamsted Experimental Station, UK).

3.3 Results

3.3.1 Internal transcribed spacer region and large sub-unit rRNA gene

The primers ITS5 and ITS4 (White *et al.*, 1990) were used to amplify the ITS 1 and 2 regions along with the intervening 5.8S rRNA gene and short adjacent terminal segments of the LSU and SSU rRNA genes. PCR product length was approximately 550 bp and no length polymorphisms were detected by agarose gel electrophoresis amongst the 56 isolates (**Table 3.1**) tested. Digestion of each of the amplification product with eight

restriction endonucleases with four-base recognition sites (*AluI*, *DpnI*, *HaeIII*, *HhaI*, *MboI*, *MseI*, *MspI* and *TaqI*) gave identical band patterns for each enzyme. The amplification products from 32 isolates, chosen to represent the full range of geographic and host origins, were sequenced. The sequences from all the isolates were identical with one another, with the exception of that from IMI 378755 (AJ228673) which had a single T-A transition at nucleotide position 109, and with ITS sequences from other *N. galligena* isolates available from databases. The sequence of a typical isolate and the database accession numbers are given in **Fig. 3.2**. The limits of the ITS 1 and 2 and 5.8S gene regions were determined by comparison with other filamentous fungi and they were estimated to be 135, 165 and 158 bp long, respectively.

For fourteen isolates, again representing the full range of origins (see **Table 3.1**), the conserved primers NL1 and NL4 (White *et al.*, 1990) were used to generate an approximately 600 bp product from the 5'-end of the LSU rRNA gene. No length polymorphisms were detected by electrophoresis. When the products from each isolate were sequenced, all were found to be identical (see **Fig 3.3** for a representative sequence and database accession numbers).

RFLP analysis, using four restriction endonucleases (*AluI*, *HpaII*, *HinfI* and *RsaI*) to digest PCR amplification products from the unlinked β -tubulin gene intron and mitochondrial small sub-unit (mtSSU) rRNA loci from a smaller number of isolates representative of global and host diversity (see **Table 3.1** for isolates used), revealed no polymorphisms.

```

1      CCGAGTTTAC AACTCCCAA CCCCTGTGAA CATACCCATC GTTGCCTCGG CCGTGCCCGC
61     TCCGGCGGCC CGCCAGAGGA CCCCCAACT CTTGTTTTAT ACAGCATCTT CTGAGTAACA
121    CGATTAAATA AATCAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
181    GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA
241    CGCACATTGC GCCC GCCAGT ATTCTGGCGG GCATGCCTGT TCGAGCGTCA TTTCAACCCT
301    CAAGCCCCCG GGCTTGGTGT TGGGGATCGG CGTGCCCTTC GCGGCGCGCC GTCCCCTAAA
361    TCTAGTGGCG GTCTCGCTGC AGCTTCCTCT GCGTAGTAGC ACACCTCGCA CCGGAAGAGC
421    AGCGCGGCCA CGCCGTTAAA CCCCCACTT CTGAAAGG

```

Fig. 3.2. Nucleotide sequence of the complementary strand of the ITS 1 and 2 regions and intervening 5.8S rRNA gene of an *N. galligena* isolate. Sequences of all 32 isolates tested (listed below with the EMBL accession numbers for the corresponding sequence) were identical with each other except that from IMI 378755 (from *Populus*) which had a T-A transition at nucleotide position 109. Isolates from *Malus* CBS100318: AJ228662, CBS 100317: AJ228663, MUCL 40716: AJ228664, MUCL 40782: AJ228665, IMI 378754: AJ228666, MUCL 40717: AJ228667, MUCL 40784: AJ228668, ICMP 13269: AJ228669, CBS 100325: AJ228670, CBS 100326: AJ228671, IMI 376406: AJ228675, MUCL 40712: AJ228676, MUCL 28137: AJ228677, IMI 376405: AJ228678, ICMP 5430-77: AJ228680, ICMP 472-87: AJ228681, ICMP 13255: AJ228682, ICMP 13256: AJ228683, IMI 378752: AJ228684, IMI 378753: AJ228685, IMI 376407: AJ228691, Ng-FV-4: AJ228688, Ng-FV-5: AJ228689, Ng-FV-6: AJ228690; from *Pyrus* MUCL 40720: AJ228672; from *Fraxinus* ATCC 11684: AJ228674, MAFF 410257: AJ228679; from *Populus* IMI 378755: AJ228673, CBS 100320: AJ228692; from *Betula* CBS 100472: AJ228686; from *Fagus* CBS 100479: AJ228687; from *Acer* CBS 100321: AJ228693.

```

1      GCTGATGACC ATTACGCCAG CATCCTTGCG AATGCGCGAA CCTCAGTCCA CCACAGGGTA
61     TTACACGACG GGCTATAACA CTCCCGAGGG AGCCACATTC CCGAAGCCTT TATCCCCCAC
121    AGCGAACTGA TGCTGGCCTG GGCCGGAAGA GTGCACCAGG GAGAACCCTG GATGATCAAC
181    CAAGCCCAAG TCTGGTCATG AGCGCTTCCC TTTCAACAAT TTCACGTACT TTTTAACTCT
241    CTTTTCAAAG TGCTTTTCAT CTTTCGATCA CTCTACTTGT GCGCTATCGG TCTCTGGCCA
301    ATATTTAGCT TTAGAAGACA TATACCTCCC ATTTAGAGCA GCATTCCCAA ACTACTCGAC
361    TCGTTGAAGG AGCTTTACAA AGGATTGGCG TCCAACCAGA CGGGGCTCTC ACCCTCTATG
421    GCGTCCCGTT CCAGGCACTC GGAAGGTACC GCACCAAAG CATCCTCTAC AAATTACAAC
481    TCGGACCCGG GGGCCAGATT TCAAATTTGA GCTGTTGCCG CTTCACTCGC CGTTACTAGG
541    CATCCTGTTG GTTTCTTTTC CTCCGCTTAT

```

Fig. 3.3. Nucleotide sequence of the complementary strand of the 5'-terminal domain of the large sub-unit rRNA gene of an isolate of *N. galligena*. Sequences of all fourteen isolates tested (listed below with the EMBL accession numbers for the corresponding sequence) were identical. Isolates from *Malus* IMI 378754: AJ005660, ICMP 5430-77: AJ005656, ICMP 9472-87: AJ005657, ICMP 13255: AJ005658, ICMP 13256: AJ005659, ICMP 13269: AJ131330, IMI 378752: AJ005661, MUCL 28137: AJ131333; from *Pyrus* MUCL 40720: AJ131331; from *Fraxinus* MAFF 410257: AJ005655; from *Populus* CBS 100320: AJ005662, IMI 378755: AJ131328; from *Acer* CBS 100321: AJ131329; from *Betula* CBS 100472: AJ131332.

3.3.2 Arbitrarily Primed PCR

DNA from all 56 *N. galligena* isolates was used successfully in PCR using both X11 and the M13 core sequence primers. The M13 primer usually generated a higher number of amplification products (typically 10-15, ranging in size from 340 bp to 2700 bp) than did X11 (4-7, 620 bp to 2450 bp). But both primers generated reproducible profiles useful for examining intra-specific variation. Profiles produced by combining the results for both primers were mostly unique with only one pair of New Zealand apple isolates (ICMP 13255 and ICMP 13256, 39 and 40 in **Fig 3.4**) being indistinguishable. Cluster analysis of the combined X11 and M13 similarity matrices suggested that there was no strong separation into clades but there did appear to be three main groups with a few more distinct isolates (*e.g.* 19, CBS 100324; **Fig 3.4**). In this analysis, isolates from non-Rosaceous hosts mostly fell into the clearest cluster (A in **Fig 3.4**), whilst those from Rosaceous hosts fell into the other two (B and C in **Fig. 3.4**). However, all the isolates in cluster A were from North America, with sub-clusters representing the USA and Canada, and this group may well be distinct for geographical rather than host reasons. The latter explanation is supported by two *Malus* isolates, CBS 100319 and CBS 100478 (49 and 52 in **Fig. 3.4**), falling into this 'North American' cluster and three isolates from non-Rosaceous hosts (IMI 378755, from *Populus* and ATCC 11684 and MAFF 410257, both from *Fraxinus*; 5, 31 and 36 in **Fig 3.4**) being placed in the clusters B and C (**Fig. 3.4**) which were comprised mostly of isolates from Rosaceous hosts. It is also supported by the fact that where several isolates from a country (*e.g.* New Zealand or Chile) other than the UK were tested, these tended to form close (but not exclusive groups (*e.g.* see isolates 37-40 in **Fig. 3.4**). This tendency for isolates from single countries to form small groups may reflect limited introductions on cultivated apple material from a centre of diversity (presumably somewhere in the old world, although it has been suggested that *N. galligena* is native to North America (Mahoney *et al.*, 1999; L. Bernier, Universite Laval, personal communication, 1997)). That the eight isolates from Canada formed two distinct groups (Ng-FV-4, Ng-FV-5, Ng-FV-6 (from British Columbia) and IMI 376407 (Nova Scotia) all from *Malus*, 45-48 in **Fig 3.4**; CBS 100319, CBS 100320, CBS 100321 and CBS 100478, two from *Malus* and one each from *Acer* and *Populus* (all

from Nova Scotia, except CBS 100319 from Quebec; 49-52 in Fig 3.4), could represent the introduction to that country of two distinct populations of this fungus. Possibly supporting the opposite explanation that the clustering may be more host-based is the fact that the two Canadian *Malus* isolates (CBS 100319 and CBS 100478) in the 'North American' group were collected from orchards either adjacent to a sugar maple (*Acer saccharum*) plantation, or in the vicinity of natural hardwood stands containing both *Acer* and *Populus* spp., respectively (G. Braun, Agriculture Canada; L. Bernier, Université Laval, personal communications, 1997).

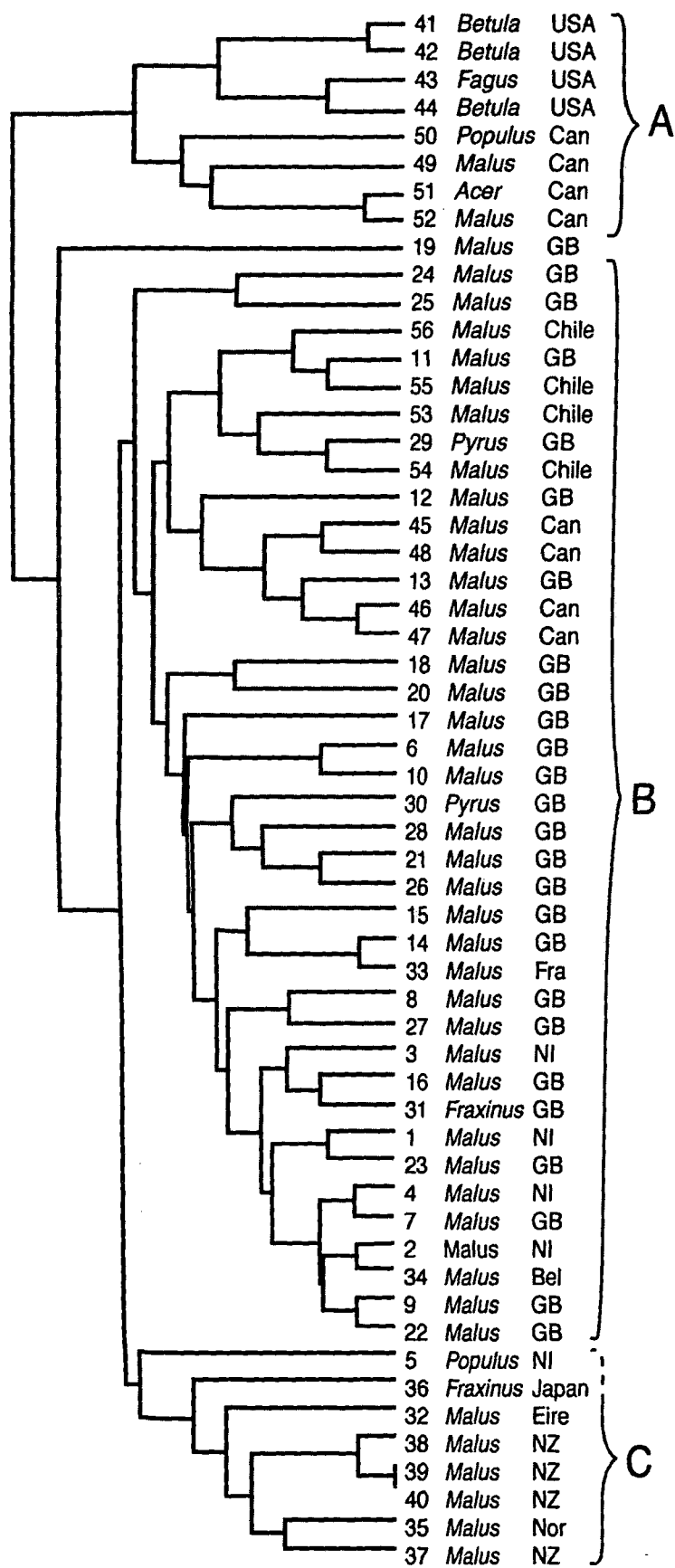


Fig. 3.4. Cluster analysis of 56 isolates of *N. galligena* using combined results of arbitrarily primed PCR using two primers (X11 and bacteriophage M13 core sequence) with host genus from which originally isolated and country of origin. Braces indicate clusters as discussed in the text. Numbers refer to isolates as listed in **Table 3.1**. Countries of origin given either in full or abbreviated as follows: Bel, Belgium; Can, Canada; Fra, France; GB, Great Britain; NI, Northern Ireland (Ulster); Nor, Norway; NZ, New Zealand; USA, United States of America.

3.3.3 IGS PCR-RFLP

Using the *Fusarium*-derived primers PN11 and PN22 in PCR, a single product exhibiting length polymorphism (ranging in approximate size from 2.45-3.15 Kbp), was amplified from all 56 isolates of *N. galligena* tested (see Fig. 3.5). This product was presumed to represent the rRNA gene operon IGS region, including about 490 bp of the 3'-end of the large sub-unit rRNA gene, and this was confirmed by direct comparison of short sequences generated from both ends of the PCR product from three representative isolates (IMI 378754, ICMP 13275 and MUCL 40782) with published sequences through database searches. The *Nectria*-derived sequences (accession numbers AJ243077 (ICMP 13275), AJ243078 (IMI 378754) and AJ243079 (MUCL 40782) (each from the 5'-end); AJ243080 (ICMP 13275), AJ243081 (IMI 378754) and AJ243082 (MUCL 40782) (each from the 3'-end)) showed greatest similarities with designated fungal IGS accessions.

The length polymorphisms seen in the PN11/PN22 PCR products could be broadly correlated with two characters, host and country of origin. Isolates from Rosaceous hosts (*Malus* and *Pyrus* combined) varied between 2.6 and 2.95 Kbp but with 41 of the 47 (87%) falling in the range 2.75 to 2.9 Kbp. The majority of isolates from all other hosts were spread evenly in the range 2.45 to 2.85 Kbp but with one isolate (from *Fraxinus* in Japan) at 3.15 Kbp. These results might suggest some specialisation in isolates from Rosaceous hosts. However, isolates from the United Kingdom and Eire also showed limited variability, falling in the range 2.7 to 2.95 Kbp (with 27 of the 36 (75%) in the range 2.75-2.85 Kbp). Isolates from other countries as a whole were more variable at 2.45 to 3.15 Kbp. Taking countries by region supported a geographic/introduction based explanation for the distribution of variation. PCR products from all eight isolates from the southern hemisphere (Chile and New Zealand) were either 2.85 or 2.9 Kbp. From North America, the four Canadian *Malus* isolates (45-48) falling in cluster A by RFLPs of the IGS region (see Fig. 3.7) and by arbitrarily primed PCR (and perhaps representing one introduction) gave a 2.9 Kbp product. Conversely, the other Canadian and the US isolates, six out of eight of which were from non-Rosaceous hosts, ranged from 2.45 to

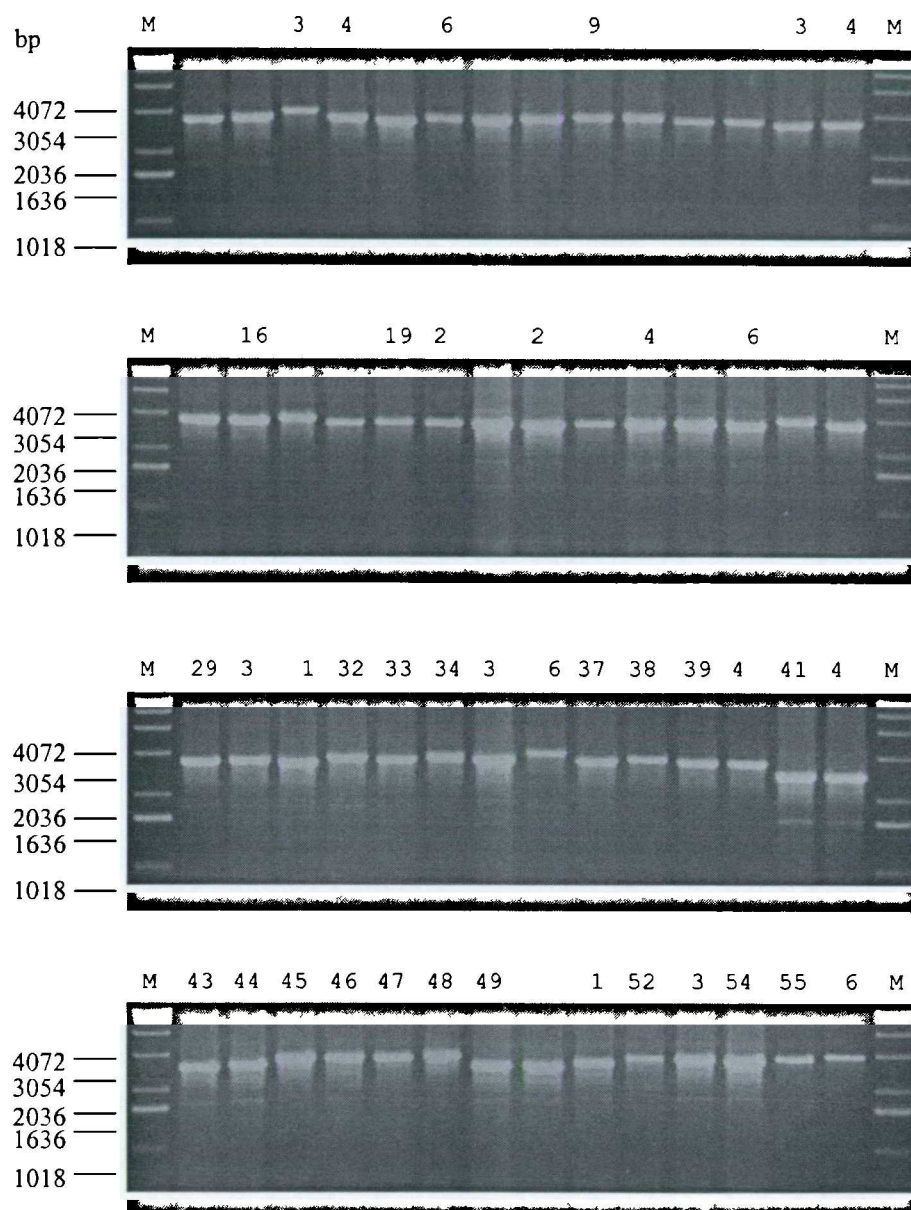


Fig. 3.5. PCR amplification of the rRNA IGS region using primers PN 11 and PN 22 (see point 3.2.3.1. for further details) for all 56 isolates from the world wide collection of *N. galligena* isolates. M: ϕ X174 *Hinf*I size marker (Promega). Lanes numbered 1 to 56 as designated in Table 3.1.

2.9 Kbp, possibly representing an older, genetically more diverse population or one not specialised for Rosaceous hosts.

Digestion of the PCR products from all 56 isolates with a panel of restriction endonucleases having four base recognition sites (*AluI*, *HaeIII*, *HhaI*, *HinfI*, *MboI*, *MspI*, *RsaI* and *TaqI*) generally revealed easily scored RFLP profiles of between 3 to 8 fragments upon electrophoresis. DNA fragments of less than 120 bp were not considered as they were not clearly resolved by electrophoresis in 20 g l⁻¹ Metaphor agarose gels. An example of the RFLP profiles produced by direct restriction enzyme digestion of IGS PCR products from all 56 isolates with *MboI* is given in **Fig. 3.6**. Depending on the restriction endonuclease used, 10 to 27 distinct IGS PCR-RFLP profiles were revealed within the world-wide collection of isolates. Taken overall, RFLP analysis revealed 50 distinct rRNA IGS haplotypes with most haplotypes represented by only a single isolate. As with the arbitrarily primed PCR, cluster analysis of a total of 196 polymorphic RFLP fragments did not give strong differentiation into clades (**Fig. 3.7**) although two clusters could be defined with a number of more distinct isolates. The smaller cluster (A in **Fig. 3.7**) corresponded closely with cluster A from the arbitrarily primed PCR in being largely from non-Rosaceous hosts and almost entirely from North America but differed in including a *Fraxinus* isolate from Japan (MAFF 410257, 36 in **Fig. 3.7**). The larger cluster (B in **Fig. 3.7**) comprised entirely isolates from Rosaceous hosts. Using the IGS marker, four isolates, two each from non-Rosaceous and Rosaceous hosts (MAFF 410257, *Fraxinus*, Japan, IMI 378755, *Populus*, Northern Ireland, CBS 100317 *Malus*, Northern Ireland and IMI 376405; 3, 5, 31 and 35 in **Fig. 3.7**) did not easily fit the two main clusters. In general, the geographic correlation seems less clear with the IGS marker than with the arbitrarily primed PCR markers (the isolates from Chile, Canada and New Zealand in clade B all being less closely clustered for each country with the IGS marker than with the arbitrarily primed PCR, *cf.* **Fig. 3.7** with **Fig. 3.4**). Isolates which shared identical haplotypes usually came from the same country (*e.g.* 2 and 4 from Northern Ireland or 45-47 from the Fraser Valley, British Columbia, Canada) but not always (isolates 48 and 55 were from Canada and Chile respectively).

3.3.4 Application to Aylesham and Budding Trial Isolates

3.3.4.1 Aylesham Trial

A total of 45 isolates were obtained from 80 cankers sampled across the trial at the final recording date (2 October 1996) and DNA prepared. No isolates were recovered from trees which originated from nurseries IV or X, but numbers typically ranged from 2 to 8 of the canker lesions on trees from other nurseries (see Table 3.2). Each isolate was assigned an IGS haplotype and an M13 genotype, defined by the combination of patterns obtained with the three restriction endonucleases, and M13 mini-satellite amplification profiles, respectively. Chi-squared analysis was used to test the difference between sources, and geographic provenance, in the distribution of both isolate IGS haplotypes and M13 genotypes made from active lesions across each trial site.

Table 3.2. Incidence of 13 IGS PCR-RFLP composite haplotypes of *N. galligena* isolates obtained from cankers on apple trees distributed across the Aylesham trial in relation to nursery source and their geographic origin.

Nursey	I	II	III	V	VI	VII	VIII	IX	Total
Origin	Europe	UK	UK	Europe	UK	UK	Europe	Europe	/
Haplotype									
A	0	0	6	0	1	0	2	2	11
B	3	3	0	0	0	1	2	0	9
C	1	1	0	1	0	2	0	0	5
D	1	0	0	1	1	0	0	1	4
E	0	0	0	2	0	0	0	0	2
F	0	0	0	0	0	0	0	2	2
G	0	0	0	0	0	3	1	1	5
H	0	1	0	0	0	0	0	1	2
I	1	0	0	0	0	0	0	0	1
J	0	1	0	0	0	0	0	0	1
K	0	1	0	0	0	0	0	0	1
L	0	0	0	0	0	1	0	0	1
M	0	0	0	0	0	0	0	1	1
Total	6	7	6	4	2	7	5	8	45

PCR amplification of the IGS region was achieved for each isolate obtained with amplification products ranging in size from 2.7 to 3 Kbp. Direct restriction digestion of each IGS product with *Hinf*I, *Hae*III and *Hha*I revealed 13 composite haplotypes, designated A-M (see **Table 3.2**). Of these haplotypes, five (I, J, K, L and M) were represented by only one isolate. The remaining haplotypes (A-H) were represented by two or more isolates, and with one exception, isolates from trees originating in the same nursery were assigned to more than one haplotype. The exception was Nursery III from which all six isolates were found to conform to haplotype A

The data shown in **Table 3.2** was subjected to Chi square analysis (**Table 3.3**). When data from all nurseries was included the distribution of haplotypes between them differed significantly ($p = 0.01603$). However, when the 6 isolates from Nursery III were excluded from the analysis no significant difference was indicated. Additionally, there was no difference in the distribution of haplotypes of isolates from trees raised in the UK compared with those raised in mainland Europe.

Table 3.3. Chi square analysis of combinations of IGS PCR-RFLP haplotype frequencies from given nursery sources and geographic origins as detailed in **Table 3.2**.

Source of variation	χ^2	df	p
All haplotypes	114.1	84	0.01603
All haplotypes and all sources except source III	85.30	72	0.1353
Geographic origin of nurseries	11.31	12	0.5023
Geographic origin of nurseries less unique haplotypes	6.245	7	0.5115

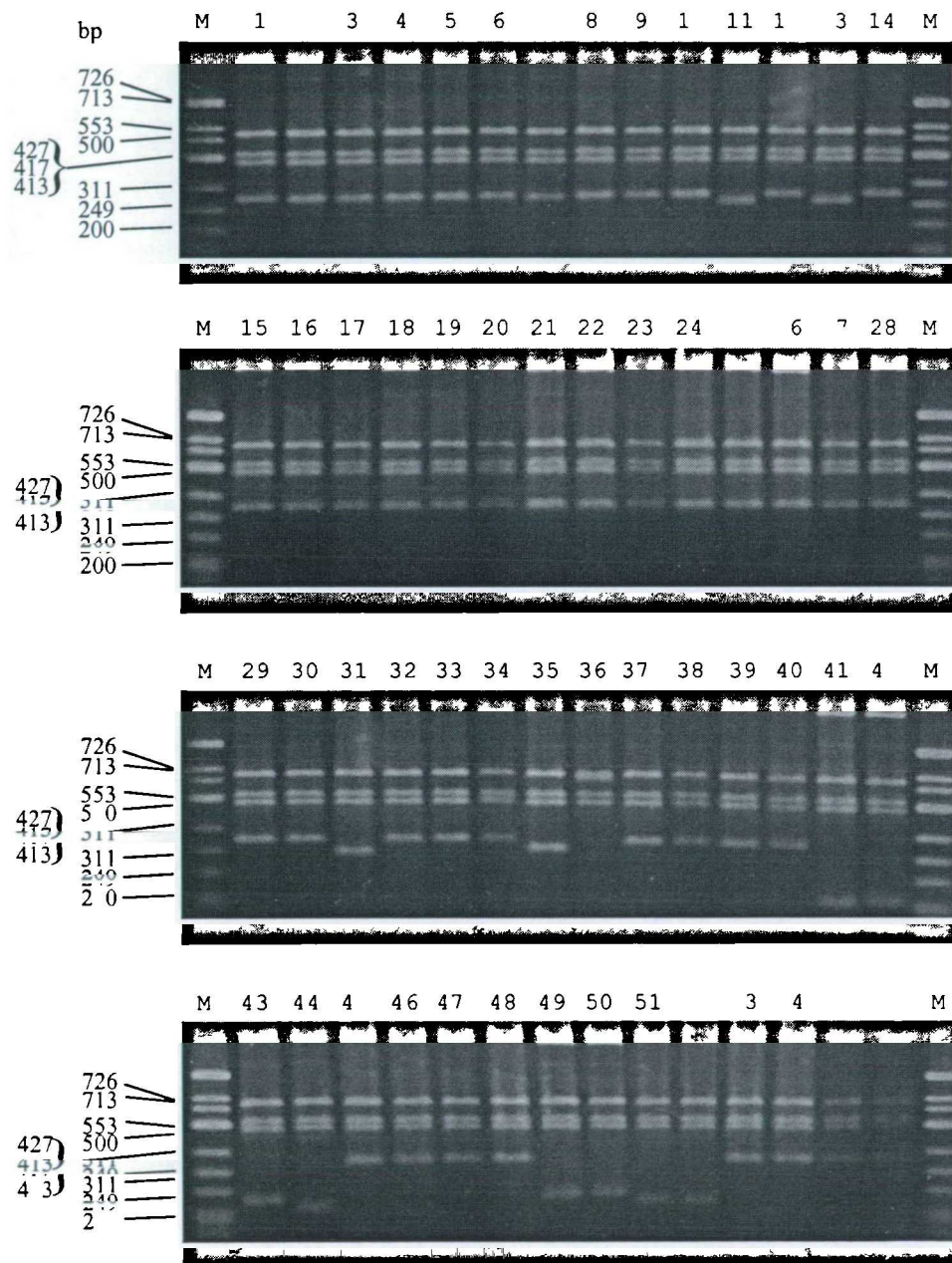
Mini-satellite based PCR fingerprinting of all 45 isolates using the core sequence of M13 bacteriophage as primer revealed 23 genotypes, of which 4 occurred more than twice and 14 were unique. The much wider disparity of genotypes relative to sample size was insufficient for reliable statistical analysis in distinguishing differences in nursery

source or origin across the trial. However, 5 out of 9 instances of M13 genotype A were from nursery source III (corresponding to 6 out of 11 instances for IGS PCR-RFLP haplotype A (see **Table 3.2**)) reinforcing the almost exclusive association of nursery source III with a distinctive clonal isolate.

