

Actinorhizal symbioses: diversity and biogeography

David R. Benson, Brian D. Vanden Heuvel and Daniel Potter

6.1 Introduction

The actinobacterial genus *Frankia* encompasses sporulating filamentous bacteria (actinomycetes) that fix N_2 ; they are defined by their ability to induce N_2 -fixing root nodules on a broad range of 'actinorhizal plants'. Actinorhizal plants, in turn, are defined by their ability to form root nodules when in symbiosis with *Frankia*. Within the root nodule, *Frankia* fixes nitrogen that is transported to the host plant in amounts sufficient to supply most of the plant's nitrogen requirements. This symbiosis allows actinorhizal plants to invade and proliferate in soils that are low in combined nitrogen. Although similar in outcome, the symbiosis differs markedly from the rhizobium–legume symbiosis. The overall nodule architecture more closely resembles a foreshortened lateral root rather than a unique plant organ, and the plants have evolved a variety of mechanisms to modulate the levels of free O_2 that would otherwise inactivate nitrogenase (Benson and Silvester, 1993). In common with legumes, however, the plants belong to the 'nitrogen-fixing Clade' within the Rosid I lineage initially described by Soltis *et al.* (1995).

Since the first successful and confirmed isolation of a *Frankia* strain in 1978 (Callaham *et al.*, 1978), many studies have addressed the diversity and distribution of *Frankia* strains in root nodules, and some have dealt with the biogeographic distribution of strains and plants. It has become clear that the existing biogeographic patterns of *Frankia* strain distribution can be viewed as resulting from adaptation by both plants and *Frankia* strains within a geographic mosaic of environments developed over millions of years. To sort out factors that control the distribution of frankiae, one must know the host ranges of strain groups, the richness (number of unique strains) and evenness (representation of each unique strain) components of strain diversity in nodules in nature and the geographical distribution of both plants and frankiae.

This chapter focuses on the broad patterns of *Frankia* strain distribution and diversity as they relate to host plant distribution across a geographical mosaic of environments. It begins with some of the issues that arise in studying the biogeography of the symbiosis, followed by a brief overview of the phylogenetic relationships among actinorhizal plants and among *Frankia* strains. Finally, information will be presented concerning the biogeography of the symbioses, and the diversity of *Frankia* strains that participate in symbiosis in each plant family. The chapter will conclude with a discussion of basic principles that are emerging.

6.2 Practical aspects of studying *Frankia* strain diversity

Several issues must be considered when discussing *Frankia* strain diversity and distribution in natural environments in relation to the plant hosts. These include but are not limited to local patterns of strain distribution in soils, including strain dominance and response to edaphic factors, regional patterns of plant and microbe distribution and global patterns imposed on the plants and microorganisms by climatic and geological changes. Patterns of symbiotic compatibility between plants and microorganisms are a function of the natural distribution of both partners across a geographical mosaic of environments (Benson and Clawson, 2000).

While patterns of diversity and distribution do occur, and can be identified with some effort, conceptual difficulties arise when studying the biogeography of actinorhizal symbioses. First is the problem, common to bacteriological studies, of defining a *Frankia* strain. A variety of markers have been used to study the ecological diversity of *Frankia* strains but at different levels of resolution (reviewed in Benson and Silvester, 1993; Schwencke and Caru, 2001). These markers range from simple phenotypic traits like sporulation within nodules to protein pattern and isoenzyme analysis to PCR-RFLP and DNA sequencing. Most recent studies have used the variability of 16S rRNA genes amplified by the PCR from isolates and nodules (for example, Benson *et al.*, 1996; Clawson and Benson, 1999a, b; Clawson *et al.*, 1997, 1998; Ritchie and Myrold, 1999), or PCR-RFLP patterns of variable intergenic regions of *nif* or rRNA genes present in nodules (for example, Jamann *et al.*, 1992, 1993; Lumini *et al.*, 1996; McEwan *et al.*, 1994; Rouvier *et al.*, 1996; Simonet *et al.*, 1991), or repetitive extragenic palindromic-PCR (Rep-PCR) (for example, Jeong and Myrold, 1999; Murry *et al.*, 1997). The resolution of these latter approaches is limited by the variability in the DNA used for analysis. However, it is possible to organise strains into closely related groups that are presumed to share more biological similarity within groups than between more distantly related groups.