3.3.4.2 Budding Trial

Despite successive attempts at isolation from all 118 cankered lesions collected from across this trial only 14 successful isolations were achieved, representing a particularly low recovery frequency. Molecular analysis of these 14 isolates by IGS PCR-RFLP revealed 6 haplotypes (B, C, D, E, F and J) distributed across the trial, 2 of which, E and J, were unique. However, due to the sample size and clear lack of differences in haplotype distribution, preliminary evaluation of this limited data set was insufficient for reliable statistical analysis. Similarly, the 5 M13 genotypes (1 unique) were also insufficient for reliable statistical analysis.

Fig. 3.6. PCR amplified rRNA IGS region digested with DNA restriction endonuclease *Mbo*I (see point 3.2.4 for further details) for all 56 isolates from the world wide collection of *N. galligena* isolates. M ϕ X174 *Hinf*I size marker (Promega). Lanes numbered 1 to 56 as designated in **Table 3.1**.



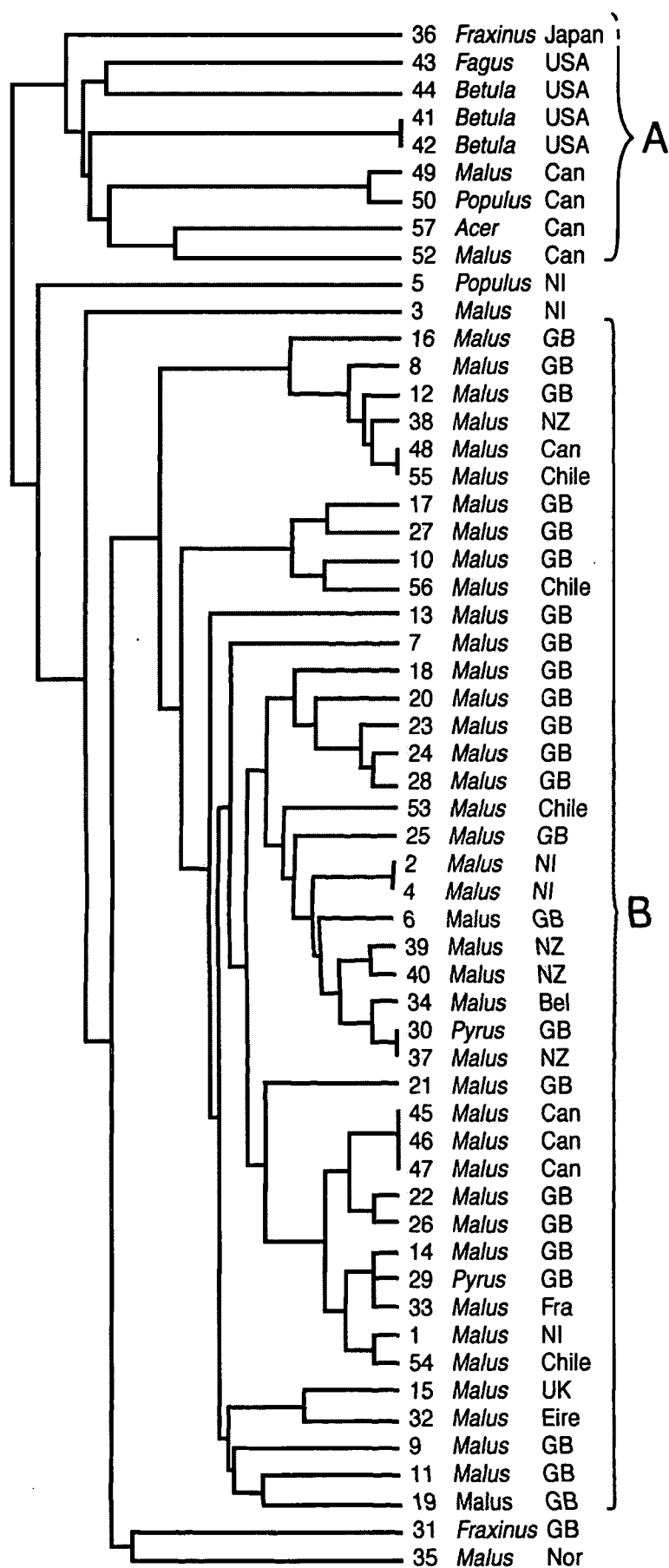


Fig. 3.7. Cluster analysis of 56 isolates of *N. galligena* using RFLP analysis of IGS amplicon with host genus from which originally isolated and country of origin. Braces indicate clusters as discussed in the text. Numbers refer to isolates as listed in **Table 3.1**. Countries of origin given either in full or abbreviated as follows: Bel, Belgium; Can, Canada; Fra, France; GB, Great Britain; NI, Northern Ireland (Ulster); Nor, Norway; NZ, New Zealand; USA, United States of America.

3.4 Discussion

A major consideration when applying molecular approaches to assessing inter- and intra-specific variation is to choose those procedures which reveal levels of discrimination between genotypes appropriate to the objective of the intended study, in this case understanding population structure in *N. galligena*. The current work was aimed at evaluating distinct PCR-based methods that utilise either segments of a specific tandemly repeated locus, the rRNA gene, or a much wider sample of variability in the genome as a whole. To this end, PCR-RFLP analysis of the internal transcribed (ITS), with sequence analysis of the ITS and the 5' end of the LSU RNA gene for a representative sub-set of isolates, and, separately, of the inter-genic spacer (IGS) regions of the rRNA genes, were compared with arbitrarily primed PCR using a random decamer primer and PCR using a mini-satellite related M13 bacteriophage derived primer for assessing variation between isolates.

No RFLPs were detected in the ITS when using the restriction endonucleases tested here and, for the sub-set examined, virtually no sequence divergence within this region (being limited to a single change in one isolate), regardless of original host or geographical provenance. This absence of variation excluded this gene segment from being developed as an intra-specific marker for molecular ecological application within *N. galligena*. As would be expected from the lack of variation within the ITS, no variation was found within the 5'-terminal sequence obtained from the LSU rRNA gene. This segment included the D1 and D2 domains which are less highly conserved than some other parts of the gene and which have been used to study divergence amongst closely related species (Edel *et al.*, 1996; Guadet *et al.*, 1989; O'Donnell, 1993; Qu *et al.*, 1988; Sheriff *et al.*, 1994). The high degree of intra-specific sequence homogeneity observed at these loci, as well as in the unlinked β -tubulin gene intron and mtSSU, suggests that they may be useful as species-specific genetic markers in molecular systematic and phylogenetic studies of the Coccinea group or the wider circumscription of the *Nectria* genus. It may also be possible to develop species-specific primers for diagnostic applications.

Both arbitrarily primed PCR analysis using a random decamer and PCR with an M13 derived primer differentiated among isolates of *N. galligena*. Both methods are well established informative techniques for evaluating genetic diversity in fungi and are probably affected by similar types of molecular changes within the genome. (Polymorphisms with random oligomer primers will result from insertions/deletions, inversion or nucleotide substitutions disrupting or rearranging primer annealing sites or by giving measurable length variation in the target DNA between primer sites; the M13 derived primer is presumably mainly revealing variation, again induced by insertion/deletion or inversions, in the spacing between closely placed, *i.e.* up to 1-2 Kbp apart, short tandem repeat sequences rather than changes in the primer sites *per se.*) As the net effect of giving a rapid estimate of overall similarity between fungal genomes is comparable for both primer types and there were no obvious major differences in the results given by the two approaches, the results are given in a combined format. Using these methods, the collection studied was clearly genetically heterogeneous with no obvious dominance by any clonal lineage. All but two of the isolates (ICMP 13255 and ICMP 13256, both from *Malus*, New Zealand) comprised unique genotypes, suggesting sexual recombination is important in both global and regional populations.

Similarly, PCR-RFLP analysis of the IGS differentiated among the isolates and the results were generally consistent with the arbitrarily/M13 primed PCR results although indistinguishable genotypes occurred more frequently with IGS-RFLP analysis than with the latter technique. These indistinguishable genotypes were not only associated with common host and country of origin (*e.g.* CBS 100472 and CBS 1004723, both from *Betula*, USA) but also diverse countries (IMI 376407 and IMI 376385, both from *Malus* but from Canada and Chile, respectively). In this particular collection, no isolates with different hosts gave identical haplotypes but such isolates may have been found if more had been tested. The level of discrimination observed when using eight restriction endonucleases to digest the PCR product was not greatly reduced when only the four most informative restriction enzymes (*Hae*III, *Hinf*I, *Hha*I and *Taq*I) were used and using just these enzymes would reduce the effort required to type isolates. The PCR-

RFLP analysis of rRNA IGS approach appears robust and has already been successfully transferred to other laboratories collaborating in studies of *N. galligena* populations.

The basis of the variation observed within *N. galligena* is not clear and the overall data presented here may be interpreted to support a population structure based on either host preference or geographic origin. The lack of variation within the ITS/LSU rRNA gene (5'-terminal end) supports the retention of *N. galligena* as a single relatively homogenous species, including those derived from *Fraxinus* spp. (exemplified here by MAFF 410257 and ATCC 11684). This is in agreement with recently reported results from the USA where a distinct molecular technique was used to show that *N. galligena* isolates formed a clade (with little intra-clade variability) quite distinct from *N. coccinea* var. *coccinea* and *N. coccinea* var. *fraxinus* isolates (Mahoney *et al.*, 1999). Within *N. galligena*, isolates derived from *Fraxinus* hosts have been proposed as a distinct variety (var. *major*) by Wollenweber (1924) although Booth (1959) was unable to separate these morphologically. In the study reported here, there was no evidence that the two *Fraxinus* isolates formed a distinct clade, being clearly separated from each other in both cluster analyses. In Northern Ireland, cross-inoculation experiments with an isolate from *F. excelsior* and one from apple showed that the former isolate caused an atypical infection symptom on apple, but that the apple-derived isolate was unable to induce lesions on *Fraxinus* (Flack and Swinburne, 1977). In Canada, it has been demonstrated that isolates derived from a range of North American hardwood species were unable to induce any necrosis on *F. americana* (Plante and Bernier, 1997), consistent with much earlier findings of Spaulding *et al.* (1936). Some degree of host specificity was also reported by Richter (1928). Although characterisation of any host preferences requires further investigation, the overall homogeneity of ITS and LSU sequence data and the inability of RAPD and PCR fingerprint data to distinguish these two isolates as a clade separate from *Malus* and *Pyrus* derived isolates suggests that specialisation is a recent phenomenon or that it is only weak in this species.

It is clear from the results that both PCR-RFLP of the rRNA IGS region and arbitrarily primed PCR (here using combined random decamer/M13 sequence primers) reveals levels of intra-specific variation which can be used to study population structures in *N. galligena*. However, the PCR-RFLP procedure of the rRNA IGS developed in this study is technically very robust, simple and reliable, and relatively unaffected by minor variations in experimental conditions (as compared to arbitrarily primed PCR). These attributes make this approach the molecular method of choice for population studies with *N. galligena* and for inter-laboratory transfer of both protocols and data.

As there was a six-fold difference between the highest and lowest incidence of *Nectria* canker between nursery sources at the final recording date of the Aylesham Trial, an attempt to resolve whether these infections occurred at source during propagation was made using the techniques developed here. Application of the IGS PCR-RFLP method to isolates obtained from canker material (derived from Chapter 2), it was possible to categorise isolates into one of 13 haplotype groups, a wider diversity than was detected by Brown *et al.*, (1994). With one exception, trees from each nursery yielded more than one haplotype, while Chi square analysis of haplotype distribution indicated that this was related to the nursery of origin of the trees. However, this was due exclusively to the nursery with only one haplotype (Nursery III, haplotype A). As most isolates obtained from trees from all sources, apart from Nursery III, were classified in disparate group this could represent infections from a mixed population of *N. galligena* either in the nursery or orchard environment. This would require a much larger sample size for assessment than achieved here. Unfortunately, the low rate of isolate recovery from this material (a problem encountered by others, Anagnostakis and Ferrandino, 1998), and subsequent small sample size, precluded a more extensive molecular appraisal. However, the low recovery frequency of *N. galligena* isolates highlights the limitations of molecular ecological or epidemiological applications, especially of tree pathogens, where large sample sizes are usually required.

Chapter 4. Molecular detection of *Nectria galligena* in apple wood

4.1 Introduction

The results of Chapter 2 has indicated that young trees may be symptomlessly infected during propagation and that these trees may be a source of canker infection following orchard establishment. In the absence of a reliable selective media, conventional diagnosis by direct isolation is often hindered by the presence of other, often faster growing, endophytic fungi (Anagnostakis and Ferrandino, 1998; A. Berrie, Horticulture Research International, personal communication, 1997) such as *Fusarium lateritium* and *Botrytis cinerea* (Li, 1995). In order to determine the frequency of occurrence of the pathogen in propagation material a sensitive diagnostic method is required.

PCR-based assays are widely used to detect plant pathogenic fungi (e.g. *Phoma tracheiphila*, Rollo *et al.*, 1990; *Gaeumannomyces graminis*, Henson *et al.*, 1993; *Polymyxa betae*, Mutasa, *et al.*, 1995; *Tilletia indica*, Ferreira *et al.*, 1996), and may be highly specific (e.g. races within formae speciales of *F. solani* (Crowhurst *et al.*, 1991) and *F. oxysporum* (Chiocchetti *et al.*, 1999)). The internal transcribed spacer regions (ITS 1 and 2) of the nuclear rRNA repeats are frequent targets for this approach (e.g. *Verticillium* spp., Nazar *et al.*, 1991; *Cylindrocladium floridanum*, Hamelin *et al.*, 1996; *Spongospora subterranea*, Bulman and Marshall 1998; *Plasmodiophora brassicae*, Faggian *et al.*, 1999; *Ophiostoma piceae*, Kim *et al.*, 1999) being readily accessible by direct PCR amplification with universal primers designed to highly conserved flanking regions (White *et al.*, 1990) and are present in high copy number (often 100-200 per fungal haploid genome (Russel *et al.*, 1984)).

The present study describes the development of an improved species-specific PCR based diagnostic assay than previously reported (Brown *et al.*, 1993), incorporating an internal positive control to identify false negatives, and allowing template quantification, for the detection and identification of *N. galligena* from infected material.

4.2 Materials and Methods

4.2.1 Fungal cultures

Endophytic fungal species were isolated concomitantly during studies on *N. galligena* in infected apple wood tissues, as described in Chapter 3, point 3.2.1. (Table 4.1), and maintained on 12 g l⁻¹ Malt Extract Agar (MEA) (Oxoid, USA) supplemented with Augmentin™ (containing amoxycillin sodium and potassium clavulanate) (SmithKline Beecham Pharmaceuticals, UK) at 0.6 mg ml⁻¹. Strains of *F. lateritium* were maintained on Spezieller Nährstoffarmer Agar plus yeast extract (SNAY) (Nirenberg, 1976). All endophytic species as listed in Table 4.1 were deposited with the International Collection of Microorganisms on Plants (ICMP), Auckland, New Zealand. Three additional isolates (MUCL 40720, MAFF 410257 and CBS 100321) from Chapter 3 were included for evaluation of primer specificity and sensitivity. High molecular weight DNA preparations were made from cellophane grown mycelial mats as described in Chapter 3, point 3.2.2. Bacterial cultures were maintained on Standard Nutrient Agar (Oxoid, USA) and genomic DNA obtained by freeze-thawing of cells in sterile Milli-Q AR grade water followed by extraction with an equal volume of Tris-washed phenol (Fisher Scientific): chloroform: pentan-2-ol (25:24:1).

4.2.2 DNA extraction from plant tissue

DNA extraction from fresh plant leaf tissue collected from the National Fruit Collections, Brogdale, Kent, UK, followed a modified version of the procedure of Doyle and Doyle (1990): two pieces of fresh young leaf tissue, harvested by enclosing in the cap of a 1.5 ml eppendorf tube, were macerated with a disposable plastic pestle in 300 µl of extraction buffer (30 g l⁻¹ CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris HCL (pH 8.0), 10 g l⁻¹ PVP-40T (soluble)) and 20 µl of 2-mercaptoethanol until the tissue formed a homogenous slurry. If necessary, 100 mg of fine acid washed sand was added to aid grinding. A further 400 µl of extraction buffer was then added, the contents mixed thoroughly and extracted with 300 µl of chloroform:octanol (24:1 v/v). Following centrifugation (2 min, 11,600 X g), the aqueous supernatant was transferred to a clean sterile microfuge tube and re-extracted twice with 300 µl of chloroform:octanol (24:1 v/v). The aqueous phase

was transferred to a clean sterile microfuge tube, approximately 600 μ l of cold (-20° C) propan-2-ol added and mixed gently. After 15-30 minutes at room temperature, DNA was pelleted by centrifugation (5 min, 11,600 X g). The supernatant was discarded and the pellet washed in 1 ml of 70 % ethanol at 4° C overnight. The pellet was finally collected by centrifugation (5 min, 11,600 X g), and after discarding the ethanol wash, air dried at room temperature.

Table 4.1. Fungal pathogenic and endophyte species associated with apple, including *N. galligena*, *N. cinnabarina*, bacterial strains, and apple, pear and quince material used in this study.

Species	Code	Origin	EMBL No.	Ch1/Ch2	ITS5/ITS4
<i>Nectria galligena</i>	IMI 378754	Wye College	AJ228666	+	+
<i>Nectria cinnabarina</i>	IMI 362479	IMI ⁷	AJ009264	-	+
<i>Aureobasidium pullulans</i>	ICMP 13346	Wye College	AF121282 ¹	-	+
<i>Penicillium expansum</i>	ICMP 13360	Wye College	AJ269846 ³	-	+
<i>Penicillium expansum</i>	ICMP 13361	Wye College	-	-	+
<i>Penicillium expansum</i>	ICMP 13362	Wye College	-	-	+
<i>Epicoccum nigrum</i>	ICMP 13352	Wye College	AJ269841	-	+
<i>Trichoderma viride</i>	ICMP 13366	Wye College	AJ269845	-	+
<i>Trichoderma viride</i>	ICMP 13367	Wye College	-	-	+
<i>Phoma</i> sp.	ICMP 13364	Wye College	-	-	+
<i>Phoma</i> sp.	ICMP 13365	Wye College	-	-	+
<i>Cladosporium herbarum</i>	ICMP 13348	Wye College	AJ244227 ¹	-	+
<i>Cladosporium herbarum</i>	ICMP 13349	Wye College	-	-	+
<i>Cladosporium herbarum</i>	ICMP 13350	Wye College	-	-	+
<i>Cladosporium herbarum</i>	ICMP 13351	Wye College	-	-	+
<i>Mucor piriformis</i>	ICMP 13359	Wye College	AJ269842	-	+
<i>Phomopsis</i> sp.	ICMP 13556	Wye College	AJ269844	-	+
<i>Alternaria alternata</i>	ICMP 13557	Wye College	U05195 ^{1,2}	-	+
<i>Fusarium</i> sp.	ICMP 13353	Wye College	-	-	+
<i>Fusarium lateritium</i>	ICMP 13355	Wye College	AJ269848	-	+
<i>Fusarium lateritium</i>	ICMP 13356	Wye College	AJ269849	-	+
<i>Fusarium lateritium</i>	ICMP 13357	Wye College	AJ269850	-	+
<i>Nectria</i> sp.	ICMP 13358	Wye College	AJ269851	-	+
<i>Botrytis cinerea</i>	ICMP 13347	Wye College	Z73765 ^{1,2}	-	+
<i>Pestalotia</i> sp.	ICMP 13363	Wye College	AJ269843	-	+
<i>Venturia inaequalis</i>	IMI 273208	HRI ⁸	-	-	+
Unidentified	ICMP 13368	Wye College	-	-	+
Unidentified Bacterial sp.	-	Wye College	-	-	N/A
<i>Bacillus subtilis</i>	-	Wye College	-	-	N/A
<i>Malus domestica</i> ⁴	-	NFC ⁹	-	-	+
<i>Pyrus communis</i> ⁵	-	NFC ⁹	-	-	+
<i>Cydonia oblonga</i> ⁶	-	NFC ⁹	-	-	+

¹Additional 5.8S rRNA gene sequences, including flanking ITS 1 and ITS 2 regions, taken from the sequence databases as representative of species quoted but not of given isolate.

²GenBank database sequence ID.

³ITS 1 region only.

⁴*Malus domestica* (cvs. Spartan, Royal Gala, Golden Delicious, Jonagold, Bramley's Seedling, Cox's Orange Pippin, Worcester Pearmain, Discovery and Gala; rootstocks, M9, M25, M26, M27, MM106, and MM111).

⁵*Pyrus communis* (cvs. Conference and Comice Bodson).

⁶*Cydonia oblonga* rootstocks Quince A and Quince C.

⁷IMI, CABI Bioscience, Egham, Surrey, United Kingdom.

⁸HRI, Horticulture Research International, East Malling, West Malling, Kent, United Kingdom.

⁹NFC, National Fruit Collections, Brogdale, Faversham, Kent, United Kingdom.

4.2.3 DNA extraction from lignified tissue

High molecular weight DNA was extracted from lignified apple wood tissue according to a modified procedure of Torres *et al.* (1993): approximately 3 cm length pieces of fresh or freeze dried apple or pear wood shoots were ground for 10 min in a ball mill (Glen Creston Ltd., Stanmore; mixer/mill 8000) using steel ball bearings, or by hand in liquid nitrogen with a pestle and mortar. DNA from milled/ground tissue was extracted in 750 μ l extraction buffer (20 g l⁻¹ CTAB, 10 g l⁻¹ polyvinyl pyrrolidone PT-40, 100 mM Tris-HCL (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 0.5 mM metabisulphite, with the addition of 4 ml l⁻¹ β -mercaptoethanol just prior to use) in a 1.5 ml microfuge tube. 100 μ l of chloroform:octanol (24:1 v/v) was added to the mixture and the whole incubated at 65° C for 30 minutes. After cooling it was further extracted by the addition of 500 μ l chloroform:octanol (24:1 v/v), vortexed briefly, and centrifuged (10 min, 11,600 X g). The aqueous phase was transferred to a clean sterile microfuge tube and extracted twice with an equal volume of Tris-washed phenol (Fisher Scientific): chloroform: pentan-2-ol (25:24:1), allowing 10 minute incubation at room temperature for each extraction. Following centrifugation (10 min, 11,600 X g), the supernatant was again transferred to a clean sterile tube and two-thirds volume water saturated diethyl ether added and vortexed briefly. The mixture was centrifuged (2 min, 11,600 X g), the supernatant discarded and the extraction repeated. Finally, an equal volume of cold (-20° C) propan-2-ol (BDH) was added, mixed gently and centrifuged (10 min, 11,600 X g). Pellets were washed in 70 % ethanol and air dried at room temperature.

4.2.4 Rapid DNA extraction from lignified tissue

A rapid procedure for the preparation of PCR amplifiable template from infected woody tissue was formulated. Thin wooden shavings, made with a pencil sharpener for shoots up to three years old or directly with a scalpel for older tissues, were placed in a 1.5 ml tube with approximately 5-600 μ l of extraction buffer (Torres *et al.*, 1993). DNA was eluted via repeated freeze/thawing in liquid nitrogen/65° C, respectively, or by incubation in a boiling bath for up to two minutes. Following centrifugation (2 min, 11,600 X g), extracts were passed through a Sepharose® spin dialysis column. Aliquots of dialysate were used directly in PCR assays.

Fungal and plant (fresh leaf and lignified tissue) DNA was resuspended in sterile Milli-Q AR grade water and stored at -20° C. The concentration and quality of DNA extracts were determined electrophoretically or spectrophotometrically (Sambrook *et al.*, 1989).

4.2.5 PCR amplification conditions

The rRNA internal transcribed spacer regions (ITS 1 and 2) and 5.8S rRNA gene from a range of fungal endophytes were amplified with universal primers ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990). PCR reaction conditions were; 5 ng template DNA (at a standard concentration of 0.5 ng/ μ l), 0.1 mM of each primer, 100 mM each of dNTP, (Pharmacia, Sweden), 1.25 units Biotaq DNA polymerase (Bioline, UK), 10 μ l 10X PCR reaction buffer (160 mM (NH₄)₂SO₄, 670 mM Tris-HCL (pH 8.8 at 25° C), 1 ml l⁻¹ Tween-20) and 1.5 mM MgCl₂ (Bioline, UK), and sterile Milli-Q AR grade water in 0.2 ml Apex thin walled polypropylene tubes (Alpha Laboratories, UK). Amplification conditions, using a Perkin-Elmer 9700 thermal cycler (Norwalk, USA) comprised an initial denaturation (3 min/ 94° C) followed by 30 cycles of 1 min/94° C, 1 min/55° C and 1 min/72° C with a final post extension of 72° C/10 min. For diagnostic PCR with primers Ch 1 and Ch 2 conditions were identical except an annealing temperature of 63° C was used. Appropriate controls were always included in all experiments.

Post-amplification, PCR products were assessed by electrophoresis of 5-10 μ l of reaction mix in a 10 g l⁻¹ agarose (Biogene, UK, 1 X TBE (89 mM Tris-borate, 2 mM EDTA at pH 8.0) gel. Following ethidium bromide staining, gels were photographed over UV light.

4.2.6 DNA sequencing

Purified PCR products were sequenced without cloning as previously described in Chapter 3, point 3.2.6. Sequences generated were submitted to the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk>) (Table 4.1).

5.2.7 Primer design

EMBL sequence accession AJ228666 representative of the ITS of 32 isolates of diverse global and host origin from Chapter 3 were aligned with the same region from other closely related *Nectria* species, mostly from the *Nectria Coccinea* group (*N. ditissima*, *N. coccinea*, *N. punicea*, *N. fuckeliana*, and *N. radicicola* (see Chapter 6)), with *N. cinnabarina*, and from several endophytic species (isolated during the course of this research). Two primers, Ch 1 5'-AAC CCC TGT GAA CAT ACC CAT C-3' (forward) and Ch 2 5'-GTG GCC GCG CTG CTC TTC CG-3' (reverse), exhibiting maximum 3' specificity to *N. galligena*, were designed using the PrimerSelect 3.05 module of DNASTar (Lasergene, Inc., Madison, USA) following the general concepts as addressed by Dieffenbach *et al.* (1995). From the sequence information, the predicted amplification size was 412 bp.

4.2.8 Primer specificity and sensitivity

Specificity of primers Ch 1 and Ch 2 for *N. galligena* was assessed by PCR against 5 ng of purified DNA from the 56 isolates as described from Chapter 3, **Table 3.1**, and closely related species (as outlined in point 4.2.7), including *N. cinnabarina*, and a range of predominantly endophytic fungal species (**Table 4.1**). The primer pair ITS 4 and ITS 5 were used to verify that extracts were suitable for amplification. Sensitivity of the standard PCR assay was determined by testing 5 and 10 fold serial dilutions of genomic DNA of *N. galligena* DNA (isolates IMI 378754, MUCL 40720, MAFF 410257, and CBS 100321, see **Table 3.1**).

4.2.9 Southern blot and hybridizations analysis

Southern blot analysis essentially followed a modified one step alkaline transfer method as described by Read and Mann (1985). Following electrophoresis through agarose gels as described in point 4.2.5., PCR products were transferred to positively charged nylon membrane (Boehringer, Mannheim) by capillary transfer in the presence of 0.4 M NaOH. Post-transfer, membranes were washed once in 1X SSC and stored at -20° C until required. Pre-hybridization (6 X SSC, 5 X Denhardt's solution, 1 g l⁻¹ SDS and 100 µg ml⁻¹ denatured salmon sperm DNA) was for 1 hour at 65° C with two post-hybridization washes for 30 minutes in 1 X SSC and 1 g l⁻¹

SDS at 65° C. The probe, prepared from the PCR amplification of *N. galligena* (isolate IMI 378754) genomic DNA with primers Ch 1 and Ch 2, was labelled with α -³²P adenosine 5'-triphosphate (ICN Pharmaceuticals Ltd., Basingstoke, UK) using DNA Polymerase I Large (Klenow) Fragment (New England Biolabs (UK) Ltd.) following the procedure described by Feinberg and Vogelstein (1984). Hybridization and subsequent detection on Kodak XAR film using intensifying screens were carried out according to standard approaches (Sambrook *et al.*, 1989).

4.2.10 Generation of a heterologous internal standard

A heterologous DNA template incorporating 5' and 3' termini identical with the *N. galligena* primer pair was generated according to the general approaches of Uberla *et al.* (1991) and Forster (1994). DNA from *Solanum tuberosum* cv. Desiree was subjected to relaxed PCR amplification using an annealing temperature of 39° C allowing multiple unspecific priming and the generation of a range of sized PCR products. A faint fragment, slightly larger to that of the specific diagnostic PCR product for *N. galligena* was excised from the gel and incubated at 4° C in 1 ml TE (10 mM Tris-HCL (pH 8.0), 1 mM EDTA (pH 8.0)) overnight. A 5 μ l aliquot of the elute was subjected to two PCR cycles at the same low annealing stringency followed immediately by 30 cycles of high annealing stringency as described in point 4.2.5. The PCR fragment was purified through Sepharose® CL-6B (Pharmacia) and cloned into pGEM-T (Promega). Plasmids containing the internal standard DNA fragment were harvested and purified from overnight cultures using the Qiagen Plasmid Midi Kit system (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. Aliquots of purified internal standard were amplified at high stringency in the presence of primers Ch 1 and Ch 2, singly and in combination, to confirm the presence of each primer specific recognition sequence. Stocks of resulting plasmid DNA were diluted in sterile Milli-Q AR grade water and stored at -20° C until required.

A standard curve was then generated. Initially, tests were carried out to determine the concentration of internal standard DNA template that would yield approximately equal or slightly less amplification product when co-amplified with 5 ng of fungal DNA. Two concentrations of competitor fragment were assessed over a 0.5 μ g to 5

pg range of fungal genomic target DNA under stringent PCR conditions. The ratio between the amounts of the two amplicons was determined by densitometric scanning of photographic negatives using a Sharp JX-325 high resolution colour scanner and the ImageMaster™ (version 1.0) module 1D software (Pharmacia Biotech). The relationship for each dilution series of *N. galligena* target DNA and internal control, the PCR product ratio (*N. galligena* product/internal control product) and the amount of fungal DNA was determined.

4.3 Results

4.3.1 Additional sequence data and fungal species identification

High molecular weight DNA prepared from the fungal species listed in **Table 4.1** gave an amplification product with universal primers ITS 5 and ITS 4 (White *et al.*, 1990) of approximately 500 to 600 bp. Amplicons were sequenced and the data used to aid identification by database comparisons (BLAST searches at <http://www.ncbi.nlm.nih.gov>).

4.3.2 Primer pair design

Previous work has shown that the internal transcribed spacer regions of *N. galligena* exhibits a high level of sequence conservation, irrespective of host or geographic origin (Chapter 3). Multiple sequence alignments were made between the sequence of the representative *N. galligena* isolate and those of related species, endophytes, pathogens and saprophytes likely to appear in, or on apple and pear tissues (see species sequence accessions in **Table 4.1**). Considerable sequence divergence between closely related species was found. However, closer analysis using PrimerSelect showed that not all divergent locations offered sufficient 3' end specificity for *N. galligena* across all species and that base composition for optimal primer design, necessary for specific and efficient priming of target DNA, were inadequate in all but two locations. This effectively eliminating the production of further primer pairs for the development of a nested PCR approach.

Two primers Ch 1 and Ch 2 (nucleotide positions 21 to 44, and 428 and 450 (as numbered beginning with the 5'-most nucleotide position, see **Fig. 4.1**), or positions

20 to 40, and 412 and 431 (from EMBL accession AJ228666), respectively), were designed and synthesised. For discrimination against *N. coccinea* var. *faginata*, *N. coccinea*, *N. punicea* and *N. fuckeliana* sequence differences were placed at the 3' end of the primer Ch 2, with further base differences, including an indel and base conversion, from position 5-7. For *N. ditissima* this was effectively achieved by a single deletion at position 5 from the 3' end of Ch 2.

4.3.3 Primer pair specificity and PCR sensitivity

Under empirically defined optimised conditions (annealing T_m 63° C), primers Ch 1 and Ch 2 yielded an amplification product of the predicted size from DNA prepared from 56 isolates of diverse host and global origin (see Chapter 3, Fig. 3.1). In practical applications, this method has been used to assist species identification in more than 300 isolates as part of a series of parallel epidemiological studies. DNA from closely related species, other fungi concomitantly recovered during attempts to isolate *N. galligena* from apple wood, bacteria and several rosaceous host DNA (Table 4.1) did not give amplification (for example see Fig. 4.2 (A), lanes 2, 3, 4 and 5). However, DNA from all fungal samples gave products with universal primers ITS 5 and ITS 4 (White *et al.*, 1990), indicating the presence of amplifiable DNA (see Table 4.1). PCR amplification of dilution series of purified DNA from differing isolates, ranging from 500 ng to 5 pg resulted in visible product on ethidium bromide stained agarose gels down to 25 pg of target template. Southern hybridization of PCR products did not lower the detection limit (see Fig. 4.3).

4.3.4 Detection of *N. galligena* in apple wood

Total DNA extracted from naturally infected canker lesions from woody apple and pear tissues produced a product of the predicted sized PCR fragment (412 bp) with diagnostic primers Ch 1 and Ch 2 (Fig. 4.2 (A), lanes 8, 9 and 10). Hybridization analysis, using α -³²P labelled diagnostic PCR fragment from a pure preparation of *N. galligena* (isolate IMI 378754) genomic DNA confirmed the presence of target sequence from the amplified woody extracts (Fig. 4.2 (B)). Sepharose® purified freeze/thaw extracts in extraction buffer or water of wood shavings taken from cankered wood contained sufficient target template for successful PCR.

A 500 bp heterologous fragment with forced identical primer recognition sites at either end for use as an internal control in routine diagnostic PCR applications was prepared from potato DNA using both primers Ch 1 and Ch 2 under relaxed annealing conditions. The slightly larger size was chosen to be distinct from the 412 bp *N. galligena* specific PCR product in order not to disadvantage the efficient amplification of the fungal target in routine applications. Two distinct bands of predicted size were produced when internal control fragment DNA was added to *N. galligena* DNA or extracts from naturally infected canker lesions in a standard PCR assay (Fig. 4.2, lanes 6 and 10).

Wood, stained or discoloured internally, indicative of the presence of *N. galligena* infection (Li, 1995), was assessed for PCR detection as a guide to sampling procedures. Ten trees (5 each of cv. Queen Cox and cv. Royal Gala), of varying levels of infection, were identified from the Wye College Experimental Orchard. Ten samples of internally discoloured and non-discoloured wood, distal to mature cankers and ranging in age from 2 to 10 years (including rootstocks and roots), were collected from each tree in spring and DNA prepared according to the modified procedure of Torres *et al.* (1993), as described above. PCR was consistently negative from all samples tested, apart from 2 root samples exhibiting considerable internal discolouration. However, with such low levels of detection it was difficult to ascertain whether this represented genuine cryptic infection, or accidental contamination. The failure of internal standard amplification was indicative of the presence of high levels of inhibitors. Further, recovery of *N. galligena* through repeated isolation attempts from all samples, including both PCR positive root samples, were unsuccessful.

Fig. 4.1. Multiple sequence alignment of the 5.8S rRNA gene sequences and their flanking internal transcribed spacers (ITS 1 and ITS 2). The sequences are written 5' to 3' and have been arranged according to their percentage similarity to *N. galligena* reference sequences AJ228666 as determined by Clustal analysis (Chapter 6). Identity of the *N. galligena* reference sequences are indicated by a period (.) and gaps are indicated by a hyphen (-). Species-specific oligonucleotide primers Ch 1 (position 21 to 44, forward) and Ch 2 (position 428 to 450, reverse) are indicated in bold. Species sequence abbreviations are as follows: N. gal, *N. galligena* (AJ228666); N. dit, *N. ditissima* (AJ009272); N. cvf, *N. coccinea* var. *faginata* (AJ009270); N. coc, *N. coccinea* (AJ009250); N. pun, *N. punicea* (AJ009273); N. fuc, *N. fuckeliana* (AJ009274); N. rad1, *N. radicicola* (AJ007357); N. rad2, *N. radicicola* (AJ007351).

	1	11	21	31	41	51
N. gal	-CCGAGTTTA	CAACTCCC-A	AACCCCTG-T	GAACATACC-	CATCGTTGCC	TCGGCGGTGC
N. dit						
N. cvf	T.A.....				T.....	
N. coc						
N. pun	C.GAT.....		A.			
N. fuc	..T.....				T.....	
N. rad1				A	T..T.....	
N. rad2	C.T.....	T.		A	T..T.....	

	61	71	81	91	101	111
N. gal	CCGCTCCGGC	GGCCCGCCAG	AGGACCCCA	AACTCTT-GT	TT-TATACAG	CATCTT-CTG
N. dit						
N. cvf					..-AT.....	T.....
N. coc				T.	..-AT.....	T.....
N. pun				T.	..-AT.....	T.....
N. fuc			A.	.C.CT.ATT	C.GA.....	T.....
N. rad1	.T...T....	A.....	AA.	CC..TAGAT.	---T.....	T.....
N. rad2	.T.T.T....	A.....G...	AA.	CC..AAGAT.	ACA.T..A..	T.....A...

	121	131	141	151	161	171
N. gal	AGTAACACGA	TTAAA-TAAA	TCAAACTTT	CAA-CAACGG	-ATCTCTTGG	-TTCTGGCAT
N. dit						
N. cvf						
N. coc						
N. pun						
N. fuc						-C.....
N. rad1	-T..	.A..--		..A.....	G.....	G.....
N. rad2	...C.-T..	...GG.C..		..A.....	G.....	G.....

	181	191	201	211	221	231
N. gal	CGATGAAGAA	CGCAGCGAAA	TGCGATAAGT	AATGTGAATT	GCAGAATTCA	GTGAATCATC
N. dit						
N. cvf						
N. coc						
N. pun						
N. fuc						C.....
N. rad1						
N. rad2						

Continuation of Fig. 4.1 from previous page.

	241	251	261	271	281	291
N. gal	GAATCTTTGA	ACGCACATTG	CGCCCCGCC-A	GTATTCTGGC	GGGCATGCCT	GTTTCGAGCGT
N. dit						
N. cvf						
N. coc						
N. pun						
N. fuc						
N. rad1			C			C
N. rad2			C			C

	301	311	321	331	341	351
N. gal	CATTTCAACC	CTCAAGCCCC	CGGGCTTGGT	GTTGGGGATC	G----GCGTG	CCCTTCGCGG
N. dit				GAT	CCGGC...C	CT....
N. cvf				-T	CG---.C.	...CC....
N. coc				AT	CG---.CC	...CC....
N. pun				-T	CG---.CC	...CC....
N. fuc					.G--C.T.CC	...CC..G..
N. rad1				A....	-----	...CC..G..
N. rad2				A....	-----A.	...C..G..

	361	371	381	391	401	411
N. gal	CGCGCCGTCC	CCTAAATCTA	GTGGCGGTCT	CGCTGCAGCT	TCCTCTGCGT	AGTAGCACAC
N. dit	C.					
N. cvf	..G.....	..C.....			C.....	ACA
N. coc	..G.....	..C.....			C.....	ACA
N. pun	..G.....	..C.....			C.....	ACA
N. fuc	C..	C.			C.....	
N. rad1	G.T	..C...A..		T...		A.....
N. rad2	T	..C...A..	C	T...		

	421	431	441	451	461	471
N. gal	CTCGCACC	GG AAGAGCAGCG	CGGCCACGCC	GTTAAACCCC	CCACTTCTGA	AAGG
N. dit		..-.....				
N. cvf	G.T.	..-C.....				
N. coc	G.T.	..-C.....				
N. pun	G.T.	..-C.....				
N. fuc	G.T.	..-A...G...				
N. rad1	...A...T..	G-A.A.....	T.....			
N. rad2	T..	G-A.A.....	T.....	..A.....		



Fig. 4.2. Species-specificity of primers Ch 1 and Ch 2 and competitive PCR showing detection of *N. galligena* in DNA preparations from lignified tissue with internal control to increase assay reliability. (a) lane 1 and 16, 1 Kb size marker (Life Technologies); 2, *N. coccinea* (IMI 361832c); 3, *N. ditissima* (CBS 100482); 4, *N. radicicola* (IMI 376409); 5, *N. galligena* (IMI 378754); 6, *N. galligena* and internal control; 8 and 9, *Malus* and *Pyrus* cankered wood samples; 10, *Malus* cankered wood sample and internal control; 11, uncankered *Malus* wood sample; 12, uncankered *Malus* wood sample and internal control; 13 and 14, suspect infected *Malus* wood samples and internal control. Lanes 7 and 15, negative controls (SDW). (b) Confirmation of the presence of target *N. galligena* sequence from the amplification of woody extracts with Ch 1 and Ch 2 by Southern hybridization analysis. Lanes as above.

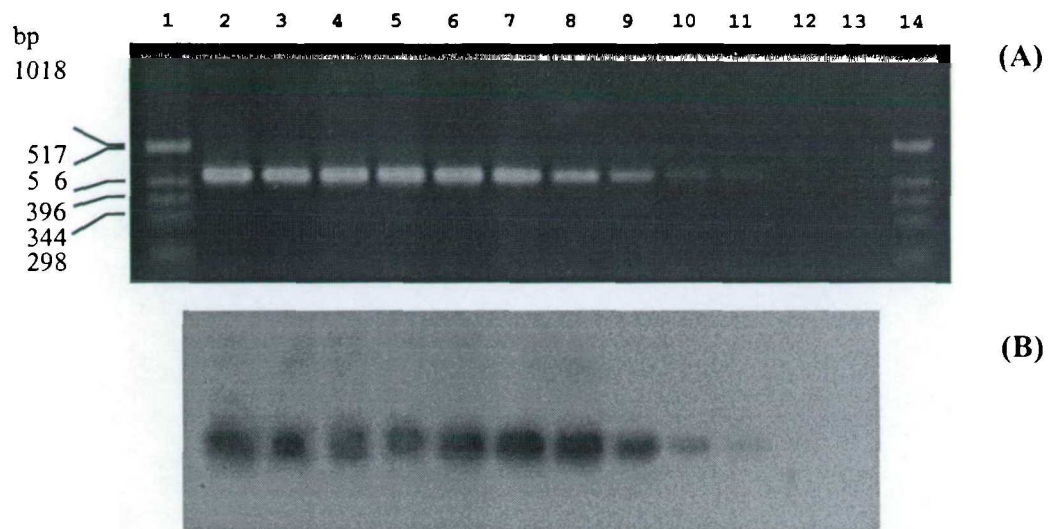


Fig. 4.3. PCR assay sensitivity using primers Ch 1 and Ch 2 and dilution series of purified *N. galligena* genomic DNA (isolates IMI 378754). (a) lanes 1 and 14, 1 Kb size marker (Life Technologies); 2-12, 5 and 10 fold serial dilutions of *N. galligena* DNA (viz. 500ng to 5 pg); 13, negative control (SDW). (b) Southern hybridization analysis of PCR amplification products from (a). Lanes as above

4.3.5 Quantitative PCR

For quantitative purposes, a calibration curve, constructed from PCR of a standard amount of internal control template and a range of *N. galligena* target DNA concentrations (Fig. 4.4) was constructed (Fig. 4.5). The fungal biomass in an infected sample can be determined from direct extrapolation from the curve of the PCR fungal target/internal control ratio of total sample extract DNA. For example, based on this calibration curve a sample from a cankered apple wood lesion in Fig. 4.2 (A) lane 10 was calculated as containing $\cong 2$ ng fungal DNA per 0.5 g of sample woody tissue.

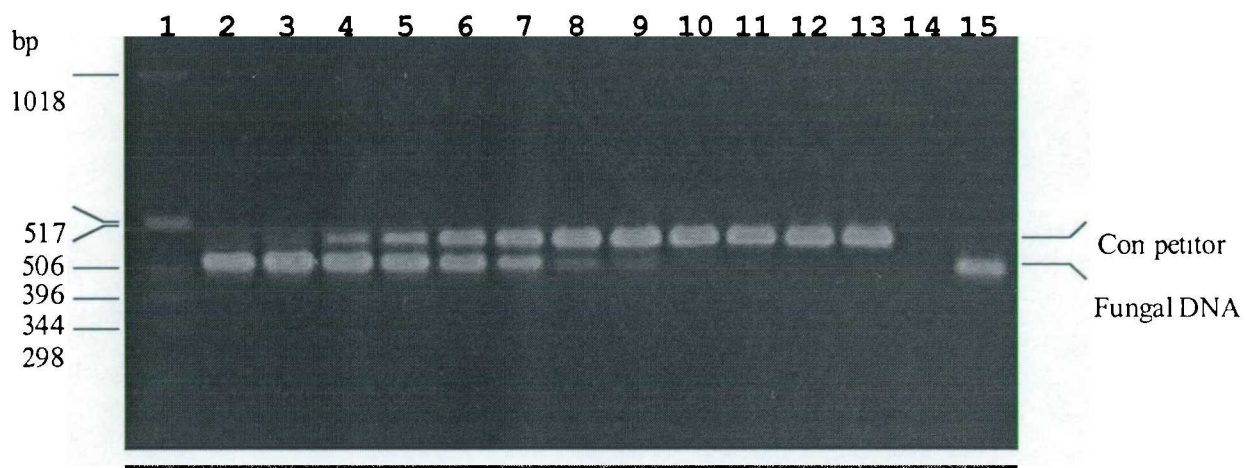


Fig. 4.4. Quantitative PCR: dilution series of *N. galligena* (IMI 378754) DNA with internal competitive fragment at 25 pg. Lane 1, 1 Kb size marker (Life Technologies); 2, 500 ng *N. galligena* DNA; 3, 250 ng; 4, 50 ng; 5, 25 ng; 6, 5 ng; 7, 2.5 ng; 8, 500 pg; 9, 250 pg; 10, 50 pg; 11, 25 pg; 12, 5 pg; 13, Competitive fragment only; 14, negative control (SDW); 15, positive control (*N. galligena* DNA).

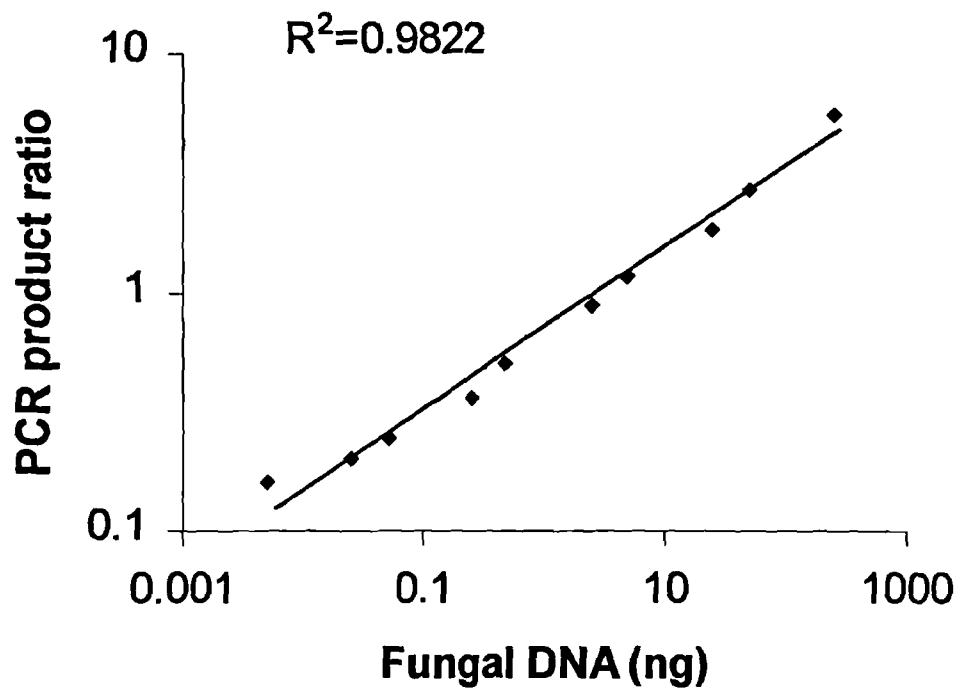


Fig. 4.5. Plot of PCR product ratio to amount of fungal DNA by competitive PCR.

4.4 Discussion

The high level of sequence homogeneity observed within the ITS region of a global collection of isolates of diverse geographic and host origin from Chapter 3 coupled with appreciable sequence divergence between closely related species across the same locus (Chapter 6) formed the basis of a molecular detection assay in this species. Two diagnostic primers, Ch 1 and Ch 2, recognising extreme 3' specificity were designed to optimal internal primer stability, GC composition and primer annealing temperatures whilst minimising potential dimerisation, hairpin duplexing and internal mis-priming according to the general concepts for optimal primer design as discussed by Dieffenbach *et al.* (1995) were designed and synthesised. Sub-optimal conditions may not only compromise good product yield, but may promote the appearance of PCR artefacts such as dimerisation, misincorporation and mismatch extension of primer template duplexes (Cobb and Clarkson, 1994).

In subsequent PCR tests, Ch 1 and Ch 2 specifically amplified an approximate 412 bp product from a large collection of *N. galligena* isolates of diverse host and global origin (Table 3.1), but not from any of the closely related *Nectria* species or fungal endophytes encountered within live apple wood material. The fungal endophytes routinely encountered within apple wood tissue during this work are mostly considered common plant pathogenic genera, responsible for a range of pre- and post-harvest diseases. The endophytic profile observed here is of similar composition and spectrum to that observed from similar material in the United Kingdom (Swinburne, 1973; Li, 1995) and from Chile (M. Lolas, Universidad de Talca, Chile; personal communication, 1998), suggesting transfer of this detection system to other apple growing areas of the world should not encounter problems of non-specificity.

Although two further locations of sequence divergence internal to the positions of Ch 1 and Ch 2 were apparent, closer inspection of their 3' specificity coupled with computer-assisted analysis of their sequence base composition excluded their exploitation for the design of a further set of primers for use in a nested PCR approach. However, due to the extent of inhibition experienced with PCR from lignified tissue extracts, the effectiveness of such an approach would not be

guaranteed. Although Hamelin *et al.* (1996) reported success of a multiplex nested approach with *Cylindrocarpon destructans* and *Cylindrocladium floridanum* from seedling pine and spruce material using species specific primers in conjunction with a universal primer (ITS 4) and fungal specific primer (ITS 1f), the tissues were juvenile seedling materials and most probably comprised very different secondary compounds than much older lignified tissue.

Despite earlier confusion regarding the identity of the causal organism of *Nectria* canker (reviewed by Booth, 1959), both *N. coccinea* and *N. ditissima* are now considered non-pathogens of rosaceous hosts, particularly apple and pear, being confined primarily to broad-leaved tree species and *Fagus* spp., respectively. Similarly, *N. fuckeliana*, *N. punicea*, and the more recently accepted taxon *N. coccinea* var. *faginata*, are primarily confined to spruce, *Rhamnus* and *Fagus* spp., respectively. However, the combined species specificity of Ch 1 and Ch 2 is much improved over that offered by a previously designed single primer ChInt (5'-GGC GGT GCC CGC TCC GGC GG-3') (Brown *et al.*, 1993), used in conjunction with universal primer ITS 4 (White *et al.*, 1990). From sequence comparisons, ChInt shows no specificity to *N. galligena* when compared to other Coccinea group species characterised in this study (including less than optimal primer parameters as stressed by Dieffenbach *et al.*, 1995), limiting its usefulness. Alternatively, Ch 1 and Ch 2 offers a much wider application, particularly on forestry tree species where *N. galligena* can infect in the presence of other Coccinea group taxa e.g. Beech Bark Disease (Houston, 1994).

Although such knowledge of restricted host ranges of these closely related species influenced the location of the specific primer set, much greater emphasis centred on the differences between *N. galligena* and *N. radicicola* in primer design. *N. radicicola* is a ubiquitous soil borne pathogen with a wide host range, including apple and pear, as well as a number of hardwood tree species. From experimental trials Braun (1991; 1995) implicated *N. galligena* as a potential component of apple replant disease in Nova Scotia. However, morphological re-evaluation and molecular characterisation of the 5.8S rRNA gene and ITS regions of strain Ch 1 (isolated from

the roots of apple cv. Beautiful Arcade from Nova Scotia in 1988, and used in both of Braun's studies), showed this isolate to be *N. radicicola* (deposited as IMI 376404). Further, *N. radicicola* (originally mis-identified as *N. galligena*) has been frequently isolated from apple roots in orchards grown on non-fumigated soils in Nova Scotia (Braun, 1991). In a parallel research programme at Wye College, *N. radicicola* has been frequently recovered from symptomless apple rootstock material (M. Lolas, Universidad de Talca, Chile; personal communication, 1998). A further isolate, IMI 375717, identified from a lesion from the a branch of an apple tree (originally supplied as *N. galligena*, strain PPRI 4895 from C. Roux, National Collection of Fungi: Culture Collection, Plant Protection Research Institute, Pretoria, Republic of South Africa, 1997) was also shown to be *N. radicicola* following morphological re-evaluation and 5.8S rRNA gene and ITS sequence characterisation. Such observations suggest the infection of apple propagating material by this species is more common than previously considered (although whether *N. galligena* is capable of infecting apple propagating material in a similar fashion is unclear).