A second difficulty is certifying that a compatible organism is absent or even present in a complex soil population. Every strain cannot be everywhere but proving that point can be difficult. Direct detection by isolation is complicated by the slow growth of *Frankia* strains on bacteriological media (10–14 days) and by their low number relative to other bacteria in the soil (Baker and O'Keefe, 1984). Therefore, *Frankia* strains have been detected, and populations assessed, by bioassay and, more rarely, by direct PCR amplification of *Frankia*-specific genes from soil. Much of this work has been reviewed previously (Benson and

Silvester, 1993; Hahn *et al.*, 1999; Lechevalier, 1994; Schwencke and Caru, 2001; Wall, 2000).

Bioassays are performed by diluting soil samples, inoculating plants, and then calculating nodulation units based on the number of nodules formed on plants per gram or cm³ of soil. The unavoidable difficulty with this type of approach is that it underestimates the number of frankiae present in the soil since only those strains capable of infecting the test plant and that actually encounter an infectible zone on the root and then form a root nodule are counted. In addition, different plant species, even within the same genus, may yield different estimates depending on their susceptibility to the local *Frankia* strains (see, for example, Huss-Danell and Myrold, 1994; Mirza *et al.*, 1994(Q7)). Nevertheless, within limits, such an approach allows comparative estimates of the number of strains in soil to be made. Estimates of frankiae populations using PCR methods have yielded some promising results (Myrold and Huss-Danell, 1994; Picard *et al.*, 1992), but the low population levels of frankiae in most soils and the questionable specificity of the primers used for analysis have limited the broad application of this approach (Normand and Chapelon, 1997).

The final problem is one of significance. That is, even if strains are defined with sufficient resolution and their geographic distribution is described, their metabolic contribution to the geographic mosaics in which they live, and their attributes that promote their distribution within the mosaic may not be obvious. The contribution of an individual bacterial strain to the environment under study is difficult to assess unless it is observable and quantifiable. To some extent, the problem of significance is less acute in the case of nitrogen-fixing symbioses where a higher organism chooses bacterial strains that are best suited to enter the symbiosis in the environment under study. Their function is known and at least part of their contribution to the soil economy can be quantified.

6.3 Taxonomy and phylogeny of actinorhizal plants and *Frankia*

6.3.1 Actinorhizal plant phylogeny

According to current taxonomy, actinorhizal plants are classified in eight families (Table 6.1). They are widely distributed, found on all continents except for Antarctica, and are a diverse group of mostly woody dicots (Table 6.1) (Baker and Schwintzer, 1990). Most members are found in temperate zones, with only a few members being found in tropical environments and a few in Arctic environments (Table 6.1). Ecologically, actinorhizal plants are usually pioneers on nitrogen-poor soils, and are frequently found in relatively harsh sites, including glacial till, new volcanic soil, sand dunes, clear cuts, and desert and chaparral (Schwencke and Caru, 2001).

Traditional taxonomic treatments suggested that actinorhizal plant families were at most only distantly related, classified in four of the six major angiosperm subclasses as delimited by Cronquist (1981) (Table 6.1). This morphological classification suggested that the actinorhizal symbiosis had evolved many times in angiosperm evolution (Mullin *et al.*, 1990). A dramatic shift in this view occurred

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Table 6.1 Classification of actinorhizal plants^a