The potential to detect *N. galligena* from lignified woody tissue was exhibited by PCR on total DNA extracts from naturally infected cankered lesions. The identity the 412 bp product was confirmed by Southern hybridization using the comparable product from an isolate of *N. galligena* grown *in vitro* as a probe. In the absence of a reliable selective media for *N. galligena*, the recovery frequency from infected cankered wood tissues by conventional plating was relatively low, typically in the range 30-40 % due, predominantly, to the emergence of faster growing endophytic fungi. Such difficulties in recovery from cankered woody tissue has been reported before (Anagnostakis and Ferrandino, 1998). By employing a molecular detection technique, such hindrances associated with these faster growing endophytes were circumvented. This approach was extended to direct PCR of Sepharose® spin dialysate of quick freeze/thaw elute of woody shavings from or adjacent to infected cankered lesions in sterile water or extraction buffer (Torres *et al.*, 1993). Coupled with the freeze/thaw elution of target template from infected tissues, PCR with the internal control fragment may serve as a quick procedure for the robust diagnosis of atypical infection symptoms. However, PCR amplification of extracts from lignified

tissues, prepared by either approach mentioned above, were not always successful, even from apparently heavily infected samples. In order to identify false negatives, probably due to the presence of inhibitors, a 500 bp heterologous internal standard incorporating identical Ch 1 and Ch 2 recognition termini was generated. When this was used, failure of the internal positive control to amplify suggested the presence of inhibitors (**Fig. 4.2. (A)**, lane 13). PCR on total woody extracts, supplemented with the internal positive control, from passage through insoluble PVP, Sepharose® dialysis or 100 or 1,000 fold dilutions did not rectify the problem, indicating the presence of high levels of inhibitors.

The direct inoculation of mature, sapling or seedling broad-leaved tree species with mycelial discs or conidial preparations to assess pathogen virulence (Plante and Bernier, 1997) or apple host resistance (van de Weg, 1987) may be difficult to interpret because of the variability in symptom development and disease progression (Dubin and English, 1974). Use of a PCR based quantitative assay in measuring localised host responses may help in these circumstances, but further evaluation of this approach as a more definitive measure in host resistance studies is required. Similarly, although the test presented here was generally able to detect *N. galligena* in cankered wood, it did not appear sensitive enough to detect fungus beyond an infected canker lesion, or was inhibited. Development of an alternative procedure to circumvent these limitations is addressed in Chapter 5.

Chapter 5. Development of magnetic capture hybridization for the improved PCR detection of *N. galligena* from lignified apple extracts

5.1 Introduction

Inhibition is an inherent feature of many PCR-based systems used for detecting plant pathogens in many hosts. It is also often a problem in the detection of soil micro-organisms. Despite the development of numerous taxon-specific or more widely applicable plant DNA extraction procedures designed to minimise co-extraction of inhibitory compounds, inhibition remains a major impediment to many routine applications, compromising both assay sensitivity and reliability. Polysaccharides, secondary plant metabolites (such as polyphenols and phenolic compounds) and ill-defined materials such as humic acids are common, PCR-inhibitory contaminants of DNA extracts made from plants and soils. A number of varied post-extraction procedures (reviewed by Bickley and Hopkins, 1999) designed to overcome inhibition and enhance PCR reliability have become established as standard purification strategies, but these are not always effective (especially where the level of inhibitors are high). Further, they may lead to a loss of target DNA.

Woody tissues contain lignin, a structurally complex cross-linked aromatic biopolymer based on variously substituted p-hydroxyphenyl propane units resistant to degradation, linked with a range of materials including polysaccharides, polyphenols, celluloses and polyglucoses, many of which are inhibitory to PCR and relatively easily extracted. For example, inhibition of PCR due to the use of wooden toothpicks to transfer bacterial or yeast colonies for direct PCR analysis of recombinant DNA has been observed (Lee and Cooper, 1995), especially where low amounts of *Taq* DNA polymerase were used. In studies on *Nectria galligena*, the cause of *Nectria* canker in apple and pear, PCR detection using species-specific primers (Chapter 4) in clearly infected lignified tissue has often been compromised by the presence of inhibitory compounds. Furthermore, in asymptomatic (or cryptic) infections, which may be implicated in dissemination of the disease (Chapter 2), the level of fungus present is usually below the limit of detection by PCR (Chapter 4).

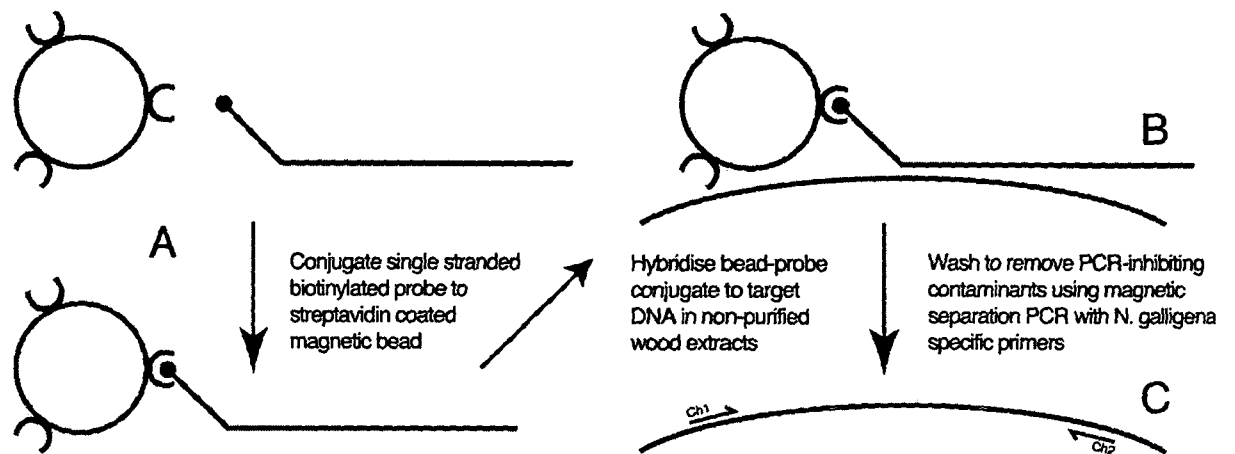
Initially developed for the detection of bacterial DNA from soil (Jacobsen, 1995), magnetic capture hybridization (MCH) is a very useful tool for the separation of specific target DNA from other DNA molecules and interfering compounds. Recently, MCH has been used to improve the PCR-detection of a nucleopolyhedrovirus from field soils (de Moraes *et al.*, 1999) and of verotoxigenic *Escherichia coli* in foods (Chen *et al.*, 1998). MCH uses solution hybridization with a specific single-stranded biotinylated probe, conjugated to streptavidin coated paramagnetic beads, to immobilize target DNA from a total DNA extract. Beads and bound DNA are collected and washed magnetically, simply and effectively removing non-target DNA and PCR inhibitors (and, if required, allowing concentration of the target molecules). Elution of the DNA is by a simple heating step which can be incorporated as part of the subsequent PCR reaction. This is shown schematically in **Fig. 5.1**. The use of MCH to purify template molecules should greatly reduce or exclude the occurrence of false negatives arising from the presence of inhibitory compounds. Because of the problem of inhibition of PCRs associated with DNA extracts from lignified apple tissues (Chapter 4), MCH offered the possibility of improved sensitivity and reliability. The objective of this work was to develop an MCH-PCR assay for *N. galligena* and demonstrate the improved detection of this fungus from woody tissue by this technique.

5.2 Materials and Methods

5.2.1 Fungal culture and DNA extraction

Growth of *N. galligena* strain IMI 375721 (isolated from a canker on an apple, cv. Carrara Brusca, at the National Fruit Collections, Brogdale, Faversham, Kent, UK) and DNA extraction from it was as described previously (Chapter 3, point 3.3.2). The concentration and quality of the DNA were estimated by spectrophotometrically (Sambrook *et al.*, 1989).

Fig. 5.1. Schematic representation of MCH-PCR assay.



5.2.2 DNA extraction from lignified tissue

Total DNA extracts from woody apple tissue samples was prepared according to a modified procedure of Torres *et al.* (1993) as described previously (Chapter 4, point 4.2.3). Final pellets were resuspended in sterile Milli-Q AR grade water and DNA concentrations quantified by electrophoretic comparison with a dilution series of bacteriophage λ DNA (Promega) and spectrophotometrically (Sambrook *et al.*, 1989). DNA concentrations were adjusted to a standard level and the preparations stored at -20° C.

5.2.3 Design and synthesis of Biotin-labeled capture probe

An 81 bp oligonucleotide was designed to be complementary to part of the ITS1 region of the *N. galligena* rRNA genes (Chapter 3, Fig. 3.2) with the aid of the programme OLIGO version 5 (Molecular Biology Insights, Inc., Cascade, CO, USA). The capture sequence extended from 14 bp downstream of the 3'-terminal nucleotide of the Ch1 primer sequence (to avoid competition during subsequent PCR tests) to the last ITS 1 nucleotide immediately upstream of the 5.8S rRNA gene (see Fig. 5.2). Further sequence from the conserved 5.8S rRNA gene was avoided in order to enhance capture-sequence specificity. Sequences and positions of diagnostic primers Ch1 and Ch2 (Chapter 5) and the oligonucleotide probe are given in Table 5.1. The capture oligonucleotide was synthesised by Genosys Biotechnologies Inc., Cambridge, UK, and incorporated a biotin molecule on a twelve-carbon atom spacer arm at the 5'-terminal. The 81 bp biotinylated oligonucleotide was not further purified.

5.2.4 Attachment of capture oligonucleotide to magnetic beads

In all wash/incubation steps the beads were immobilized using a Magnetic Particle Concentrator (MPC-E) (Dynal, Oslo, Norway). 400 μ l of a 10 mg ml⁻¹ suspension of superparamagnetic M-280 streptavidin (covalently coated) polystyrene beads (Dynal A.S., Oslo, Norway) was transferred to a 1.5 ml microfuge tube and washed three times with 400 μ l of 1 X PBS (0.05 M phosphate buffer with 9 g l⁻¹ NaCl (pH 7.3)) containing 1 g l⁻¹ (SDS), to remove preservative, followed by a single wash in 400 μ l

Fig. 5.2. Diagram of ITS regions 1 and 2 of the rRNA gene from *N. galligena* showing approximate positions of primers Ch 1 and Ch 2 and the 81 bp biotinylated capture oligonucleotide.

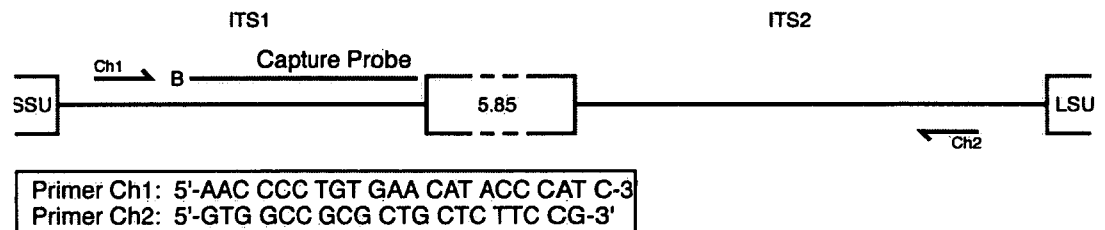


Table 5.1. Sequences and positions within the rRNA repeats of primers Ch 1 and Ch 2 (see Chapter 4) and 81 bp capture oligonucleotide used for MCH-PCR detection of *N. galligena* from lignified apple tissue.

Oligonucleotide	Postion in ITS ^a	Sequence
Ch 1	20-40	5'-AAC CCC TGT GAA CAT ACC CAT C-3'
Ch 2	412-431	5'-GTG GCC GCG CTG CTC TTC CG-3'
MCH-oligo ^b	55-135	5'-GCC CGC TCC GGC GGC CCG CCA GAG GAC CCC CAA ACT CTT GTT TTA TAC AGC ATC TTC TGA GTA ACA CGA TTA AAT AAA TCA-3'

^anumbered as for EMBL accession AJ228666, beginning with the 5'-most nucleotide sequenced.

^bthe 81 bp oligonucleotide incorporated a biotin molecule with a twelve-carbon atom spacer arm on the 5' terminus.

binding buffer (10 mM Tris-HCL, 1 mM EDTA, 1 M NaCl (pH 8.0)). Biotinylated MCH capture oligonucleotide (100 μ g) was added in 100 μ l and the mixture incubated at 25° C for 60 min. Following incubation, the beads/oligonucleotide conjugate was washed three times with 400 μ l of binding buffer and finally resuspended in 400 μ l of sterile Milli-Q AR grade water. All manipulations were performed with the use of aerosol barrier tips (Continental Laboratory Products, USA).

5.2.5 Hybridization and capture of target DNA

Hybridization of MCH probe to target template from DNA samples essentially followed Jacobsen (1995). Typically, 50 ng of DNA (in 10 μ l and denatured by heating at 99° C for 10 minutes on a Techne PHC-3 thermal cycler (Techne, Cambridge, UK)) from total preparations of woody apple tissues were added to 200 μ l of a pre-warmed (to 62° C) hybridization solution (5 X SSC, 10 g l⁻¹ low-fat dried milk powder (blocking agent), 1 g l⁻¹ N-laurylsarcosine and 0.2 g l⁻¹ SDS) followed by 100 μ g MCH-capture oligomer/bead conjugate. Samples were incubated for 4 hours at 62° C in a Techne HB-1 hybridization oven. Beads were washed once in sterile Milli-Q AR grade water and resuspended in 25 μ l of sterile Milli-Q AR grade water for use in PCR analysis.

5.2.6 PCR of capture target DNA

Following hybridisation, samples were subjected to PCR amplification using primers Ch1 and Ch2 (Table 5.1) which amplify a 412 bp fragment specific to *N. galligena* (Chapter 5). Following PCR, typically 10 μ l of each reaction mixture was analysed by gel electrophoresis in 10 g l⁻¹ agarose gel (Biogene, UK) in 1 X TBE (89 mM Tris-borate, 2 mM EDTA (pH 8.0)) at 4 V/cm for 2 hours. A 1 Kb ladder (Gibco BRL) was used as a size marker. Following ethidium bromide staining, bands were visualised and photographed over UV light.

5.3 Results

5.3.1 Hybridization probe design and streptavidin-bead conjugate preparation

Intra-specific homogeneity within the *N. galligena* rRNA gene ITS regions (Chapter 3), the repetitive nature of these genes, and the fact that species-specific primers have been developed (Chapter 5) made this the locus of choice for use in MCH-PCR. A specific complementary hybridization probe was designed to the ITS 1 region, shown to be less variable than ITS 2 when compared with other closely related *Nectria* species including *N. radicicola* (Chapter 6). Only 95 bases occur between the end of the forward primer, Ch1, and the highly conserved 5.8S rRNA gene. As MCH requires a capture molecule nested between the diagnostic primers and of sufficient length and GC content to ensure efficient hybridisation, an 81 bp biotinylated single-stranded oligomer was designed and synthesised (**Table 5.1**) (excluding the conserved 5.8S rRNA gene and the Ch1 primer recognition site, to avoid reduction in specificity or compromise in PCR efficiency through competition for the recognition site). Other than desalting, no purification of the biotinylated probe after synthesis was performed because of potentially large losses of oligonucleotide during HPLC or PAGE purification. Although purification is recommended to remove unbound biotin molecules from biotinylated oligonucleotide (since free biotin may potentially compete for binding sites on the streptavidin-coated magnetic beads, reducing the effective binding capacity of the biotinylated hybridization conjugate), spectrophotometric analysis of oligomer-bead conjugate stocks and wash supernatants from experiments indicted high levels of probe-bead binding (>80 %).

5.3.2 Improved PCR detection sensitivity

To evaluate the sensitivity of the MCH-PCR technique, 10-fold serial dilutions from 1 ng to 1 fg, of genomic DNA from *N. galligena*, strain IMI 375721, in sterile Milli-Q AR grade water were tested by MCH-PCR with primers Ch1 and Ch2. The lower limit of visual detection on an ethidium bromide stained agarose gels was approximately 10-100 fg genomic DNA, an approximately 10-100 fold increase in sensitivity over that achieved by PCR without MCH (Chapter 4).

The usefulness of the MCH in improving reliability and sensitivity of PCR for *N. galligena* from naturally infected and putatively infected lignified apple material, was demonstrated by applying it to a range of extracts from known infected and suspect samples, some of which had proved problematic in PCR without MCH: (i) two young cankers from apple trees and one from pear; (ii) a mature *Nectria* canker lesion from an 8 year old branch from an apple cv. Bramley Seedling tree; (iii) an apple (M9) rootstock suspected to be asymptotically infected from a 2 year old layer-bed; (iv) wood collected from about 10 cm beyond the leading edge of an active *Nectria* canker on a 4 year old branch of an apple cv. Queen Cox tree. As expected, PCR without MCH was successful in detecting *N. galligena* in the young cankers (Fig. 5.3, lanes 3-5). However, for the samples from older cankers or asymptomatic wood, amplification by PCR without MCH was unsuccessful (Fig. 5.3, lanes 6-8), even when post-extraction the samples were passed through Sepharose® CL-6B (Amersham Pharmacia Biotech UK) or insoluble PVP or when the DNA samples were diluted (data not shown). A standard internal positive control (Chapter 4) also did not amplify in this experiment, indicating the presence of PCR-inhibitory compounds. Following capture-hybridization with the MCH-oligomer/streptavidin-bead conjugate but without any other post-extraction treatment, amplification of an approximate 412 bp fragment by PCR using primers Ch1 and Ch2 was successful for these sample extracts (Fig. 5.3, lanes 9-11).

No attempt was made to quantify by PCR the level of *N. galligena* in each of the samples. Also, isolation of *N. galligena* from lignified tissues is not easy, and with old lesions or asymptomatic wood it is often extremely difficult, so we have no independent estimate of the fungal biomass present in the lignified tissue samples used here. However, as even the limited number of samples presented here clearly show, MCH-PCR was able to detect *N. galligena* in samples where PCR without MCH was unsuccessful, representing an overall qualitative improvement in sensitivity and reliability.

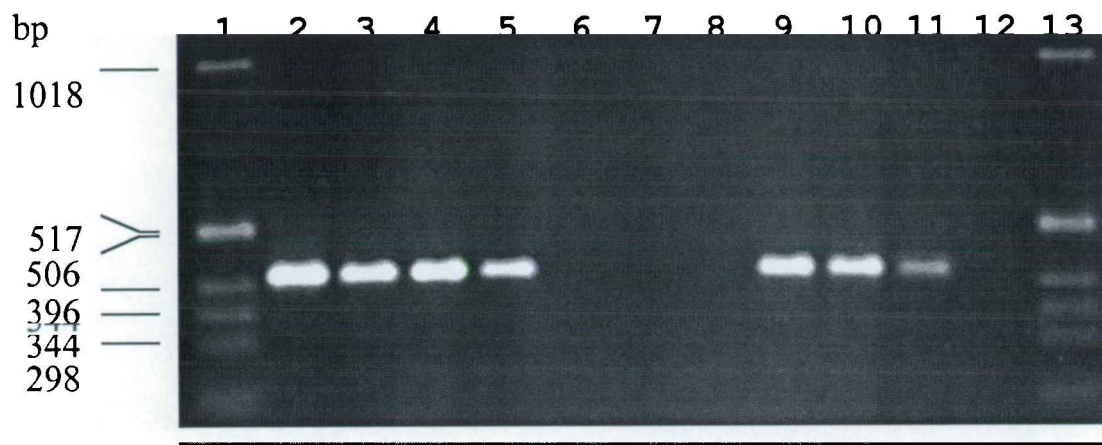


Fig. 5.3. Improved sensitivity and reliability of MCH-PCR for detection of *N. galligena* in DNA preparations from lignified tissue. Lanes 1 and 13, 1 Kb size marker (Life Technologies). Lane 2, DNA extract from *N. galligena* (IMI 375721) in culture. Lanes 3-8, PCR without MCH; lanes 3-5 are extracts from young cankers on apple *cv.* Discovery, apple *cv.* Royal Gala and pear *cv.* Conference; lane 6, extract from old canker on apple *cv.* Bramley Seedling; lane 7, extract from apple rootstock (M9) suspected of being infected asymptotically; 8, extract from wood without canker but distal to an active canker on apple *cv.* Queen Cox. Lanes 9-11, samples as lanes 6-8 but tested using MCH-PCR as described in text. Lane; 12, negative control

5.4 Discussion

The development and application of a magnetic capture hybridization procedure employing a biotinylated capture oligonucleotide for improved PCR detection of *N. galligena* from lignified apple extracts has been demonstrated. The results show a 10-100 fold increase in sensitivity for DNA from the fungus in culture and for samples from woody tissues represent a significant improvement in PCR sensitivity and reliability. Whilst routine application of MCH-PCR to the screening of materials with low or cryptic levels of infection may be limited by the additional cost and relative assay complexity, this approach may prove an effective research tool for use in studying disease aetiology and asymptomatic systemic spread within the tree. From consulting the literature, this appears to be the first report of the detection of a fungal plant pathogen using MCH-PCR.

Chapter 6. Molecular characterisation of the *Nectria coccinea* group with particular reference to *N. radicicola*

6.1 Introduction

Results from Chapters 2 and 3 indicate that young trees, particularly rootstocks, can be infected symptomlessly during propagation. How such material may become infected at source is unclear, although the results of Chapter 2 suggest the conventional model of disease spread is not exclusive. In light of these findings, determining the source of rootstock infection becomes central to understanding the biology and prospects for disease management of the pathogen in intensive apple production systems. A possible alternative source of infection within rootstock propagation systems might be through the soil, although this requires evaluation. An informative approach to this is by phylogenetic inference through analysis of sequence data from closely related taxa of known habitat and aetiology. Such an approach may be particularly useful where *in vivo* observations of the organism in question are inconclusive, or interpretation from experimental tests or trials are difficult or unreliable.

The genus *Nectria* (*sensu lato*) is large and cosmopolitan, comprising a number of groups based on perithecial and stromatal characteristics (Booth, 1959). Anamorphs are very diverse, including *Tubercularia*, *Fusarium*, *Cylindrocarpon*, *Verticillium*, *Gliocladium*, *Myrothecium*, *Volutella*, *Acremonium* and many others. Anamorph/teleomorph connections have been reported in many *Nectria* species and a broad correlation has been recognised between teleomorph group and anamorph genus (Rossman and Samuels, 1979). This improved understanding, especially combined with DNA sequence data, will probably result in *Nectria* being split into a number of more narrowly defined genera in future (Rossman *et al.*, 1999).

Nectria species with *Cylindrocarpon* anamorphs are currently split into five groups (Booth, 1959; Samuels and Brayford, 1990, 1993, 1994; Brayford and Samuels, 1993), plus some tropical species, which are so unusual that they cannot currently be assigned to a group (D. Brayford, CABI Bioscience, personal communication, 2000).

For identification of the anamorphic states, *Cylindrocarpon* spp., Booth (1966) proposed a classification system grouping taxa on the basis of microconidium and/or mycelial chlamydospore production in pure culture. This system was recognised to be 'artificial' but to serve the practical purpose of species identification (D. Brayford, CABI Bioscience, personal communication, 2000, concerning discussions with Booth). This approach was an extension of the earlier system proposed by Wollenweber (1913, 1917).

The Coccinea group, to which *N. galligena* is assigned, has a characteristic perithecial wall anatomy in vertical sections and typically a distinct stromatic development in host tissues (Booth, 1959) consisting of the species as outlined in Chapter 1, point 1.1.4. Taken together, the Coccinea group is considered one of the most economically important groups of tree infecting pathogens world-wide (Booth, 1959), responsible for a range of cankerous or shoot die-back symptoms on a wide range of woody host species, but each exhibiting a degree of host selectivity. However, the taxonomic framework as proposed by Booth (1959, 1966), although adequate for the purposes of species identification, is now generally considered 'unnatural' in a phylogenetic sense (Samuels and Brayford, 1994; D. Brayford, CABI Bioscience, personal communication, 2000) and, across the genus as a whole, unsuitable to resolve sub-generic groupings or inter-group relationships. This has resulted in the arbitrary inclusion or separation of related species for taxonomic convenience (e.g. *N. fuckeliana* exhibits atypical perithecial wall features consistent with the Mammoidea type of lateral wall, but produces abundant microconidia in young cultures consistent with Coccinea cultural characteristics).

By contrast, *N. radicicola* Gerlach and Nilsson, the most prominent member of the *Nectria radicicola* group (Samuels and Brayford, 1990), is a ubiquitous soil-borne fungus responsible for a range of severe disease conditions from a wide range of taxonomically diverse host plants (Gerlach, 1961; Booth, 1967; Domsch *et al.*, 1980). This also includes the roots of a wide range of tree species, in particular *Malus*, where it has been implicated in apple replant disease (Braun, 1991, 1995). Whether *N. galligena* infection can occur from the sub-terrain, as purported to in

Chapter 4, is unclear, although may explain possible aspects of rootstock infection from nursery sources. Although no known soil-borne phase for *N. galligena* is reported from the literature, inference from evolutionary relationships through a more 'natural' classification of the Coccinea group may assist evaluation of this hitherto largely unrecognised possible infection source. None of the Coccinea group members are currently considered soil-borne in any phase of their life-cycles, but this is an assumption based on the lack of chlamydospore production by this group of taxa (Brayford, 1992; D. Brayford, CABI Bioscience, personal communication, 2000).

Molecular data has been widely used to estimate phylogenetic relationships at varying taxonomic levels across the fungal kingdom to address a wide range of systematic questions (Bruns *et al.*, 1991), including molecular phylogeny and character evolution. This approach has been particularly useful where morphological criteria are unclear or controversial (Lee and Hanlin, 1999; Drehmél *et al.*, 1999; Chen *et al.*, 1999). Molecular markers have been shown to be useful in differentiation of species or strains at either the individual or population level. Analysis of the ITS spacer regions within the rRNA repeat genes have proved indispensable in modern fungal molecular systematics because of their degree of phylogenetic versatility and informativeness for assessing both intra- and inter-specific relationships. Further, numerous studies have shown how conservation and mild sequence variation in these spacers can be used to determine phylogenetic relationships, define species and generate species-specific probes and primers (Bruns *et al.*, 1991; Boysen *et al.*, 1996; Kuninaga *et al.*, 1997; Mugnier, 1998).

The objectives of this study were to evaluate several PCR based molecular methods to help delineate species boundaries within and between the *Nectria* Coccinea group and current *N. radicola* species concept, and to further assess their evolutionary relationships through ITS phylogenetic analysis. This approach may lead to an improved understanding of the classification of these taxa and their evolutionary histories with possible insight into aspects of their biological function, including, through phylogenetic inference with *N. radicola* sequence data, the ability of *N. galligena* to infect from a soil-borne phase. The exploitation of ITS sequence

divergence for molecular detection between these species, other than *N. galligena* already addressed in Chapter 4, are discussed.

6.2 Material and methods

6.2.1 Fungal isolates and culture conditions

The species of *Nectria* used in this study were selected to be representative of the Coccinea group (based on availability in recognised culture collections) as defined by Booth (1959) (except for *N. hederæ* and *N. punicea* var. *ilicis*). The four *N. galligena* isolates were selected from the entire range of molecular variation as observed in Chapter 3. Strains of *N. radicicola* were selected to represent wide geographic and host diversity. The provenances of isolates used are detailed in **Table 6.1**.

All species were maintained on Spezieller Nährstoffarmer Agar plus yeast extract (SNAY) (Nirenberg, 1976) slants at 4° C and grown routinely at 20° C on Malt Extract (MEA) and Potato Dextrose (PDA) 12 g l⁻¹ Agar (Oxoid, USA) media under continuous fluorescent light (Philips TLD 50W/84HF). All taxa were authenticated according to anamorphic criteria, namely colony morphology and conidial characteristics (Booth, 1966). If not previously accessioned, each taxon was deposited with a major international fungal collection (CABI Bioscience (formerly International Mycological Institute) (IMI), Centraalbureau Voor Schimmelcultures (CBS) or Mycothèque de l'Université Catholique de Louvain (MUCL)) with the express permission of suppliers.

6.2.2 DNA extraction

For the preparation of high molecular weight DNA, mycelia were grown on cellophane membrane on MEA for 6-7 days as described above and extracted according to the procedure described in Chapter 3, point 3.2.2. Extracted DNA was resuspended in Milli-Q AR grade water and stored at -20° C. Appropriate dilutions were prepared for routine PCR applications.

Table 6.1. *Nectria* species and isolates used in this study.

Species/Isolate	Code	Host	Origin	Year	Isolator
<i>N. galligena</i> MacCracken	CBS 100318	<i>Malus domestica</i>	N. Ireland	1995	A.
<i>N. galligena</i> Langrell	IMI 375721	<i>Malus domestica</i>	UK	1996	S.R.H.
<i>N. galligena</i> Langrell	MUCL 40721	<i>Pyrus communis</i>	UK	1997	S.R.H.
<i>N. galligena</i>	CBS 100320	<i>Populus grandidentata</i>	Canada	1997	P. G. Braun
<i>N. coccinea</i>	IMI 382869	<i>Acer pseudoplatanus</i>	UK	1977	S. Dennis
<i>N. coccinea</i>	IMI 361832c	<i>Ulmus glabra</i>	UK	1994	J. N. Hedger
<i>N. c. var. faginata</i> ^a	CBS 100480	<i>Fagus grandifolia</i>	Canada	1994	F. Plante
<i>N. c. var. faginata</i> ^a	CBS 100481	<i>Fagus grandifolia</i>	USA	-	D.R. Houston
<i>N. c. var. faginata</i> ^a	IMI 268212ii	<i>Fagus sylvatica</i>	USA	1982	D.R. Houston
<i>N. ditissima</i>	CBS 100482	<i>Alnus rubra</i>	Canada	-	D. Clark
<i>N. punicea</i>	CBS 150.29	<i>Malus domestica</i>	Germany	1929	H.W. Wollenweber
<i>N. fuckeliana</i>	CBS 100328	<i>Picea abies</i>	Denmark	-	-
<i>N. fuckeliana</i>	MUCL 40715	<i>Picea abies</i>	Lithuania	1995	R. Vasiliauskas
<i>N. fuckeliana</i>	CBS 100322	<i>Picea abies</i>	Lithuania	1995	R. Vasiliauskas
<i>N. fuckeliana</i>	CBS 100323	<i>Picea abies</i>	Lithuania	1995	R. Vasiliauskas
<i>N. radicicola</i>	IMI 376404	<i>Malus domestica</i>	Canada	1988	P. G. Braun
<i>N. radicicola</i>	IMI 375717	<i>Malus domestica</i>	South Africa	1993	I. H. Rong
<i>N. radicicola</i>	IMI 375718	<i>Malus domestica</i>	UK	1996	M. Lolas
<i>N. radicicola</i>	IMI 375719	<i>Malus domestica</i>	UK	1996	M. Lolas
<i>N. radicicola</i>	IMI 375720	<i>Malus domestica</i>	UK	1996	M. Lolas
<i>N. radicicola</i>	IMI 376403	<i>Alnus glutinosa</i>	UK	1995	P. J. Fisher
<i>N. radicicola</i>	IMI 376408	<i>Arbutus menziesii</i>	UK	1986	D. Rose
<i>N. radicicola</i>	IMI 061536	<i>Narcissus</i> sp.	?	1930	F. H. Feekes
<i>N. radicicola</i>	IMI 376409	<i>Pinus sylvestris</i>	UK	1995	G. MacAskill
<i>N. radicicola</i>	IMI 376412	<i>Pinus sylvestris</i>	UK	1995	G. MacAskill
<i>N. radicicola</i>	IMI 376410	Soil	UK	1995	G. MacAskill
<i>N. radicicola</i>	IMI 376411	Soil	UK	1995	G. MacAskill

^a *N. coccinea* var. *faginata*

6.2.3 Arbitrary primed PCR

Decamer Operon oligonucleotide primer X11 5'-GGA GCC TCA G-3' (Operon Technologies Inc., Alameda, CA, USA) and the M13 core sequence of mini-satellite M13 bacteriophage phage DNA derived primer (5'-GAG GGT GGC GGT TCT-3') (Stenlid *et al.*, 1994) as previously exploited in revealing intra-specific variation in *N. galligena* (Chapter 3) were used under optimised amplification conditions as previously described (Chapter 3, point 3.2.5). Reproducibility of arbitrary primed PCR reaction profiles were assessed by performing duplicate independent reactions for each isolate/primer combination. Control reactions omitting fungal template DNA were carried out in each experiment to check against contamination of reagents and reaction mixtures with non-sample DNA. Amplification products were separated by electrophoresis on 10 or 15 g l⁻¹ agarose (Biogene, UK) 1 X TBE (89 mM Tris-borate, 2 mM EDTA at pH 8.0) gels, at 4.5 V/cm for 2 hours and 5.5 V/cm for 4 hours, for RAPD and the M13 core sequence primer, respectively. Gels were stained in a solution of ethidium bromide (1 mg ml⁻¹). A 1 Kb ladder (Life Technologies) was used as a size marker. Only reproducible unambiguous amplification products were included in analysis.

6.2.4 PCR amplification of ribosomal DNA spacers

The internal transcribed spacer regions (ITS 1 and 2), including the 5.8 S rRNA gene, of the nuclear rRNA and the entire intergenic spacer region (IGS), separating each successive tandemly repeated rRNA operon copy, were amplified using primers ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990), and PN 11 (5'-GCT GGG TTT AGA CCG TCG TG-3') and PN 22 (5'-CAA GCA TAT GAC TAC TGG C-3') (Mouyna and Brygoo, 1992), respectively. Reaction and thermo-cycling conditions were as previously described (Chapter 3, point 3.2.4).

Efficacy and size determination of individual PCR reactions were assessed by electrophoresis of 5-10 µl of post amplification reaction mix on a 10 g l⁻¹ agarose (Biogene, UK) 1 X TBE (89 mM Tris-borate, 2 mM EDTA at pH 8.0) gel using a 1

Kb ladder as a size marker (Life Technologies) and quantified by direct comparison with a dilution concentration series of bacteriophage λ DNA (Promega).

6.2.5 RFLP analysis of PCR products

PCR products were digested with *MseI*, *MboI*, *MspI*, *AluI*, *HaeIII*, *TaqI*, *DpnI* and *HinfI* for the ITS 1-5.8S-ITS 2 region, and *HinfI*, *HaeIII*, *HhaI*, *TaqI*, *MspI*, *MboI*, *AluI* and *RsaI* for the IGS region as previously described (Chapter 3, point 3.2.6). Restriction fragments were separated on a 20 g l⁻¹ Metaphor agarose gel (FMC Bioproducts) in 1 X TBE (89 mM Tris-borate, 2 mM EDTA at pH 8.0) following the manufacturers preparation instructions. Electrophoresis was at 4 V/cm for 3.5 hours with 4 μ l of ϕ X174/*HinfI* fragments (0.1 μ g μ l⁻¹) as a size marker (Promega). Following ethidium bromide staining, gels were photographed over UV light.

6.2.6 DNA sequencing

Purified PCR products were sequenced without cloning using standard techniques as previously described in Chapter 3, point 3.2.8. Sequences generated were submitted to the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk>) (Table 6.2.).

6.2.7 Data evaluation

Amplification products or restriction fragment sizes were estimated by direct comparison of migration distances to known sized markers and converted into base pairs using the program DNA-FIT (Oerter *et al.*, 1990). Pairwise profile comparisons of fragments were scored on the basis of the presence or absence of bands of the same electrophoretic mobility (considered as identical gene loci) to produce similarity matrices using Jaccard's similarity coefficient, *i.e.* the number of bands shared by the pair divided by the number of bands in either or both (Sneath and Sokal, 1973), and are expressed in the text as percentage values. Phenograms were constructed using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA) and a Neighbour-Joining method and produced in PHYLIP package version 3.5c (Felsenstein, 1993).

Sequence data were aligned using the computer program CLUSTAL V (Higgins and Sharp, 1989). All phylogenetic analyses were performed using programmes within PHYLIP package version 3.5c (Felsenstein, 1993). Transition/transversion parameter ratios were calculated using PUZZLE (Strimmer and von Haeseler, 1996). DNA distance matrices produced from the sequence data (regions ITS 1 and ITS 2 individually, as well as the entire ITS 1, 5.8S and ITS 2) were calculated using the Kimura 2-parameter model (where distance is defined as the probability of nucleotide substitutions per site (Kimura, 1980)) in DNADIST. Neighbour-Joining analysis was carried out using the Neighbor program with tree topology tested with 1000 bootstrap trials.

6.3 Results

6.3.1 Arbitrarily primed PCR

Arbitrary primers X11 (RAPD) and the M13 core sequence primer, previously used to characterise intra-specific variation within *N. galligena* (see Chapter 3) were used successfully in the generation of PCR amplification profiles between all representative species of the Coccinea group and *N. radicicola* strains (except for strain IMI 375720 which failed to amplify with X11). Both primers revealed reproducible polymorphisms useful for characterising variation. As observed in *N. galligena*, the M13 primer generally generated a higher number of amplification products (typically 5-15, ranging in size from 2.85 to 0.34 Kbp), than did X11 (3-16, ranging in size from 4.3 to 0.45 Kbp), of which 75, and 62 bands were polymorphic, respectively. Using Jaccard similarity co-efficient to produce similarity matrices, cluster analysis using either Neighbour-Joining or UPGMA methods were very similar (comparative data not shown). Similarity co-efficients among isolates ranged from 4.6% to 88% for X11, and 0 to 100% for M13 (see **Fig. 6.1.** and **Fig. 6.2.**). Analysis of all pairwise similarities revealed that while there was a reasonable correlation between the two data matrices, there was a tendency for M13 similarities to be higher than those for RAPD values. Only a few pairs of isolates showed markedly differing similarities between M13 and RAPD analysis, notably *N. radicicola* strains IMI 376403 and IMI 376408. For this pair, the M13 analysis gave

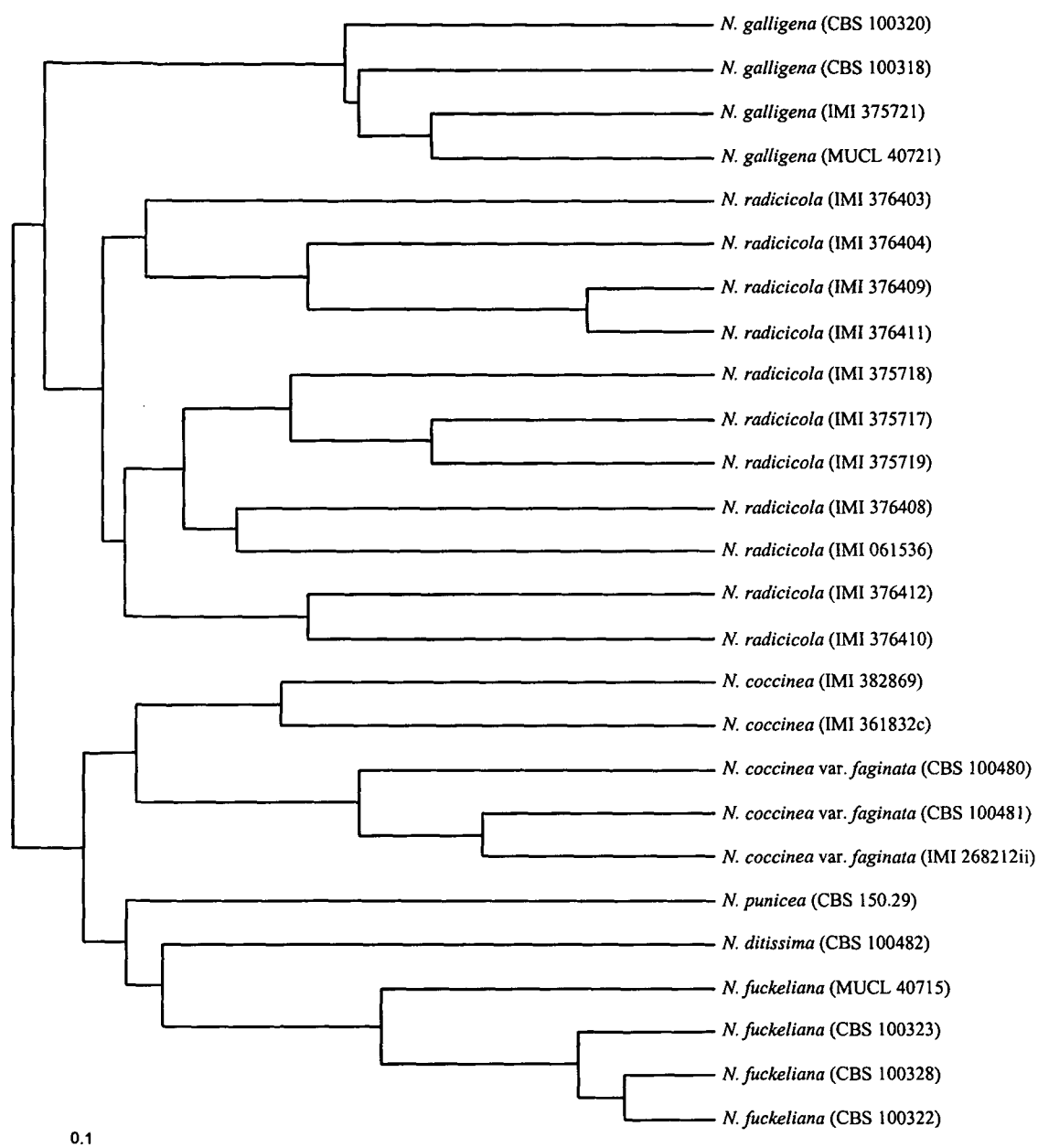


Fig. 6.1. Unrooted dendrogram constructed exclusively from RAPD data indicating the relationship among all *Nectria* Coccinea species and strains of *N. radicola* (except IMI 375720). Genetic similarities were calculated from a matrix of pairwise distances generated using Jaccards similarity co-efficient (Sneath and Sokal, 1973). Distances are shown above the dendrogram. The dendrogram was generated from the distance matrix using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA).

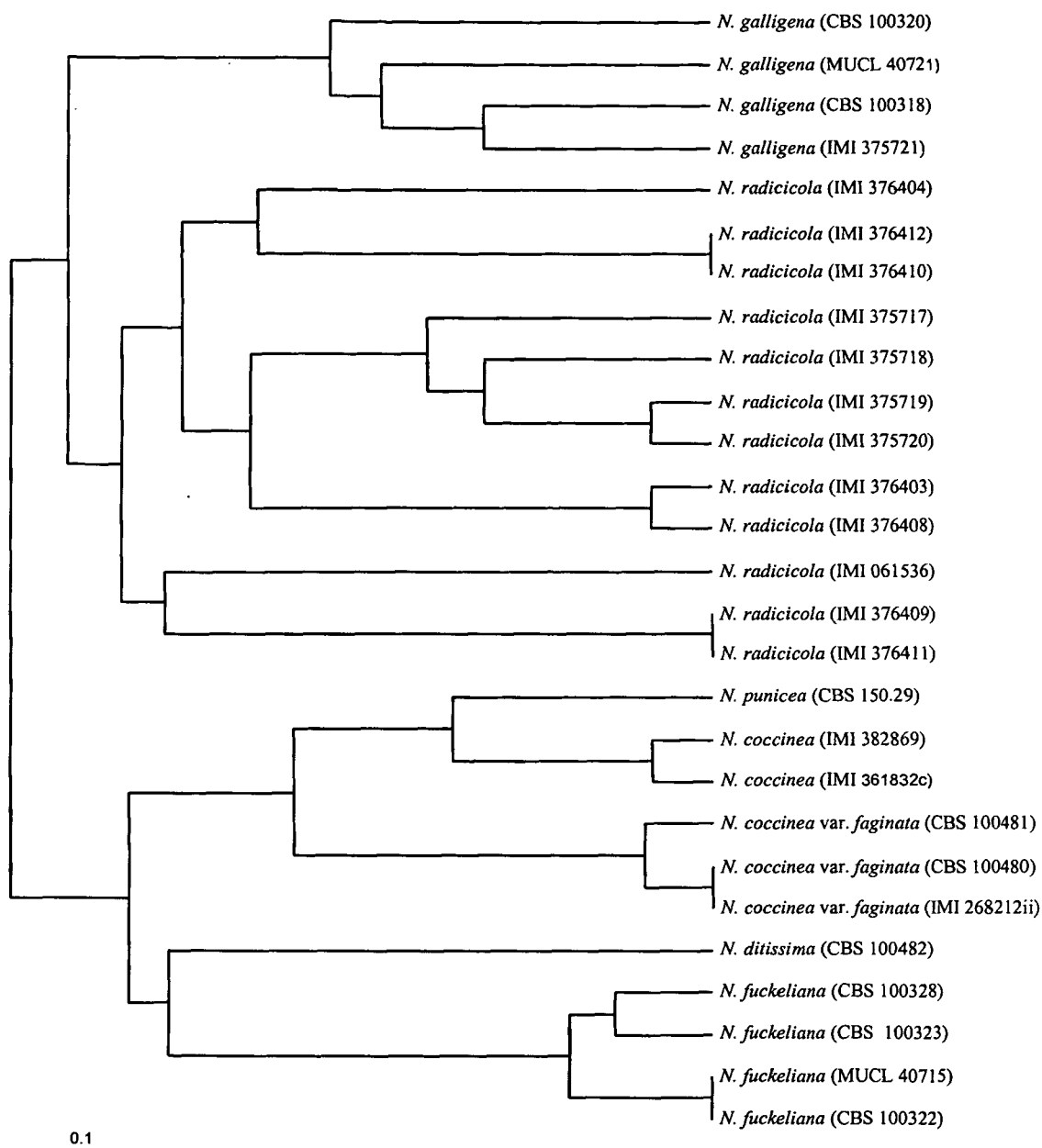


Fig. 6.2. Unrooted dendrogram constructed exclusively from M13 fingerprint data indicating the relationship among all *Nectria* Coccinea species and strains of *N. radicola*. Genetic similarities were calculated from a matrix of pairwise distances generated using Jaccards similarity co-efficient (Sneath and Sokal, 1973). Distances are shown above the dendrogram. The dendrogram was generated from the distance matrix using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA).

a similarity of 92%, whereas the RAPD analysis gave a similarity value of just 8%. Cluster analysis of the combined X11 and M13 similarity matrices revealed two predominant clusters comprising sub-groups, broadly reflecting the morphological distinction of the species assessed, with similarity among isolates in each group ranging from 0 to 90% (Fig. 6.3.). The first main cluster consisted of two sub-groups, comprising the four *N. galligena* isolates, representing the widest genotypic range as previously characterised in Chapter 3 with similarity values ranging from 42% to 64%. Each of the *N. radicicola* strains divided into two further sub-groups with similarity values ranging from 16% to 90% and 17% to 58%, respectively. No obvious host or geographic correlation to substantiate this sub-division was apparent.

The four *N. galligena* isolates used appeared separated from the other members of the Coccinea group, generally inconsistent with the overall taxonomic association of this group as proposed by Booth (1959). The reasons for this are unclear. The second main cluster comprised three sub-groups separating *N. fuckeliana*, *N. coccinea* and *N. coccinea* var. *faginata* strains (intra-specific similarity within each sub-group ranging from 63% to 84%, 61%, and 77% to 82%, respectively). Inter-cluster similarities ranged from 13% to 16% between *N. fuckeliana* and *N. coccinea* var. *faginata*, 16% to 27% between *N. fuckeliana* and *N. coccinea*, and 26% and 40% between *N. coccinea* and *N. coccinea* var. *faginata*. Based on the limited range of isolates used these analyses support the genetic separation of this latter variety from *N. coccinea*, but also the close relatedness of *N. punicea* to *N. coccinea* (similarity value of 33%). *N. ditissima* was clearly distinctive with closest affiliation for the *N. fuckeliana* cluster (similarity values ranging from 22% to 28%). Despite the levels of variation observed, the most prominent feature of these analyses was the higher level of intra-specific variation observed within *N. radicicola* (similarity values ranging from 9% to 90%) than inter-specific variation observed between the representative species of the Coccinea group (6% to 84%).

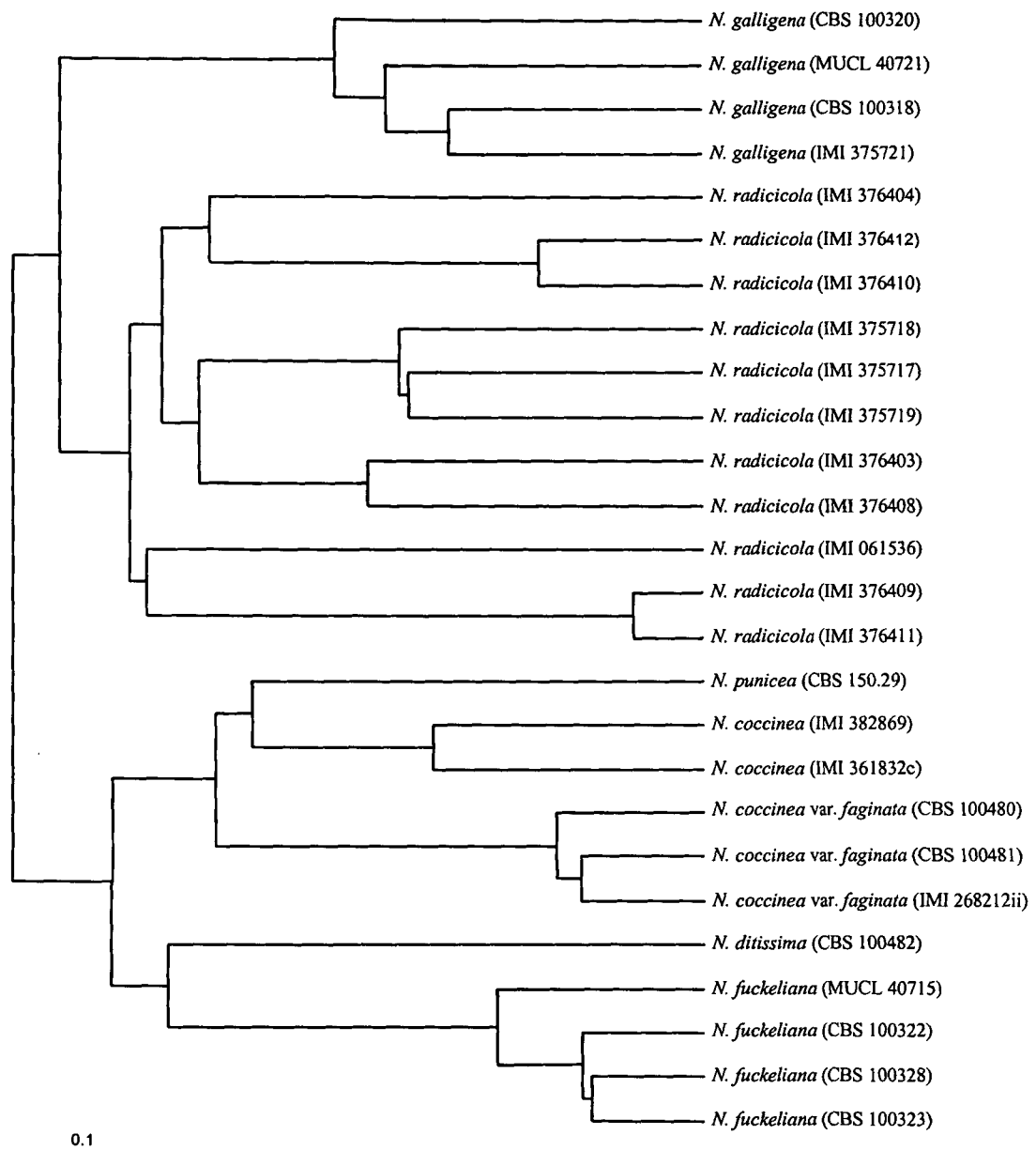


Fig. 6.3. Unrooted dendrogram constructed from combined RAPD and M13 fingerprint data indicating the relationship among all *Nectria* *Coccinea* species and strains of *N. radicola* (except IMI 375720). Genetic similarities were calculated from a combined RAPD and M13 fingerprint data matrix of pairwise distances generated using Jaccards similarity co-efficient (Sneath and Sokal, 1973). Distances are shown above the dendrogram. The dendrogram was generated from the distance matrix using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA).

6.3.2 rRNA spacer PCR-RFLP analysis

Using conserved universal primers ITS 5 and ITS 4 (White *et al.*, 1990), single PCR products were successfully amplified from all 27 isolates of the 7 species representing the entire length of the ITS 1, 5.8S rRNA gene, and ITS 2 region, including short flanking segments of the small and large sub-unit rRNA genes of the rRNA repeat. Each product exhibited length homogeneity of approximately 550 bp as observed by gel electrophoresis. By comparison, the IGS, amplified with primers PN11 and PN22, amplified single stranded products exhibiting length heterogeneity ranging in size from 2.35 to 3.1 Kbp for all strains (except *N. radiculicola* strains IMI 375720 and IMI 061536 which failed to amplify). Some degree of intra-specific IGS length polymorphism, as previously detected in the wider survey of a world-wide collection of *N. galligena* (Chapter 3), was observed in the strains of *N. fuckeliana* and *N. radiculicola* studied. However, the number of isolates tested from each species was too few to draw any meaningful correlation between host or geographic origin. Direct RFLP analysis of either spacer region broadly separated individual species into their morphologically recognised groups, consistent with the results obtained with the arbitrarily primed PCR, but with differing levels of intra and inter-specific resolution.

Digestion of the amplified ITS with restriction endonucleases *Hinf*I, *Hae*III, *Mse*I and *Msp*I produced 1-3, 2-4, 3-4 and 2-4 DNA fragments per isolate, representing 2, 3, 3 and 6 DNA fragment patterns generated per enzyme, respectively. No one enzyme was sufficient to resolve all species. Digestion with a further four restriction enzymes, *Mbo*I, *Taq*I, *Alu*I and *Dpn*I, each with four base recognition sites, revealed no restriction sites within this region.

Combining all ITS restriction fragments data, cluster analysis of all 27 polymorphic bands clearly separated species of the Coccinea group from *N. radiculicola* strains, but was insufficient to resolve all species within the Coccinea group (Fig. 6.4.). Cluster analysis (using UPGMA with Jaccard similarity co-efficient derived matrix) were identical with cluster analysis generated with Neighbour-Joining analysis and analysis generated using Match similarity co-efficient derived matrices (data not

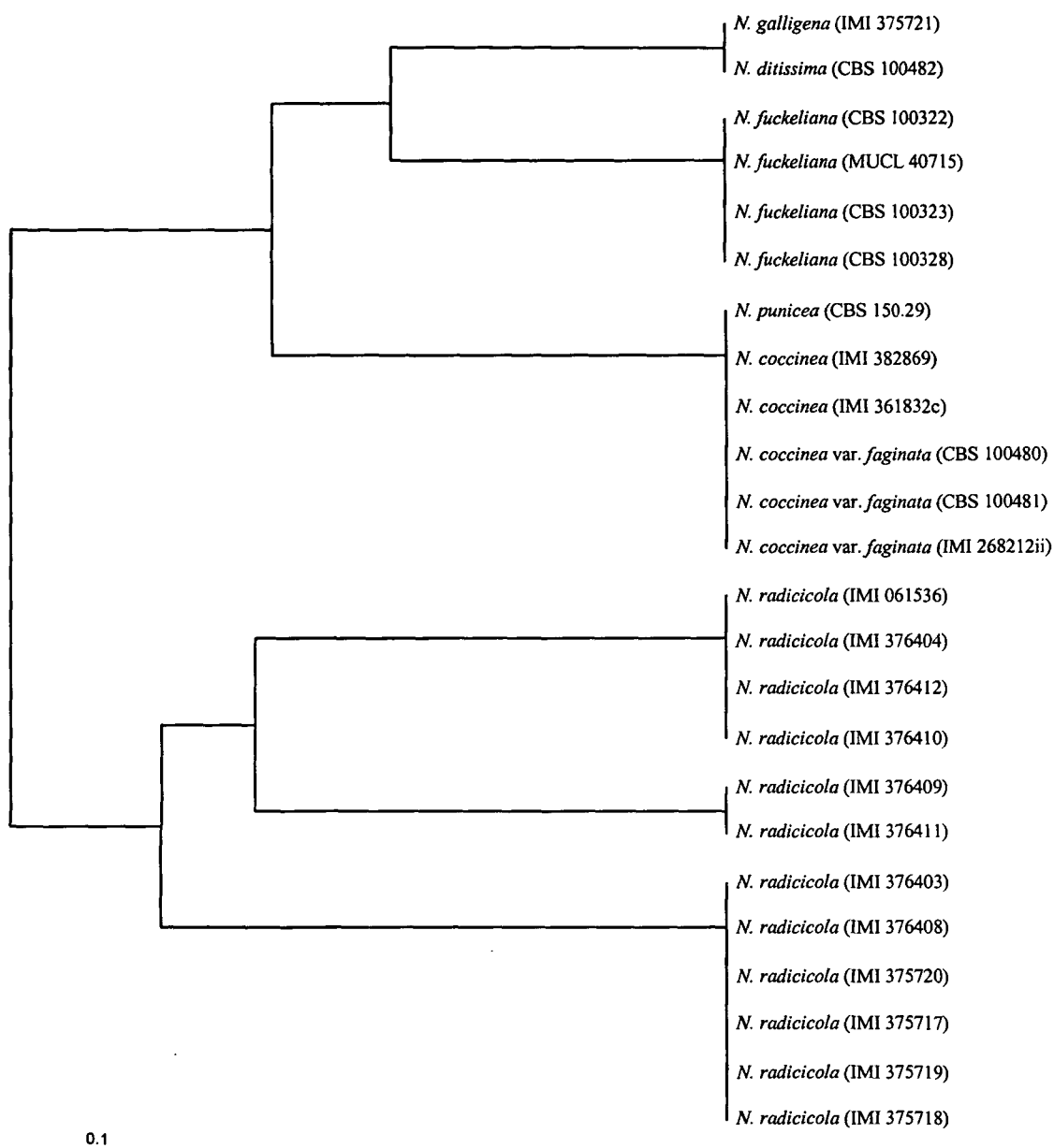


Fig. 6.4. Unrooted dendrogram constructed exclusively from ITS PCR-RFLP data indicating the relationship among all *Nectria Coccinea* species and strains of *N. radicicola*. Genetic similarities were calculated from a matrix of pairwise distances generated using Jaccards similarity co-efficient (Sneath and Sokal, 1973). Distances are shown above the dendrogram. The dendrogram was generated from the distance matrix using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA).

shown). The Coccinea cluster comprised three sub-groups of which *N. galligena*, indistinguishable from *N. ditissima*, formed one group, separate from the *N. coccinea*, *N. coccinea* var. *faginata* and *N. punicea* isolates (50%), which also formed an indistinguishable group. Only the *N. fuckeliana* strains separated as a distinct homogenous group, exhibiting a closer affinity for *N. galligena* and *N. ditissima* (62%) than for the *N. coccinea* and *N. punicea* species (47%). Of the *N. radiculicola* strains, three distinct intra-specific sub-groups were revealed. Intra-specific similarity values (ranging from 31% to 46%) were, as observed with analysis of arbitrarily primed PCR results, much less than inter-specific values exhibited between species of the Coccinea group, and exhibited no obvious geographical or host correlation.

In contrast, PCR-RFLP analysis of the IGS of all strains with restriction enzymes *MspI*, *HaeIII*, *HhaI*, *TaqI*, *RsaI*, and *HinfI* produced a much larger and more complex range of restriction profiles revealing easily scorable bands of between 3 and 8 resolvable fragments upon electrophoresis. Species represented by more than one strain all exhibited unique restriction profiles for each of the six enzymes used, except for *N. radiculicola* strains IMI 376409 and IMI 376411 which were identical. Cluster analysis of a total of 250 polymorphic bands (only fragments larger than 120 bp were taken into consideration as fragments smaller than this were not clearly resolved by 2% Metaphor electrophoresis), combined from all IGS restriction data, maintained the main separation of Coccinea group species from *N. radiculicola* strains, as observed with the ITS-RFLP analysis, but exhibiting much greater levels of intra and inter-specific resolution within both main clusters resolved, particularly within the *N. radiculicola* strains (Fig. 6.5.). The *N. coccinea* var. *faginata* strains formed a group (51% to 77%) within the range or similarity observed for the two *N. coccinea* strains (36%). As with the combined arbitrary primed PCR analysis, the clear distinction of *N. ditissima* was maintained but exhibited closest affiliation with *N. punicea*, which in turn was closest to the *N. coccinea* and *N. coccinea* var. *faginata* cluster. The separation of the *N. fuckeliana* strains were maintained as a distinct cluster with intra-specific similarity ranging from 38% to 76%, but with comparable relatedness to Coccinea group species (10% to 18%) as observed with the arbitrary

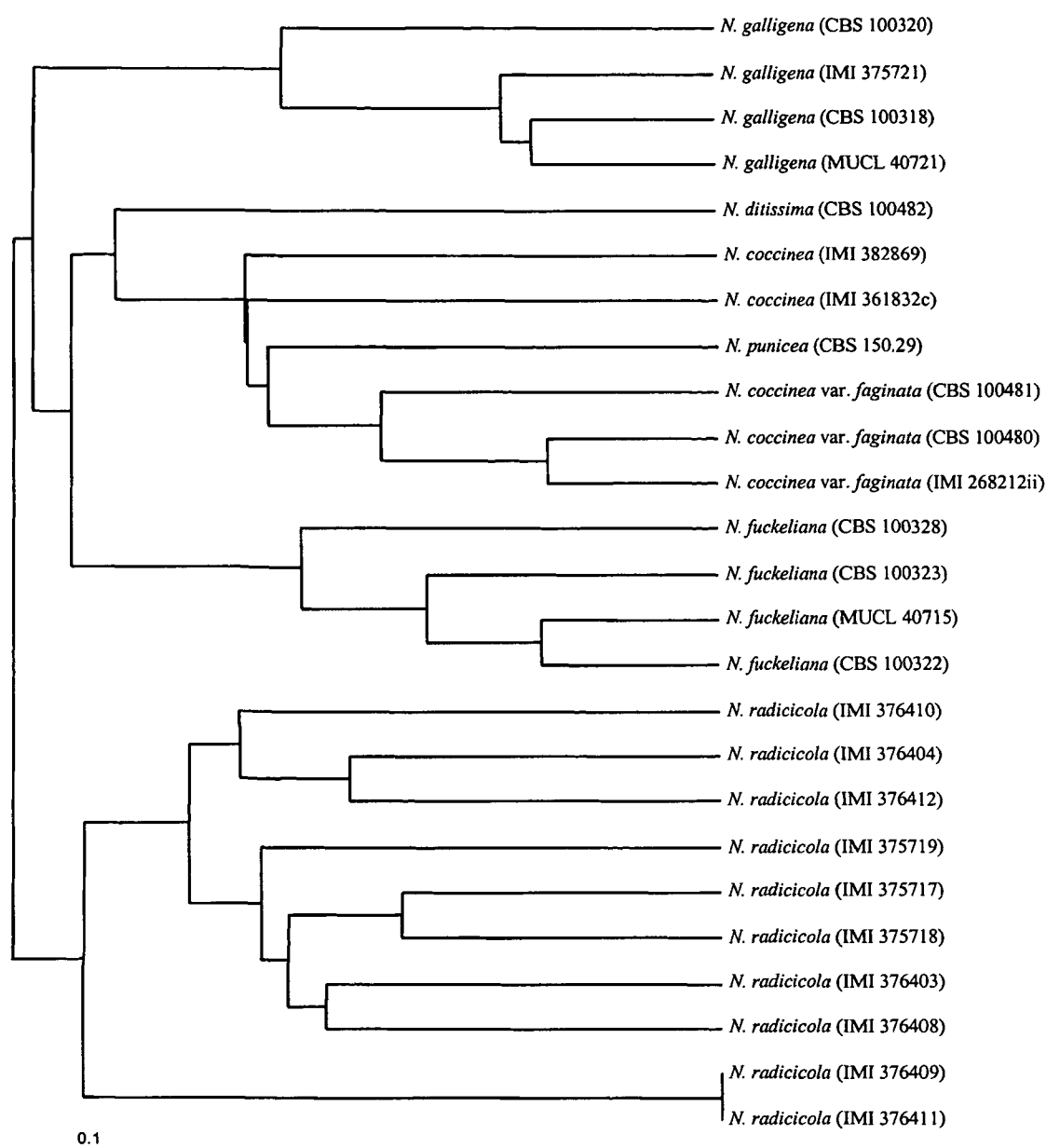


Fig. 6.5. Unrooted dendrogram constructed exclusively from IGS PCR-RFLP data indicating the relationship among all *Nectria Coccinea* species and strains of *N. radicicola* (except IMI 375720 and IMI 061536). Genetic similarities were calculated from a matrix of pairwise distances generated using Jaccards similarity co-efficient (Sneath and Sokal, 1973). Distances are shown above the dendrogram. The dendrogram was generated from the distance matrix using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA).

primed PCR analysis. As with the arbitrary primed PCR results, the four *N. galligena* isolates (40% to 75%) clustered independently of the main Coccinea group species, exhibiting greatest affiliation for *N. fuckeliana* strains (10% to 15%), *N. coccinea* (IMI 382869) (8% to 11%) and *N. punicea* (8% to 9%). Again, the separation of the *N. galligena* isolates beyond the range of genetic variation of the Coccinea group observed was notable with these analyses. Intra-specific variation within the *N. radicicola* strains, divided into three further sub-groups, ranged in similarity values from 12% to 100% with no obvious host or geographic correlation to substantiate this sub-division. Again, the most prominent feature of these analyses was the higher level of intra-specific variation observed within *N. radicicola* strains (similarity values ranging from 12% to 100%) than inter-specific variation (3% to 77%) observed between the representative species of the Coccinea group.

6.3.3 Combined arbitrarily primed and rRNA spacer PCR-RFLP analysis

As with the arbitrary primed data, analysis of all pairwise distances between the RAPD, M13 and IGS data matrices (except for *N. radicicola* strains IMI 376403 and IMI 376408) revealed a reasonable consistency between the three systems used despite differences in the basis of variation being exploited by the methods used. M13 analysis tended to give higher similarities for the more similar species than RAPD or IGS PCR-RFLP analysis. In turn, IGS PCR-RFLP analysis had a tendency for slightly higher similarities than RAPD analysis. Some notable examples of such differences were detected between *N. radicicola* strains IMI 376403 and IMI 376408 (RAPD, 8%, IGS PCR-RFLP, 47%, and M13 fingerprinting analysis, 92%) and for *N. fuckeliana* strains CBS 100328 and CBS 100323 between IGS PCR-RFLP and M13 fingerprint analysis (38% and 87%, respectively). Cluster analysis of RAPD, M13 and IGS PCR-RFLP combined Jaccard generated distance matrices retained the general relationships as calculated for IGS PCR-RFLP data (singly) and combined RAPD and M13 generated results, with only slight modification (**Fig. 6.6.**). Again, *N. punicea* was placed closest to *N. coccinea* (32% to 33%) both separated from *N. coccinea* var. *faginata* (35% to 38%, and 34% to 40%, respectively). This is consistent with the RADP and M13 combined results but a major change from the *N. coccinea* var. *faginata* sub-group nestled within the range of similarity observed

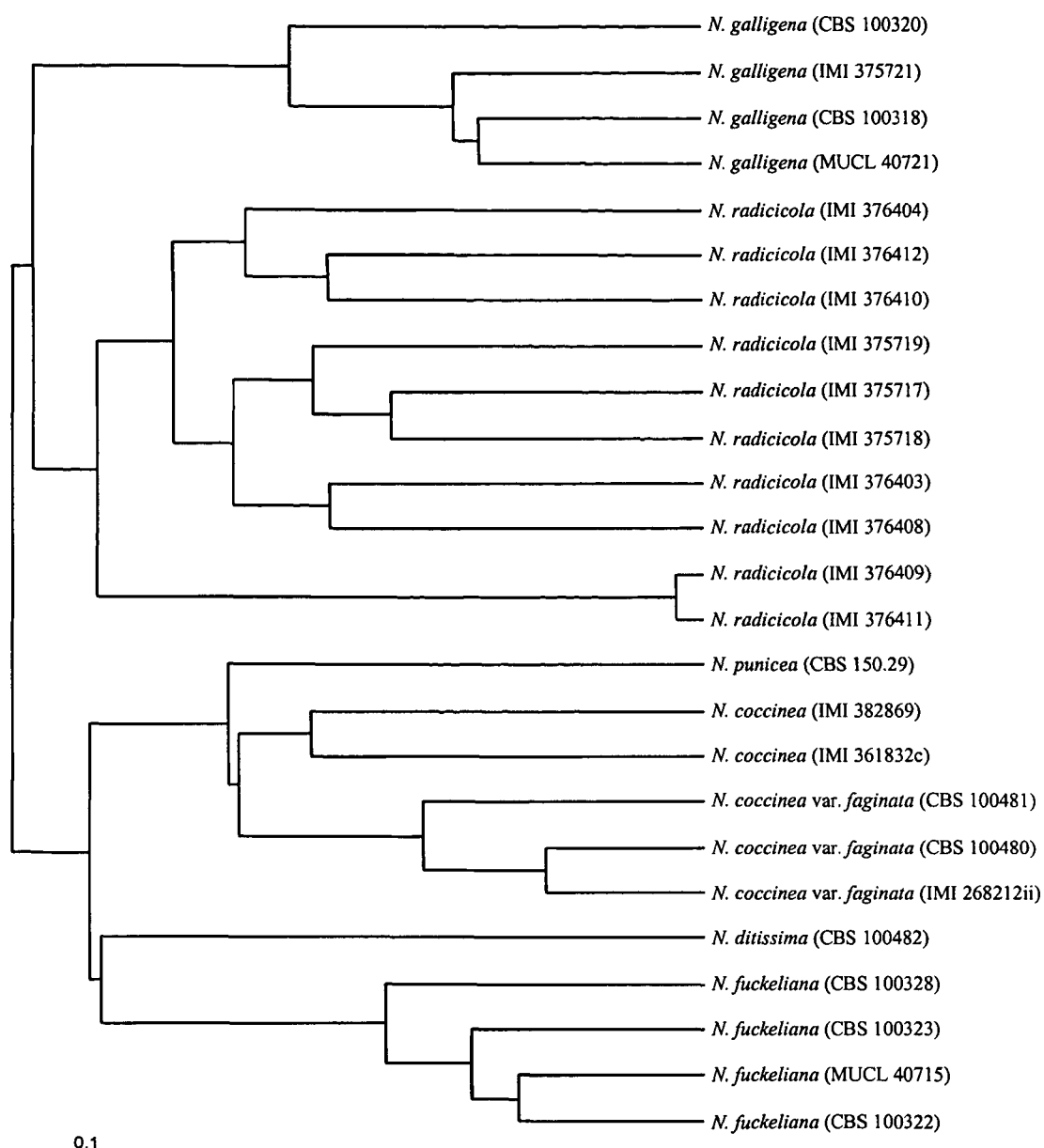


Fig. 6.6. Unrooted dendrogram constructed from combined IGS PCR-RFLP, RAPD and M13 fingerprint data indicating the relationship among all *Nectria* Coccinea species and strains of *N. radicola* (except IMI 375720 and IMI 061536). Genetic similarities were calculated from a matrix of pairwise distances generated using Jaccards similarity co-efficient (Sneath and Sokal, 1973). Distances are shown above the dendrogram. The dendrogram was generated from the distance matrix using an Unweighted Pair-Group Method with Arithmetic Means (UPGMA).

between the two *N. coccinea* strains revealed exclusively through IGS PCR-RFLP analysis. Both *N. ditissima* and the *N. fuckeliana* strains are consistent with earlier analyses, as are the *N. radiculicola* strains, although each of the four *N. galligena* strains appear almost equi-distant between the Coccinea (3% to 11%) and *N. radiculicola* (4% to 11%) clusters. Further, the higher level of intra-specific variation observed within *N. radiculicola* than inter-specific variation observed between the representative species of the Coccinea group, a prominent feature of all previous analyses, is reversed in this analysis (similarity values ranging from 13% to 57%, and 3% to 78%, respectively).

6.3.4 rRNA ITS sequence analysis

DNA sequence alignment of the entire length of the ITS 1, 5.8S rRNA gene, and ITS 2 region, generated from isolates representative of each of the seven species is shown in **Fig. 6.7**. Sequence analysis resolved the accurate lengths from each region from each isolate of each species. Length variability between ITS 1 and ITS 2 was negligible between species of the Coccinea group, but within strains of *N. radiculicola* the ITS 1 varied more than that of ITS 2 (**Table 6.2**). Similarly, the 5.8S rRNA coding region exhibited sequence length homogeneity in all Coccinea derived sequences as did the same region in the *N. radiculicola* strains, except both groups differed slightly in length (159 and 163 bp, respectively). Coccinea species represented by more than one isolate, such as *N. coccinea* (2), *N. coccinea* var. *faginata* (3), and *N. fuckeliana* (2), each exhibited complete intra-specific sequence identity (see footnote to **Table 6.2**), irrespective of host or geographical location, in contrast to the variability detected within *N. radiculicola* strains. Overall the internal transcribed spacers contained most of the sequence variation with the nucleotide composition of the ITS 1 more variable than that of ITS 2. Variations ranged from single base pair changes to multiple changes representing deletion and insertion events. One exception to this general nature of variation was the presence of an apparent 20bp insertion sequence in *N. cinnabarina* (IMI 362479: AJ009264) from position 62 to 81 (**Fig. 6.7**), consisting almost entirely of GC residues. The sequence showed no distinct homology with any sequence contained within the databases, but does have 13 bases in common (65%) with the beginning of the ITS 2 region which

could indicate an ancestral duplication or crossover. The same 20bp sequence was also present in the ITS 1 region, with similar ITS 2 region homology, from *N. cinnabarina* strain NRRL 20484 with just one nucleotide base undetermined (the corresponding sequence L36616 was obtained from the databases). Sequence alignment across all species (shown in Fig. 6.7.), revealed a total of 156 variable sites (31.2%), out of a total 500 positions. The ITS 2 regions exhibited between 0 and 20.5% sequence divergence. In contrast, the ITS 1 regions exhibited much higher sequence divergence ranging from 0 to 39.7%. As expected, the sequence of the 5.8S rRNA coding region was highly conserved across all species and contained only 9 variable sites (5.6%). **Table 6.3.** shows the sequence divergence and genetic distance calculated between species across the entire ITS 1, 5.8S, ITS 2 region.

Inter-specific genetic distances were estimated according to the Kimura 2-parameter parameter model within the DNADIST package in PHYLIP (version 3.5c) of the entire internal transcribed spacer region sequence (ITS 1, ITS 2 and 5.8S rRNA gene) between species pairs (**Table 6.3.**). Inter-specific genetic distances within the Coccinea group, although relatively small, were comparable to the extent of genetic distance observed intra-specifically within *N. radiculicola*, consistent with the PCR marker data suggesting that *N. radiculicola* is a highly genetically variable taxon.

Using *N. cinnabarina* (AJ009264) as an outgroup (type species of the genus (Rossman *et al.*, 1999)), phylogenetic analysis of the internal transcribed spacer region sequence data was performed using the Kimura 2-parameter model within the PHYLIP (Felsenstein, 1993) package DNADIST. Despite relatively small inter and intra specific distances (**Table 6.3.**), these analyses separated all the isolates of the seven species into two primary clades, with two *N. radiculicola* strains (IMI 376409 (AJ007353) and 94-1685 (ITS variant C) forming a third grouping. Each division was supported by relatively strong bootstrap values (Fig. 6.10.). All of the Coccinea group isolates appeared to form a monophyletic group supported by calculated genetic distance values, distinct from *N. radiculicola*, comprising three sub-clades largely separated along morphological criteria (Booth, 1959). The *N. coccinea* strains,

Fig. 6.7. Multiple sequence alignment of the 5.8S rRNA gene sequences and their flanking internal transcribed spacers (ITS 1 and ITS 2) of strains listed in **Table 6.2.** using GeneDoc (Nicholas *et al.*, 1997). The sequences are written 5' to 3' and have been arranged according to their percentage similarity to *N. galligena* reference sequences AJ228666 as determined by CLUSTAL V analysis. Identity of the *N. galligena* reference sequences are indicated by a period (.), gaps are indicated by a hyphen (-) and non-determined nucleotide bases as N. Species sequence abbreviations are as follows: N.gal 1, *N. galligena* (IMI 378754: AJ228666); N.gal 2, *N. galligena* (IMI 378755: AJ228673); N.dit, *N. ditissima* (CBS 100482: AJ009272); N.coc, *N. coccinea* (IMI 382869: AJ009268); N.cvf, *N. coccinea* var. *faginata* (CBS 100480: AJ009270); N.pun, *N. punicea* (CBS 150.29: AJ009273); N.fuc, *N. fuckeliana* (CBS 100323: AJ009275); N.rad 1, *N. radiculicola* (94-1685: ITS variant C); N.rad 2, *N. radiculicola* (IMI 376409: AJ007353); N.rad 3, *N. radiculicola* (IMI 376404: AJ007357); N.rad 4, *N. radiculicola* (IMI 061536: AJ007354); N.rad 5, *N. radiculicola* (IMI 375719: AJ007356); N.rad 6, *N. radiculicola* (IMI 375717: AJ007355); N.rad 7, *N. radiculicola* (IMI 376404: AJ007351); N.rad 8, *N. radiculicola* (IMI 376408: AJ007352); N.rad 9, *N. radiculicola* (UAMH 4907: AF172261); N.rad 10, *N. radiculicola* (94-0001: ITS variant A); N.rad 11, *N. radiculicola* (89-0283: ITS variant B); N.cin, *N. cinnabarina* (IMI 362479: AJ009264).

	1	11	21	31	41	51
N.gal 1	-CCGA-GTTT	ACAACCTCCC-	AAACCCCTG-	TGAACATACC	--CATCGTTG	CCTCGGCGGT
N.gal 2	-....-				--.....	
N.dit	-....-				--.....	
N.coc	-....-				--.....	
N.cvf	-T.AGA....				--T.....	
N.pun	-....T....			A.....	--.....	
N.fuc	-..T.-....				--T.....	
N.rad 1	-....-				T-AT..T....	
N.rad 2	-....-				A-T..T....	
N.rad 3	-....-				A-T..T....	
N.rad 4	C.T.-....	T			A-T..T....	
N.rad 5	-....-				A-T..T....	
N.rad 6	C.T.-....	T			A-T..T....	
N.rad 7	C.T.-....	T			A-T..T....	
N.rad 8	C.T.-....	T			A-T..T....	
N.rad 9	-....-				A-T..T....	
N.rad 10	-....-				A-T..T....	
N.rad 11	-....-				A-T..T....	
N.cin	-....-				TTT.CT....	.T.....G

	61	71	81	91	101	111
N.gal 1	G-----	-----	-CCCGCTCCG	GCGGCCCGCC	AGAGGACCCC	CAAACCTTTG
N.gal 2	-----	-----	-----	-----	-----	-----
N.dit	-----	-----	-----	-----	-----	-----
N.coc	-----	-----	-----	-----	-----	-----T
N.cvf	-----	-----	-----	-----	-----	-----T
N.pun	-----	-----	-----	-----	-----	-----T
N.fuc	-----	-----	-----	-----	-----	A..C.CT.AT
N.rad 1	-----	-----	..T.T....	A.A.....	-----	A..C.CT-GA
N.rad 2	-----	-----	..T.T....	A.A.....	-----	A.A.CC..AAGA
N.rad 3	-----	-----	..T..T..	..A.....	-----	A.A.CC..TAGA
N.rad 4	-----	-----	..T.T.T..	..A.....	-----	A.A.CC..AAGA
N.rad 5	-----	-----	..T.T.T..	..A.....	-----	A.A.CC..AAGA
N.rad 6	-----	-----	..T.T.T..	..A.....	-----	A.A.CC..AAGA
N.rad 7	-----	-----	..T.T.T..	..A.....G.	-----	A.A.CC..AAGA
N.rad 8	-----	-----	..T.T.T..	..A.....	-----	A.A.CC..AAGA
N.rad 9	-----	-----	..T..T..	..A.....	-----	A.A.CC..TGAT
N.rad 10	-----	-----	..T..T..	..A.....	-----	A.A.CC..TGAT
N.rad 11	-----	-----	..T..T..	..A.....	-----	A.A.CC..TGAT
N.cin	ATCGCCCCGG	CGCCCTCGGG	C..G.AC..A	.GC.....	G.....	A.A.CT..TG.T

Continuation of Fig. 6.7. from previous page.

	121	131	141	151	161	171
N.gal 1	TTTTAT--AC	AGCATCTT-C	TGAGTAACAC	GATTAA--AT	AAATCAAAAC	TTTCAA-CAA
N.gal 2	--..	..A.....-		--..		-...
N.dit	--..			--..		-...
N.coc	G.....T-..	..T.....-		--..		-...
N.cvf	G.....T-..	..T.....-		--..		-...
N.pun	G.....T-..	..TG.....-		--..		-...
N.fuc	..C.GAAT..	..T.....-		--..		-...
N.rad 1	..AC..TT.A	GAAG.....-	AC.	--..		-...
N.rad 2	..AC..TT.A	GAAG.....-	-C.	--..		A...
N.rad 3	..--..TT..	..T.....-	-T	..A..--..		A...
N.rad 4	..AC..TT..	..T.....A.	C.-T	GGTC		A...
N.rad 5	..AC..TT.A	T.-.	C.-T		C.....	A...
N.rad 6	..AC..TT.A	A.A.	C.-T		C.....	A...
N.rad 7	..AC..TT.A	..T.....A.	C.-T	..GG.	C.....	A...
N.rad 8	..AC..TT.A	..T.....A.	C.-T	..AGG.	C.....	A...
N.rad 9	..--..AT..	..T.....-	-T			-...
N.rad 10	..--..AT..	..T.....-	-T			-...
N.rad 11	..--..AT..	..T.....-	-T			-...
N.cin	.CC.T.----	-TGAT.CT.	G.T..	A.GC..--..	T.....	-...

	181	191	201	211	221	231
N.gal 1	CGG-ATCTCT	TGG-TTCTGG	CATCGATGAA	GAACGCAGCG	AAATGCGATA	AGTAATGTGA
N.gal 2						
N.dit						
N.coc						
N.cvf						
N.pun						
N.fuc		..-C.....				
N.rad 1						
N.rad 2	..G.....	..G.....				
N.rad 3	..G.....	..G.....				
N.rad 4	..G.....	..G.....				
N.rad 5	..G.....	..G.....				
N.rad 6	..G.....	..G.....				
N.rad 7	..G.....	..G.....				
N.rad 8	..G.....	..G.....				
N.rad 9						
N.rad 10						
N.rad 11						
N.cin						

	241	251	261	271	281	291
N.gal 1	ATTGCAGAAT	TCAGTGAATC	ATCGAATCTT	TGAACGCACA	TTGCGCCCGC	C-AGTATTCT
N.gal 2						
N.dit						
N.coc						
N.cvf						
N.pun						
N.fuc		C.				
N.rad 1						T.....
N.rad 2						.C.....
N.rad 3						.C.....
N.rad 4						.C.....
N.rad 5						.C.....
N.rad 6						.C.....
N.rad 7						.C.....
N.rad 8						.C.....
N.rad 9						
N.rad 10						
N.rad 11						
N.cin						C...

Continuation of Fig. 6.7. from previous page.

	301	311	321	331	341	351
N.gal 1	GGCGGGCATG	CCTGTTTCGAG	CGTCATTTC	ACCCTCAAGC	CCC--CGGGC	TTGGTGTGG
N.gal 2						
N.dit						
N.coc						
N.cvf						
N.pun						
N.fuc						
N.rad 1						
N.rad 2		C.....				
N.rad 3		C.....				
N.rad 4		C.....				
N.rad 5		C.....				
N.rad 6		C.....				
N.rad 7		C.....				
N.rad 8		C.....				
N.rad 9						
N.rad 10						
N.rad 11						
N.cin					TT.....	

	361	371	381	391	401	411
N.gal 1	GGATCG----	GCGTGCCCTT	CGCGGCGCGC	CGTCCCCTAA	ATCTAGTGGC	GGTCTCGCTG
N.gal 2						
N.dit	..GATCCGGC	..C.....C	T.....	..C.....		
N.coc	...ATCG---	..CC...CC	G..	C..		
N.cvf	...TCG---	..C...CC	G..	C..		
N.pun	...TCG---	..CC...CC	G..	C..		
N.fuc	G--C	.T.CC...CC	..G.....	..C.....	..C.....	
N.rad 1		..A...TCC	--GC..C..			
N.rad 2		..A...TCC	--GC..C..			
N.rad 3	A.....	CC	..G.....	..G.T..C..	..A.....	
N.rad 4	A.....	..A...C	..G.....	..G.T..C..	..A.....	
N.rad 5	A.....	..A...CC	..G.....	..T..C..	..A.....	..C.....
N.rad 6	A.....	..A...CC	..G.....	..T..C..	..A.....	..C.....
N.rad 7	A.....	..A...C	..G.....	..T..C..	..A.....	..C.....
N.rad 8	A.....	..A...C	..G.....	..T..C..	..A.....	..C.....
N.rad 9	A.....	CC	..G.....	..G.T..C..	..A.....	
N.rad 10	A.....	CC	..G.....	..NN.T..C..	..A.....	
N.rad 11	A...N.---	CC	..G.....	..NG.T....	..A.....	
N.cin	GCCT	...GCGT..G	ACGCCGTG..	..G...G..		

	421	431	441	451	461	471
N.gal 1	CAG-CTTCCT	CTGCGTAGTA	GCACACCTCG	CACCGGAAGA	GCAGCGCGGC	CACGCCGTTA
N.gal 2						
N.dit						
N.coc		..C.....	..ACA...	..G.T...-C		
N.cvf		..C.....	..ACA...	..G.T...-C		
N.pun		..C.....	..ACA...	..G.T...-C		
N.fuc		..C.....		..G.T...-A	..G.....	
N.rad 1	T...-			..T..G-A	A.....	
N.rad 2	T...-			..T..G-A	A.....	
N.rad 3	T...-		A.....A	..T..G-A	A.....T...	
N.rad 4	T...-			..T..G-A	A.....T...	
N.rad 5	T...-			..T..G-A	A.....T...	A.
N.rad 6	T...-			..T..G-A	A.....T...	A.
N.rad 7	T...-			..T..G-A	A.....T...	A.
N.rad 8	T...-			..T..G-A	A.....T...	G.
N.rad 9	T...-			..T...-A	A.....T...	
N.rad 10	T...-			..T...-A	A.....T...	
N.rad 11	T...-			..T...-A	A.....T...	
N.cin	T..T.C...			-AC	TT.....	

Continuation of **Fig. 6.7.** from previous page.

	481	491
N.gal 1	AACCCCCAC	TTCTGAAAGG
N.gal 2		
N.dit		
N.coc		
N.cvf		
N.pun		
N.fuc		
N.rad 1	A..	T
N.rad 2	A..	
N.rad 3		
N.rad 4		
N.rad 5		
N.rad 6		
N.rad 7		
N.rad 8		
N.rad 9		
N.rad 10		
N.rad 11		
N.cin	AACT	GT-

Table 6.2. Lengths of both internal transcribed spacer regions (ITS 1 and ITS 2) and 5.8S rRNA gene, including EMBL accession number, for each sequence characterised.

Species/Isolate	Code	Length (bp)			EMBL Accession N ^o
		ITS 1	ITS 2	5.8S	
<i>N. galligena</i>	IMI 378754	135	165	158	AJ228666 ^a
<i>N. galligena</i>	IMI 378755	135	165	158	AJ228673 ^{a,b}
<i>N. coccinea</i>	IMI 382869	135	165	159	AJ009268 ^c
<i>N. coccinea</i>	IMI 361832c	135	165	159	AJ009250 ^c
<i>N. coccinea</i> var. <i>faginata</i>	CBS 100480	136	164	159	AJ009270 ^d
<i>N. coccinea</i> var. <i>faginata</i>	CBS 100481	136	164	159	AJ009271 ^d
<i>N. coccinea</i> var. <i>faginata</i>	IMI 268212ii	136	164	159	AJ009251 ^d
<i>N. ditissima</i>	CBS 100482	134	168	159	AJ009272
<i>N. punicea</i>	CBS 150.29	137	164	159	AJ009273
<i>N. fuckeliana</i>	CBS 100328	136	166	159	AJ009274 ^e
<i>N. fuckeliana</i>	CBS 100323	136	166	159	AJ009275 ^e
<i>N. radicicola</i>	IMI 376404	132	164	163	AJ007357
<i>N. radicicola</i>	IMI 375717	139	164	163	AJ007355
<i>N. radicicola</i>	IMI 375719	136	164	163	AJ007356
<i>N. radicicola</i>	IMI 376403	140	164	163	AJ007351
<i>N. radicicola</i>	IMI 376408	141	164	163	AJ007352
<i>N. radicicola</i>	IMI 061536	141	164	163	AJ007354
<i>N. radicicola</i>	IMI 376409	136	162	163	AJ007353
<i>N. radicicola</i>	94-0001	132	164	159	ITS variant A ^f
<i>N. radicicola</i>	89-0283	132	164	159	ITS variant B ^f
<i>N. radicicola</i>	94-1685	136	162	159	ITS variant C ^f
<i>N. radicicola</i>	UAMH 4907 ^g	134	167	156	AF172261 ^h
<i>N. cinnabarina</i>	IMI 362479 ⁱ	154	170	159	AJ009264

^aPreviously characterised in Chapter 3.

^b*N. galligena* sequence AJ228673 exhibited a T-A transition at nucleotide position 109.

^cBoth AJ009268 and AJ009250 are identical.

^dAJ009270, AJ009271 and AJ009251 are identical.

^eBoth AJ009274 and AJ009275 are identical.

^fITS Sequence data taken from Hamelin *et al.* (1996). FASTA formatted sequence data kindly supplied by R. C. Hamelin, Canadian Forest Service, Quebec.

^gUniversity of Alberta Microfungus Collection and Herbarium. Strain isolated from roots of *Poa pratensis*, Atlanta, USA.

^hSequence derived from databases.

ⁱStrain obtained from D. Brayford, CABI Bioscience, Egham, UK (formerly International Mycological Institute).

including *N. punicea*, formed a closely related group supported by a statistically significant branch (100%), grouping *N. coccinea* with *N. punicea* (0.0044), maintaining the separation of *N. coccinea* from *N. coccinea* var. *faginata* (0.011), supporting the identity of *N. coccinea* var. *faginata* as a distinct taxon (Lohman and Watson, 1943) (see **Table 6.3.**). The *N. fuckeliana* clade (both strains (CBS 100328: AJ009274, CBS 100323: AJ009275) exhibiting complete sequence identity) appeared ancestral to the Coccinea group, as indicated by genetic distance comparisons with each Coccinea group member (**Table 6.3.**). The ITS region from the two *N. galligena* isolates, previously shown to be highly conserved intra-specifically (see Chapter 3) were most closely related to *N. ditissima*, differing by 1.3-1.6% (0.0177-0.02). The *N. radicicola* strains (genetic distance of 0.0724 from the closest related *N. coccinea* species) formed three distinct sub-clades exhibiting greater intra-specific diversity (ranging from 0 to 18% and 0 to 0.1741 for sequence divergence and genetic distance, respectively) than inter-specific diversity observed between the Coccinea group species, comprising a more extensive and complex range of nucleotide changes. From the genetic distances calculated (**Table 6.3.**), the smaller *N. radicicola* grouping (comprising strains IMI 376409 (AJ007353) and 94-1685 (ITS variant C) from the phylogenetic analysis) appeared ancestral to the Coccinea group species (**Fig. 6.10.**).

All sequences generated during the course of this study were submitted to the EMBL Nucleotide Sequence Database (see **Table 6.2.**).

Table 6.3. Matrices of the Genetic Distance between the entire ITS 1, 5.8S, ITS 2 sequence data of species/isolate pairs as estimated using the Kimura 2-parameter model within the PHYLIP (version 3.5c) DNADIST package (Felsenstein, 1993) (lower left) and percentage divergence as calculated using CLUSTAL V (Higgins and Sharp, 1989) analysis (upper right).

	N. gal 1*	N. gal 2	N. dit	N. coc	N. cvf	N. pun	N. fuc	N. rad 1	N. rad 2	N. rad 3	N. rad 4	N. rad 5	N. rad 6	N. rad 7	N. rad 8	N. rad 9	N. rad 10	N. rad 11	N. cin
N. gal 1*	0	0.2	1.3	3.8	4.3	3.8	4.3	9.5	9.5	8.8	9.9	9.9	10.4	10.4	10.1	5.3	5.1	4.8	13.3
N. gal 2	0.0022	0	1.6	3.8	4.3	3.8	4.3	9.2	9.2	8.7	9.9	10.2	10.6	10.4	10.1	5.3	5.1	4.8	13.3
N. dit	0.0177	0.0200	0	4.1	5.0	4.0	5.0	10.0	10.0	9.5	10.4	10.8	11.2	11.2	10.9	6.1	5.8	5.6	13.8
N. coc	0.0430	0.0431	0.0406	0	0.7	0.9	5.0	10.8	10.2	9.5	10.7	11.0	11.2	11.2	10.9	6.5	6.3	6.6	14.1
N. cvf	0.0478	0.0478	0.0523	0.0110	0	1.8	5.0	11.0	10.2	9.2	10.9	11.0	11.4	11.4	11.2	6.8	6.6	6.8	13.8
N. pun	0.0432	0.0432	0.0431	0.0044	0.0177	0	5.2	11.5	11.0	10.7	12.2	12.0	12.4	12.7	12.4	6.8	6.5	6.8	14.9
N. fuc	0.0592	0.0593	0.0662	0.0666	0.0715	0.0693	0	10.7	10.5	8.8	10.9	11.2	11.6	11.7	11.4	6.0	5.8	5.5	14.9
N. rad 1	0.0823	0.0797	0.0924	0.1058	0.1110	0.1034	0.1100	0	1.6	6.7	6.4	6.2	6.2	6.4	6.2	7.5	7.3	7.0	14.4
N. rad 2	0.0722	0.0697	0.0821	0.0927	0.0953	0.0903	0.0942	0.0247	0	5.2	4.7	4.5	4.5	4.7	4.5	8.0	7.8	8.0	14.2
N. rad 3	0.0644	0.0645	0.0765	0.0844	0.0845	0.0872	0.0864	0.0776	0.0548	0	2.0	3.1	3.1	3.1	2.9	3.4	3.4	3.9	15.6
N. rad 4	0.0717	0.0718	0.0815	0.0944	0.0970	0.0973	0.0886	0.0794	0.0519	0.0221	0	2.2	2.2	1.7	1.7	5.2	5.3	5.8	16.7
N. rad 5	0.0742	0.0767	0.0865	0.0971	0.0996	0.0974	0.0985	0.0744	0.0472	0.0313	0.0242	0	0.2	0.9	0.9	5.7	5.5	6.3	17.4
N. rad 6	0.0767	0.0792	0.0891	0.0997	0.1023	0.1001	0.0962	0.0770	0.0496	0.0336	0.0219	0.0043	0	0.9	0.9	6.2	6.0	6.7	18.1
N. rad 7	0.0791	0.0792	0.0915	0.1022	0.1048	0.1051	0.0986	0.0818	0.0542	0.0336	0.0174	0.0131	0.0108	0	0.4	6.2	6.0	6.7	17.8
N. rad 8	0.0766	0.0767	0.0890	0.0996	0.1022	0.1024	0.0960	0.0793	0.0518	0.0313	0.0174	0.0130	0.0108	0.0043	0	6.0	5.7	6.5	17.5
N. rad 9	0.0500	0.0501	0.0619	0.0746	0.0746	0.0773	0.0692	0.0751	0.0625	0.0201	0.0625	0.0386	0.0409	0.0432	0.0409	0	0.0	0.4	14.0
N. rad 10	0.0478	0.0479	0.0598	0.0724	0.0725	0.0751	0.0670	0.0729	0.0603	0.0202	0.0603	0.0364	0.0387	0.0411	0.0387	0.000	0	0.4	14.1
N. rad 11	0.0456	0.0456	0.0551	0.0725	0.0726	0.0752	0.0647	0.0706	0.0628	0.0247	0.0628	0.0434	0.0458	0.0481	0.0457	0.0044	0.0044	0	13.8
N. cin	0.1376	0.1374	0.1586	0.1622	0.1651	0.1538	0.1722	0.1511	0.1401	0.1596	0.1622	0.1656	0.1714	0.1741	0.1711	0.1486	0.1494	0.1467	0

*N. gal 1, *N. galligena* (IMI 378754: AJ228666); N. gal 2, *N. galligena* (IMI 378755: AJ228673); N. dit, *N. ditissima* (CBS 100482: AJ009272); N. coc, *N. coccinea* (IMI 382869: AJ009268); N. cvf, *N. coccinea* var. *faginata* (CBS 100480: AJ009270); N. pun, *N. punicea* (CBS 150.29: AJ009273); N. fuc, *N. fuceliana* (CBS 100323: AJ009275); N. rad 1, *N. radicola* (94-1685: ITS variant C); N. rad 2, *N. radicola* (IMI 376409: AJ007353); N. rad 3, *N. radicola* (IMI 376404: AJ007357); N. rad 4, *N. radicola* (IMI 061536: AJ007354); N. rad 5, *N. radicola* (IMI 375719: AJ007356); N. rad 6, *N. radicola* (IMI 375717: AJ007355); N. rad 7, *N. radicola* (IMI 376404: AJ007351); N. rad 8, *N. radicola* (IMI 376408: AJ007352); N. rad 9, *N. radicola* (UAMH 4907: AF172261); N. rad 10, *N. radicola* (94-0001: ITS variant A); N. rad 11, *N. radicola* (89-0283: ITS variant B); N. cin, *N. cinnabarina* (IMI 362479: AJ009264).
+Number of mismatches were obtained from pairwise alignments.

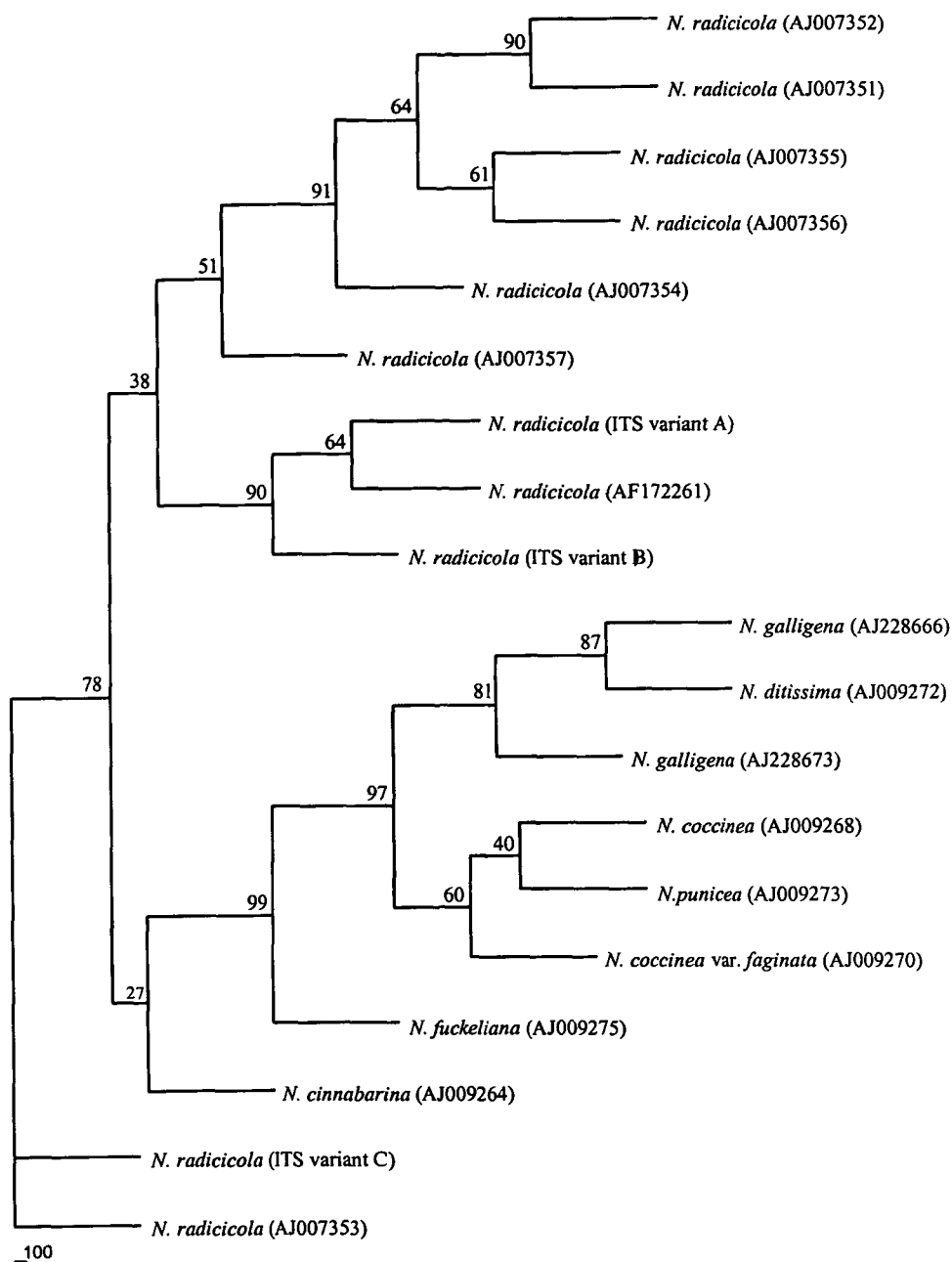


Fig. 6.8. Rooted phylogenetic tree indicating the relationships between *Nectria* Coccinea species and *N. radicola* strains based exclusively on rRNA ITS 1 sequence data. The phylogenetic tree was created using neighbour-joining (Neighbour package) from genetic distance values created with the Kimura 2-parameter model (DNADIST package) obtained from PHYLIP (version 3.5c). Confidence limits of the branches were created in a bootstrap analyses from 1000 replicates and are given as percentiles of the total number of replicates.

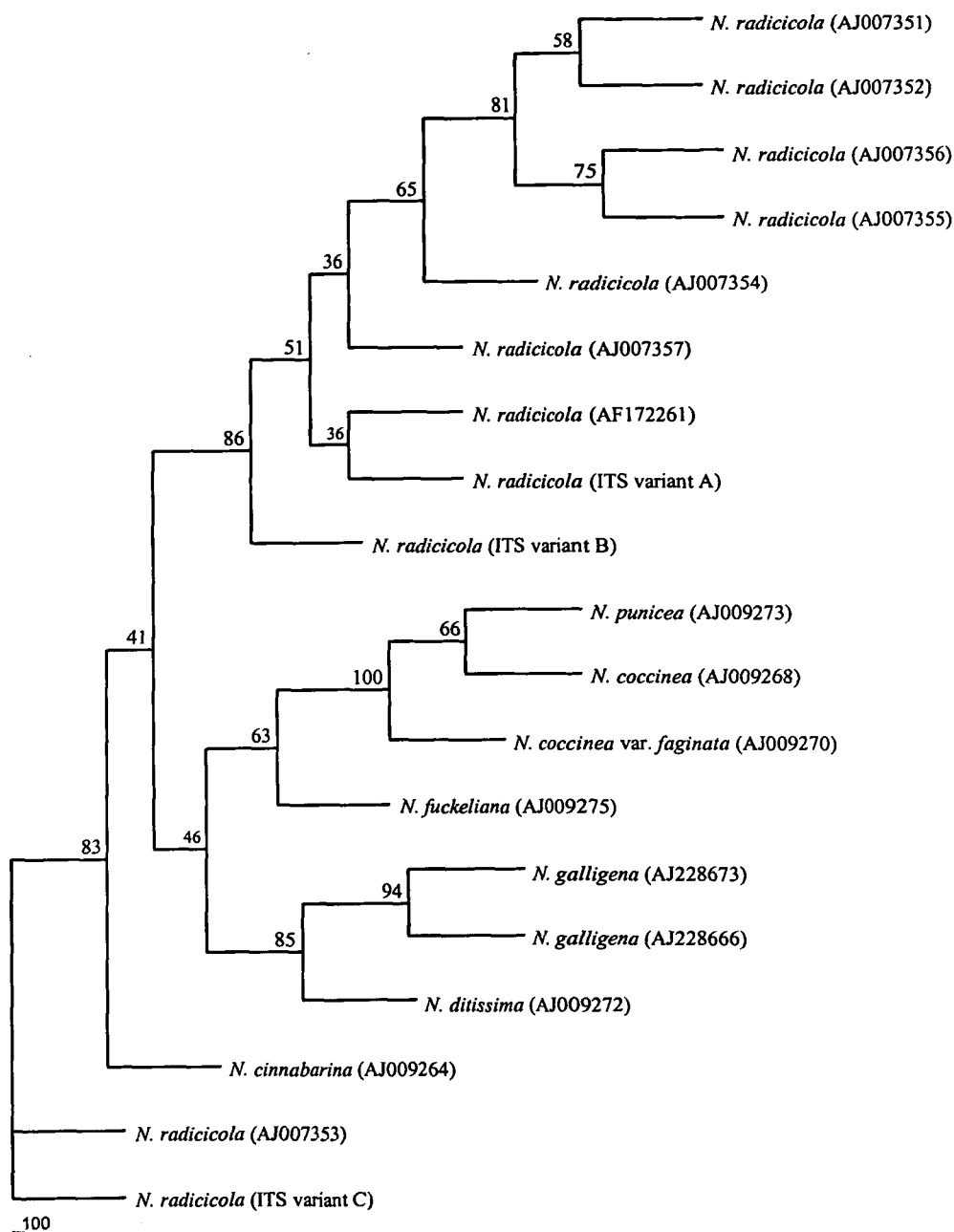


Fig. 6.9. Rooted phylogenetic tree indicating the relationships between *Nectria* Coccinea species and *N. radicola* strains based exclusively on rRNA ITS 2 sequence data. The phylogenetic tree was created using neighbour-joining (Neighbour package) from genetic distance values created with the Kimura 2-parameter model (DNADIST package) obtained from PHYLIP (version 3.5c). Confidence limits of the branches were created in a bootstrap analyses from 1000 replicates and are given as percentiles of the total number of replicates.

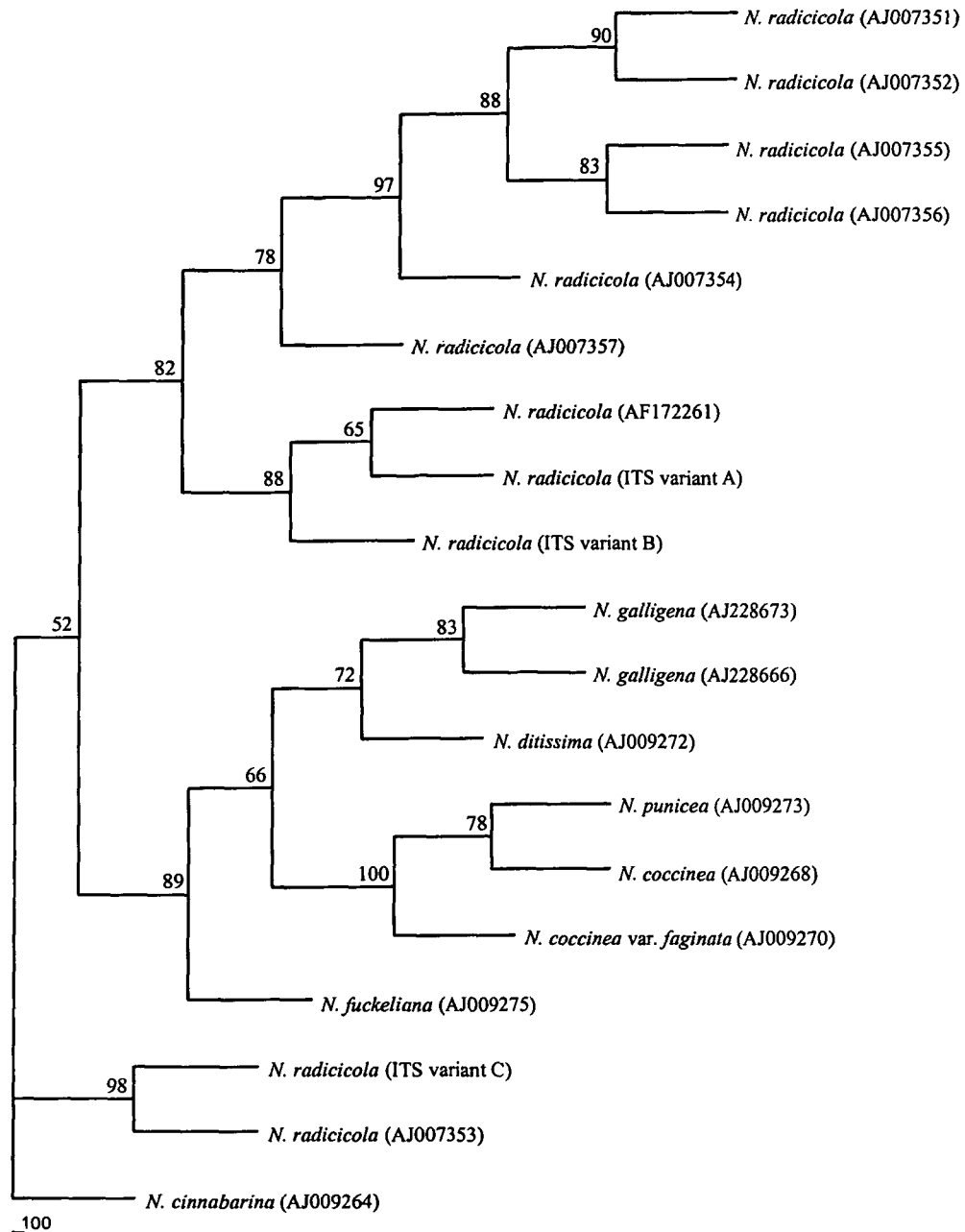


Fig. 6.10. Rooted phylogenetic tree indicating the relationships between *Nectria* Coccinea species and *N. radicola* strains based on rRNA (ITS 1-5.8S-ITS 2) sequence data. The phylogentic tree was created using neighbour-joining (Neighbour package) from genetic distance values created with the Kimura 2-parameter model (DNADIST package) obtained from PHYLIP (version 3.5c). Confidence limits of the branches were created in a bootstrap analyses from 1000 replicates and are given as percentiles of the total number of replicates.

6.4 Discussion

The *Nectria coccinea* group is one of several sub-generic groupings within the genus *Nectria sensu lato* (Booth, 1959) comprising of what is arguably the most economically important collection of tree infecting fungi (Booth, 1959; Brayford, 1992). The group is separated primarily on a combination of perithecial anatomy, stromatal characteristics and production of anamorphs with abundant microconidia but lacking chlamydospores. Using a range of PCR based molecular markers previously developed for the study of intra-specific variation in *N. galligena* (see Chapter 3), and a direct ITS sequencing approach, the aims of this study were to evaluate both species delineation and phylogenetic relationships between accepted members of the group, respectively. Strains of *N. radicicola*, a largely genetically uncharacterised ubiquitous soil borne pathogen with a wide host range, responsible for root rots of diverse plants (Gerlach, 1961; Booth, 1967; Domsch *et al.*, 1980), and occasionally recovered from bark lesions from upper parts of trees, were also included for comparison and relationship assessment.

Fungal rRNA operons, comprising functionally conserved genes coding for the 5.8S, small sub-unit (SSU) and large sub-unit (LSU) rRNA genes as tandemly repeated multi-gene arrays, are particularly attractive targets for phylogenetic studies, due primarily to the differing rates of evolution between the functionally conserved genes and associated non-coding spacer regions (Hillis and Dixon, 1991). Although not considered to evolve independently (Dover, 1986), a process of concerted evolution is proposed for sequence homogenization of these multi-array operons within sexually reproducing populations by either unequal crossing over or gene conversion events, or a combination of both (Dover, 1986). The systematic versatility of rRNA operon spacer regions within (ITS) and between (IGS) successive repeats have previously been shown to differ inter- (Harrington and Wingfield, 1995; Edel *et al.*, 1996), and sometimes, intra-specifically (Edel *et al.*, 1995; Appel and Gordon, 1995) where they have also been used in population analysis. The internal transcribed spacers, although transcribed, play no structural role in the mature ribosome. Under less evolutionary constraints, selection pressure to eliminate mutations is considered much less. However, it is increasingly clear that due to their relatively rapid

evolution ITS regions may vary among or within species (Bruns *et al.*, 1991), being phylogenetically versatile for assessing relationships at varying taxonomic levels. ITS sequence based phylogenies continue to be widely exploited across the fungal kingdom.

The much larger IGS, particularly the non-transcribed section, has by comparison shown considerable intra-specific variation in fungi (Spreadbury *et al.*, 1990; Vilgalys and Gonzalez 1990; Kim *et al.*, 1992; Appel and Gordon, 1995; Appel and Gordon, 1996) and other species (Hillis and Davis, 1988). Less variation occurs in the transcribed region prior to the small sub-unit gene (King *et al.*, 1993). The phylogenetic utility of the IGS region depends on the mechanisms by which it evolves (Appel and Gordon, 1996), but is still useful in species characterisation, especially identification, delineation and intra-specific variation.

In addition to the use of single primed arbitrary reactions (SPARs) such as randomly amplified polymorphic DNA (RAPDs), or mini or micro-satellite based primers, in population studies or epidemiological applications, these techniques also provide a useful approach both in species identification and in assessing genetic relationships between different fungal taxa (Meyer *et al.*, 1993; Tommerup *et al.*, 1995; Freeman and Rodriguez, 1995).

ITS-RFLP analysis employing the range of restriction endonucleases quoted in this study exhibited insufficient resolution in differentiating species. In ITS PCR-RFLP only a limited amount of sequence information is used and can be compounded by insertions and deletions, although a higher resolution can be achieved by using the complete sequence. By contrast, IGS-RFLP analysis, using eight restriction enzymes as specified, resolved all species, with a further degree of intra-specific variation (although, as previously mentioned, the phylogenetic utility of this region also depends on the mechanisms by which it evolves and is further compounded by insertions and deletions events). Similarly, both arbitrarily primed PCR analysis using a random decamer and PCR with the M13 derived 'core' sequence as primer (Stenlid *et al.*, 1994) grouped species in a consistent fashion, despite the fundamental

basic differences in mechanisms of molecular change being exploited in each approach. Overall, results were broadly consistent between each method allowing presentation in combined formats (except for ITS PCR-RFLP data which exhibited relatively few polymorphic bands overall). From the results obtained, molecular characterisation via PCR fingerprinting and rRNA spacer RFLP analysis are clearly useful and informative approaches to species identification and delineation in the isolates of *Nectria* species studied here. However, the wider separation of the *N. galligena* isolates from the rest of the Coccinea group, including *N. ditissima* is unclear, particularly where ITS sequence phylogeny (and ITS PCR-RFLP data) grouped them more closely. This may be an anomaly of marker data integration from more than one electrophoretic separation of restriction or arbitrarily primed profiles of the different species studied. However, all data sets were generated from the results of multiple electrophoretic separations, comprising different combinations of species in each separation. Further, each amplification product, or PCR restriction fragment, from each separation was routinely sized as described in point 6.2.7. as standard.

Such observations, including the nature of the mechanisms of molecular changes exploited by each marker method, coupled with the extent and type of genetic material assessed with these methods, although useful in species and intra-specific characterisation, limit their usefulness in phylogenetic applications. Polymorphisms with random oligomer primers are widely considered to result from insertion, deletions, inversion or nucleotide substitutions disrupting or rearranging primer annealing sites or by giving measurable length variation in the target DNA between primer sites, whereas the M13 derived primer is considered revealing variation between the distal ends of closely placed short tandem repeat sequences. Neither approach guarantees amplification of identical loci for direct comparison. Further, only a limited amount of sequence information is exploited in direct ITS-RFLP where the pattern of variation can be again compounded by random insertion or deletion events. Similarly, in the much larger IGS, differences are also attributed to insertion or deletions in sub-repeat arrays (Appel and Gordon, 1996), thought to result from unequal crossing over events (Dover, 1986; Hillis and Davis, 1988). Co-migrating bands from different species have previously been shown to be non-homologous

(Martin and Kistler, 1990), indicating that comparison of fragment size alone may not be useful for estimation of evolutionary change, but may be useful, as previously discussed, in species delineation and identification as experienced here. This is similar to views held by others (e.g. Felsenstein, 1993; Vandongen, 1995) who maintain the general unsuitability of marker data for phylogenetic studies and the irrelevance of statistical analysis of confidence limits of resultant dendrogram branches by bootstrapping, as they are generally considered not to satisfy the assumptions central to such calculations. However, each dendrogram produced from the different types of marker data generated in this study revealed no change in topography when subjected to such analysis.

Based on ITS sequence phylogeny, representative species of the *Nectria Coccinea* group form a relatively closely related monophyletic group to the more variable *N. radicicola*. Although the Coccinea group taxonomy is more recently considered 'unnatural' or 'artificial' (D. Brayford, CABI Bioscience, personal communication, 2000, concerning discussions with Booth) these results support the contemporary classification of these species based on perithecial anatomy and spore morphological criteria as proposed by Booth (1959, 1966), establishing a distinct evolutionary association between members of the group. However, the relationship with the more variable, perhaps more evolutionary older soil borne *N. radicicola* is less clear. Based on the genetic distances calculated (see **Table 6.3.**) both *N. galligena* isolates (IMI 378754 and IMI 378755) are more closely related to *N. cinnabarina* than *N. radicicola* strains (IMI 376409 and 94-1685) (0.1376 and 0.1374, and 0.1511 and 0.1401, respectively), and that both *N. punicea* appears almost equi-distant from *N. cinnabarina* than *N. radicicola* strain 94-1685 (0.1538). This may suggest that *N. cinnabarina* is not a suitable genetically distinctive out group and further suggests that interpretation of the topography of the phylogenetic tree should be made with care. However, supporting this phylogeny, arbitrary primed fingerprint and IGS PCR-RFLP characterisation appear consistent with the distribution and relatedness of each species and strain studied. Again, a feature of these analyses is the greater level of intra-specific variation observed in strains of *N. radicicola* than that observed inter-specifically for taxa representative of the *Nectria Coccinea* group. The *N.*

radicicola ITS data presented here is consistent with the ITS variability observed by Hamelin *et al.* (1996), reporting the existence of three ITS variants (representatives included in this study) from analysis of 11 strains isolated from *Pinus* and *Picea* spp. from across Quebec. Although the *N. radicicola* strains used here were obtained from a much wider host range and geographic origin than in the study of Hamelin *et al.* (1996) no direct correlations were apparent with the disparity of molecular data (including the fingerprint and PCR-RFLP analysis) remaining unclear. A much wider survey, employing a greater number of strains than the 12 used in this study would be required to investigate this further.

Levels of intra-specific divergence appear much greater in species exhibiting a particular broad host range e.g. *Colletotrichum gleosporioides* and *C. acutatum* (Screenivasaprasad *et al.*, 1996), *Alternaria solani* (McKay *et al.*, 1999) and in soil borne pathogens e.g. *Fusarium sambucinum* (O'Donnell, 1992) and *Rhizoctonia solani* (Kuninaga *et al.*, 1997), although Hamelin *et al.* (1996) observed no sequence divergence within the ITS of 12 strains of *Cylindrocladium floridanum* isolated from *Pinus* and *Juglans* roots from different nursery sources across Quebec. However, the range of variation observed within the *N. radicicola* data sets presented here, particularly the ITS divergence, suggests either continuous species variation or the existence of a sub-species organisation such as a species complex, possibly comprising a number of inter-sterile mating populations as observed in *N. haematococca*. Evidence for the latter interpretation would require confirmation via conventional genetic appraisal through classical inter-strain matings. However, in support of this, Samuels and Brayford (1990) recorded considerable variation in cultural and morphological characteristics of *N. radicicola* collections made from different host species from New Zealand, Indonesia and Venezuela, formally characterising several new varieties and discussing the existence of further variants.

That *N. radicicola* is phylogenetically ancestral to the Coccinea group, based on ITS sequence analysis, it is conceivable, or indeed possible, that Coccinea group species may retain hitherto unrecognised evolutionary aspects of the *N. radicicola* life cycle or habit(s) that may be of unobserved epidemiological or pathogenic significance,

despite their recorded levels of host specialisation/associations (Booth, 1959). This may be noteworthy to the results of Chapter 2, where infected rootstocks have been implicated in the commercial dissemination of *Nectria* canker in young apple trees. Rootstocks are intensively propagated in stool and layer beds, spending much of the process earthed up. The ability of *N. galligena* to both reside in, and infect from, soil could explain a hitherto unrecognised source of inoculum capable of contaminating propagating stocks. However, although informative, the relatively comparative range of genetic distances observed between all Coccinea species and *N. radicicola* strains with *N. cinnbarina* suggests this species may not be sufficiently genetically distinct for use as an out group despite its 'type species' status and that alternative species should be sought. Inclusion of sequence data representative of additional taxa, covering the wider circumscription of the genus, would further assist in refining phylogenetic resolution and revealing clearer evolutionary relationships amongst these species.

Nectria radicicola is a ubiquitous soil-borne pathogen with a wide host range, including apple and pear, as well as a number of hardwood tree species. More recently this pathogen has been found responsible for a serious necrosis of vine rootstock in France ('*Pied noir*') (Dumot *et al.*, 1999) and on Walnut in Italy (Montecchio and Causin, 1995). Experimental trials have implicated *N. radicicola* as a potential component of apple replant disease in Nova Scotia (Braun, 1991; 1995) (the identity of the causal organism of these experiments were established through both morphological and molecular evaluation as outlined in Chapter 4), and has been frequently isolated from apple roots in orchards grown on non-fumigated soils there (Braun, 1991). In parallel studies at Wye College, *N. radicicola* has been frequently recovered from symptomless apple rootstock material (M. Lolas, Universidad de Talca, Chile, personnel communication, 1998). Such observations suggest the infection of apple propagating material by this species is more common than previously considered. By contrast, two *N. radicicola* strains (IMI 375717 and IMI 376408) used in this study were originally isolated from branch bark lesions from *Malus* and *Arbutus*, respectively. These unusual isolations further highlight our

dearth of primary biological or epidemiological knowledge of these species and the type and extent of their habit ranges.

Nectria coccinea, *N. coccinea* var. *faginata* and *N. fuckeliana* are each defined as heterothallic taxa (Booth, 1966; Parker, 1976; Cotter and Blanchard, 1978) whereas *N. galligena* has been confirmed as both hetero- and homothallic by independent studies by Kruger (1974) and El-Gholl *et al.* (1986) (and earlier Lortie, 1964), complicating any degree of informative phylogenetic inference on the sexual characteristics of this species. Molecular data from Chapter 3 supports the retention of *N. galligena* as a single relatively homogenous species. However, isolates studied by Kruger (1974) were exclusively from apple (predominantly from Germany) while isolates studied by Lortie (1964) and El-Gholl *et al.* (1986) were derived from a range of hardwood species from North America. These observations suggest any further specialisation in the genetics of *N. galligena* may also be a recent phenomenon, although it is unclear whether the results of these researchers, like the nature of intra-specific variation characterised in *N. galligena* in Chapter 3, are based on host preference or geographic origin.

Despite distinct monophyly in representative species of the *Nectria* Coccinea group exhibited here, sufficient inter-specific sequence divergence exists within both ITS regions for the possible design of taxon-specific primer pairs, including sufficient divergence between *N. galligena* and *N. ditissima*, where the level of sequence divergence was 0-0.7% and 3.8% for ITS 1 and 2, respectively (despite *N. ditissima* being considered a non-pathogen of rosaceous hosts). Similarly, despite much wider ITS divergence observed in *N. radicola* strains examined, conserved intra-specific sub-regions in both ITS 1 and 2, distinct from other characterised *Nectria* spp. ITS sequences were observed. The level of inter-specific ITS sequence divergence, but localised intra-specific conservation in both regions in all strains, appears sufficient for the design of further diagnostic primer pairs, including ITS exclusive nested primer pairs, a similarity of 92%, whereas the RAPD analysis gave a similarity value of just 8%. Cluster analysis of the combined X11 and in addition to those of Hamelin *et al.* (1996).

Additional taxa, covering the wider circumscription of the genus, including additional ITS sequences from other authenticated species (e.g. *Fusarium* spp., and *Calonectria* spp.) will be sampled in the future in order to increase phylogenetic resolution, furthering our understanding of the evolutionary biology of this genus and evaluation of morphological criteria and biological function.

Chapter 7. General Discussion

7.1 Epidemiology

The current epidemiological model for the spread of *Nectria* canker is based on the pioneering work of Wiltshire (1921, 1922). This model makes the assumption that each lesion is the result of separate infection events and that these are initiated by the arrival of external inoculum, disseminated by rain or wind from neighbouring hosts, onto a susceptible surface such as a freshly exposed leaf scar. Whilst this model continues to inform current control strategies (Xu and Butt, 1994) there are reasons for concluding that it is incomplete. For example, the model fails to account for the intensity and non-random distribution of infection particularly in young orchards planted in sites remote from sources of inoculum (unpublished observations; T. Swinburne, Wye College, personal communication, 1994; D. Rosenberger, Cornell Hudson Valley Laboratory, personal communication, 1996). Additionally, some growers report that the application of protective fungicide sprays in autumn and spring fail to achieve the measure of control that the model would predict (Lovelidge, 1995).

These observations led Li (1995) and Brown *et al.* (1994) to attempt to identify the source of inoculum leading to severe infections in young plantations. Using morphometric analysis and RFLP techniques they concluded that at least some orchard infections could be linked to the nurseries in which the trees were propagated, implying that infection *per se* occurred at one or more of the production stages. Bennett, working at East Malling Research Station (now Horticulture Research International, East Malling) in the 1960's had reached a similar conclusion, and obtained a reduction in the number of maiden trees with canker by treating the bud sticks with benomyl prior to grafting (quoted in Howard *et al.*, 1974).

If nursery infection is indeed the origin of some cankers, which appear in young orchards 1-5 years after planting out, then this carries two further implications. Firstly, the period of latency must be much longer than hitherto envisaged, i.e. several months (Crowdy, 1949). Secondly, those cankers developing on branches

distal to the first years growth from such infections must be the result of inoculum carried asymptomatically within the tree. Evidence that *N. galligena* can be present within the vascular system of branches without inducing symptoms has been obtained using direct isolation techniques (Li, 1995), immunofluorescent microscopy (Dewey *et al.*, 1995), and PCR detection, as reported in this thesis, from otherwise healthy looking tissue.

The research described in this thesis was undertaken as part of a larger collaborative programme, with the intention of confirming whether or not infection in the nursery can lead via a symptomless phase to disease in the orchard. To help address this objective, two further aims included the development of molecular research tools for *N. galligena* strain discrimination and the detection of symptomless infection in apple trees. A phylogeneic appraisal of the *Nectria Coccinea* group based on ITS sequence analysis, was also conducted.

7.2 Confirmation of latent infection

The Aylesham and Budding Trials (Chapter 2) both provided additional confirmation that at least some part of the initial infection observed in young orchards can originate in the nursery. The data from the Aylesham Trial showed that there were significant differences between the percentage of infected trees propagated in some of the nurseries. Whilst this may reflect differences in the frequencies of initial, latent infection in trees received from different nurseries in the first year, other explanations are possible. For example, the rootstocks or scions used in different nurseries to produce maiden trees may have clonal variation affecting resistance or susceptibility. Additionally, there may have been variation in vigour between the batches of trees produced by the different nurseries, again affecting resistance. Ideally, the results obtained would have been confirmed by the molecular typing methods developed in Chapter 3 to compare the isolates from the trial. In the event, poor recovery of isolates from infected wood prevented this, and the apparent relationship between molecular type and nursery of origin depended on the data from one nursery alone, which cannot provide convincing proof. Indeed, the presence of more than one molecular type from isolates from trees from most nurseries could indicate that there

were external sources of inoculum in this orchard or several types within the nursery of origin. Interpretation of such data is made even more difficult by the not infrequent practice of budwood and/or rootstock exchange by commercial nurseries.

To control the variables which hampered the Aylesham trial, current trials have overseen the deliberate exchange of rootstocks and budwood between collaborating nurseries, and the trees produced have been planted at sites in one of three UK apple growing regions. This should have the merit of both estimating the frequency of infection within the nurseries (at least for the year in which the experiment was begun) and identifying the location of the initial infection court within the tree using the molecular typing techniques developed here. The Budding trial described here provided strong evidence that the rootstocks produced by different nurseries contain symptomless infection which can be 'transmitted' later to the developing scion. Further evidence implicating the rootstock as the site of initial infection comes from experiments conducted in collaboration with Dr. B. Howard (Horticulture Research International, East Malling), in parallel with the experiments described in this thesis. The trial is not yet concluded, but preliminary results indicate that the application of relatively small numbers of conidia to scars made by removing side buds from rootstocks prior to budding leads to the development of cankers on the scions two, three and up to four years later.

If further experiments confirm that nursery infection contributes significantly to the frequency of disease in the orchard, then this may enable the development of new control strategies. An obvious way of achieving this would be through certification procedures comparable to those of that have largely eliminated virus infection from fruit trees under the EMLA scheme. This will require a reliable method of diagnosis and detection of infection in the absence of overt symptoms. Such techniques would also facilitate the identification of the route by which infection become established during the propagation process.

7.3 Molecular detection

Of the diagnostic methods tested so far, direct isolation of *N. galligena* in the absence of a selective medium, and the substantial microflora present in apple wood, was found to be too difficult (Chapter 4; Li, 1995). In the experiments reported here, it was also found difficult to obtain isolates from older lesions, which contained many secondary organisms. The monoclonal antibody method developed by Dewey *et al.* (1995) was also tested in the earlier course of work at Wye College, but was found to lack the necessary sensitivity or specificity to reliably detect the pathogen in woody tissues. These preliminary experiments indicated that this was due to cross reaction with wood or microfloral compounds which interfered with the specificity of the monoclonal antibody used.

To overcome these difficulties attempts were made to develop a rapid, species-specific PCR method coupled with methods for the preparation of total DNA from woody tissue. Two primers, specific to *N. galligena*, were designed from analysis of sequence divergence of the ITS spacers between *N. galligena* and closely related species. Under stringent PCR conditions, primers Ch 1 (5'-AAC CCC TGT GAA CAT ACC CAT C-3') and Ch 2 (5'-GTG GCC GCG CTG CTC TTC CG-3') amplified a 412 bp fragment specific to *N. galligena* DNA. Coupled with methods for the preparation of total DNA from woody tissue, these developments led to the formulation of an assay for the detection of target *N. galligena* DNA from infected lignified host tissue. However, compounds inhibitory to the PCR compromised the reliability and sensitivity of the assay, a feature commonly encountered in many PCR-based plant disease diagnostic procedures. For this reason a 500 bp heterologous internal standard, incorporating identical specific primer recognition sites, was developed for inclusion in individual reaction tests as an internal positive control to help discriminate against false negatives due to inhibition.

However, failure of the internal standard to amplify in routine applications, and occasionally with heavily infected cankered material, indicated high levels of inhibitory compounds. Further, this approach did not appear sensitive enough to detect fungus beyond an infected lesion, where internal infection, presumably at

much lower levels, was suspected. Such limitations were effectively circumvented by the incorporation of a further purification step based on magnetic capture hybridisation technology. Employing this approach, target DNA is immobilised, and effectively separated from a total DNA extract via solution hybridization with a highly specific, single-stranded biotinylated probe (designed to the highly conserved ITS 1 region of *N. galligena*), conjugated to streptavidin coated paramagnetic beads. The bead-probe conjugate and bound DNA assemblage are immobilised magnetically, with non-target extraneous DNA and unwanted co-precipitated compounds, including PCR inhibitors, effectively removed by simple washing steps (simultaneously concentrating target molecules). Target elute can then be used directly in subsequent conventional PCR diagnostic reactions. As the results showed a 10-100 fold increase in sensitivity for DNA from the fungus in culture and for samples from woody tissues, including from asymptomatic tissue, this represented a significant improvement in PCR sensitivity and reliability. Overall, the incorporation of this purification step should greatly reduce or exclude the occurrence of false negatives arising from the presence of inhibitory compounds. Theoretically, the protocols developed here will enable routine screening of nursery material to assess the frequency of symptomlessly infected stocks.

Li (1995) indicated that symptomless infection is not continuously distributed, but also that recovery frequency is seasonally dependent. If, as has already been discussed, the pathogen is carried passively within the xylem, it will be essential to identify the optimal time within the season and the most likely location within tissues to sample to give the best chance of detection. However, due to increased sensitivity, a degree of caution in respect of the interpretation of the PCR results to the final expression of the disease, within the productive life span of the individual tree, would be required. The 10-100 fold increase in sensitivity offered by incorporation of magnetic capture hybridisation technology may allow detection well below the expression threshold of symptomless infection. As no data exists on the level, or levels, of disease threshold of such infection, this would require evaluation. The use of Spartan budwood, from residual trees planted out from the Budding Trial (Chapter

2), and which showed no symptoms of canker expression after several seasons, could be used as indicator material for this purpose.

7.4 Population structure and possible centre(s) of origin of *N. galligena*

Although developed for the evaluation of intra-specific variation in *N. galligena* for molecular ecological applications, interpretation of the population structure of the global selection of isolates used in this study, remains unclear. One interpretation, that distribution fits a geographical structure is supported by the closer genetic association of rosaceous derived isolates, regardless of their geographical origin, than those derived from non-rosaceous hosts. This could be explained by large scale global dissemination of the disease, most likely through the inter-continental movement of infected propagating stocks. As all temperate growing regions of the world record the incidence of the disease, particularly those heavily planted during periods of colonisation from the old world, this tends to support this possibility. An alternative explanation, that the population structure is based on host preference, is also inconclusive.

Although *N. galligena* is polyphagous, results from detailed sequence and PCR-RFLP characterisation of the rRNA ITS, 5' end LSU and unlinked loci, of representative global isolates support the retention of this taxon as a single, relatively homogenous species. Only *Fraxinus* derived isolates are considered host specialised to some degree, but based on the data presented here, such specialisation is probably only a relatively recent phenomenon. No reports exist for host specialisation of any other rosaceous or non-rosaceous (hardwood) derived isolates.

The distribution of *Nectria* canker in, for example, North America, may have resulted from the cross infection of native hardwood species from contaminated propagating stocks acting as a source of inoculum, introduced from, presumably, the old world. However, this is difficult to comprehend from the present extent of hardwood infection across the entire North American continent within the relatively short period since the start of modern colonisation. Alternatively, the much greater level of intra-specific variation observed in isolates derived particularly from North American

hardwood hosts (in particular *Fagus*, *Betula* and *Acer*) than isolates obtained from other rosaceous hosts, predominantly of European provenance, could represent a much greater evolutionary age indicative of the centre of origin of this species. This is consistent with the general views of Mahoney *et al.*, (1999) and Bernier (L. Bernier, Universitaire Laval, Quebec, personal communication, 1997) who considered that, despite its recognition as a problem on apple in Europe for centuries (Swales, 1921), *N. galligena* is indigenous to North America. Previously, *Nectria* cankers' long association with apple has led to the general assumption that its centre of origin is shared with that of the modern concept of domestic *Malus*, currently considered to be in the region bordering North Western China and modern day Kazakhstan (B. Juniper, University of Oxford, personal communication, 2000).

Although the introduction of contaminated propagating apple stocks to North America since commencement of colonisation remains possible, the alternative interpretation, evolution on hardwood hosts in North America and subsequent spread to rosaceous hosts such as apple and pear, which the data also suggests, is also plausible. However, how *N. galligena* may have left the North American continent before the intervention of man is unclear. This may have been achieved through much earlier spread on a suitable host or hosts (*Malus* or hardwood species) west from North America via an inter-continental land bridge in the region of the Bering Straits to Eurasia. Alternatively, a single Eurasian origin, exhibiting a bi-directional migration both west across the Urals and east, again across the region of the Bering Straits, may have been responsible. Inclusion of additional isolates derived from European hardwood hosts, rather than almost exclusively apple in this study, would help clarify this latter possibility.

A much larger global sample, coupled with much greater variation in isolate host and geographic origin, in particular from Asia and the Far East, may help resolve the global origin of this species. Such information may be useful in both hardwood and top fruit tree improvement breeding programmes and an increased understanding of the variability of the pathogen for improved management procedures.

7.5 Phylogeny

In view of the results of the experimental trials, and questions regarding the significance of spore mobility and the susceptibility of infection courts, it is still unclear as to how propagating nursery stocks, in particular rootstocks as implicated in this research, may become initially infected. A hitherto unconsidered infection source, that *N. galligena* could be infecting propagating stocks, particularly rootstocks, from the soil was examined *via* phylogenetic inference, comparing rRNA ITS sequences from related Coccinea group species and *N. radicicola* strains. Application of both PCR based marker techniques developed in this study clearly delineated each representative species studied. Further, taken with ITS sequence phylogeny, these analyses supported the contemporary classification of Coccinea group species as defined by Booth (1959, 1966), previously considered as 'artificial' (D. Brayford, CABI Bioscience, personal communication, 2000, concerning discussions with Booth). The latter analyses further established a distinct evolutionary association between each group species. This approach was extended to include representative strains of soil borne *N. radicicola*. Although none of the Coccinea species as outlined by Booth (1959, 1966) are considered soil borne (Brayford, 1992), phylogenetic appraisal, based primarily on ITS sequence analysis, suggests *N. radiciola*, as a continuously variable species or species complex, may be ancestral to the Coccinea group. Further, such a relationship may infer a degree of biological function, in particular, the ability to reside in, and infect from soil (as in the case of *N. radicicola*). Inclusion of additional taxa, covering the wider circumscription of *Nectria*, including *Fusarium* and *Calonectria* species, should permit improved phylogenetic resolution and help further our understanding of the phylogeny and evolutionary biology of this genus, including further assessment of possible biological function(s). If *N. galligena* were capable of the ability to infect from soil, this would have implications for the control of the disease in the orchard. For example, despite recommendations to the contrary, mulching of pruning debris, including fallen infected fruits, still a common practice in many commercial plantations, may serve as a ready source of inoculum on a continually seasonal basis. This may render replanting of previously productive orchard land as impractical, in addition to the phenonemon of apple replant disease. More importantly, this would

have a number of further implications for contaminated nursery propagated stocks, in particular the need for their elimination from production for effective control, relying on the methods of detection as discussed earlier.

Research papers based on chapters 2, 3, 4 and 5 submitted for publication

Langrell, S. R. H., D. J. Barbara, and T. R. Swinburne. (1998). Molecular detection and quantification of *Nectria galligena* from apple wood, abstr. 3.3.50. *In* Abstracts of the 7th International Congress of Plant Pathology. International Society for Plant Pathology.

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