Subclass ^b	Order ^c	Family	# nodulated genera/total # of genera ^d	Genus	Distribution of genus ^e
Hamamelidae	Fagales	Betulaceae	1/6	<i>Alnus</i>	n. temperate, higher elevations in C. and S. America, n. Africa, Asia
		Casuarinaceae	4/4	<i>Allocasuarina</i> <i>Casuarina</i> <i>Ceuthostoma</i>	Australia Old World tropics Philippines, Borneo, New Guinea
		Myricaceae	2/3	<i>Gymnostoma</i> <i>Comptonia</i> <i>Myrica</i>	Malaysia to W. Pacific e. N. America nearly cosmopolitan (not Mediterranean, Australia)
Rosidae	Rosales	Elaeagnaceae	3/3	<i>Elaeagnus</i> <i>Hippophae</i> <i>Shepherdia</i>	Europe, Asia, N. America temperate Eurasia N. America
		Rhamnaceae ^f	7/55	<i>Ceanothus</i> <i>Colletia</i> <i>Discaria</i> <i>Kentrothamnus</i> <i>Retanilla</i> <i>Trevoa</i> ^g <i>Cercocarpus</i> <i>Chamaebatia</i> <i>Dryas</i> <i>Purshia</i> ^h	N. America, esp. California s. S. America s. S. America, Australia, New Zealand S. America (Bolivia, Argentina) S. America (Peru, Chile) S. America (Andes) w. N. America California, Baja California circumboreal, arctic – alpine w. N. America
Magnoliidae	Cucurbitales	Coriariaceae	1/1	<i>Coriaria</i>	Mexico to S. America, w. Mediterranean
Dilleniidae		Datisceae	1/1	<i>Datisca</i>	w. N. America, s. Asia

^a Compiled after Baker and Schwintzer (1990), Swensen (1996), Benson and Clawson (2000), and Schwencke and Carú (2001).

^b According to the classification of Cronquist (1988).

^c According to the classification of the Angiosperm Phylogeny Group (1998); all of these orders fall in the 'Eurosoid I' group of eudicots.

^d Number of nodulated genera over the total number of described genera in the family

^e Compiled from Mabberley (1988) and from the International Plant Names Index (www.ipni.org).

^f *Adolphia* may be actinorhizal, but has not been confirmed (Cruz-Cisneros and Valdés, 1991).

^g *Talguenea* should be combined under *Trevoa* (Tortosa, 1992).

^h *Purshia* and *Cowania* have been combined under *Purshia* (Henrickson, 1986).

with the publication in 1993 of the first extensive molecular phylogeny for angiosperms using sequences from the chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) (Chase *et al.*, 1993). This phylogeny placed all actinorhizal angiosperms in the 'Rosid I' Clade, later termed 'Eurosoids I' (Angiosperm Phylogeny Group (APG) (1998); *Figure 6.1*). Furthermore, the two families in which symbiotic relationships with *Rhizobium* and related bacteria occur, Fabaceae (containing the legumes) and Cannabaceae

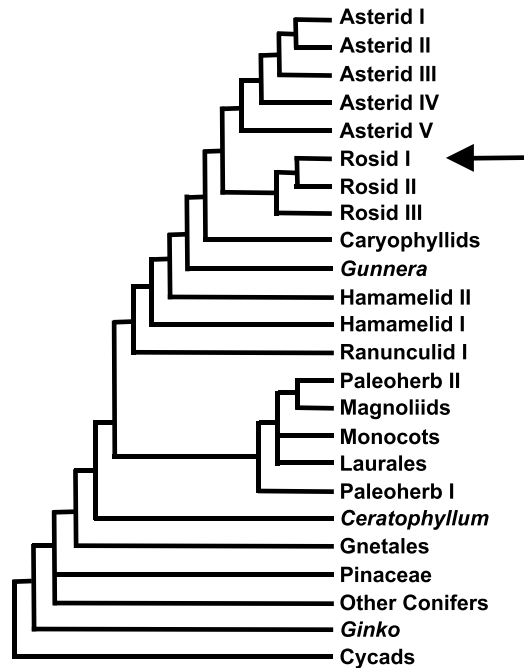


Figure 6.1. A summary of the major Clades recovered in the strict consensus of 3900 most parsimonious trees based on an alignment of 499 rebel sequences representing angiosperm diversity. Adapted and used with permission from the annals of the Missouri Botanical Gardens (Chase *et al.*, 1993). The arrow highlights the Rosid I Clade where all nitrogen-fixing plants, including all actinorhizal and rhizobial plants occur.

(in which only members of the genus *Parasponia* engage in symbiotic nitrogen fixation with rhizobia), are also included in this Clade. This clustering led to the suggestion that the predisposition to form symbiotic nitrogen fixing root nodules may have evolved only once in flowering plants (Soltis *et al.*, 1995).

Later authors examined the phylogenetic relationships of actinorhizal plants in more detail, and provided more rigorous analyses of the origin and evolution of the actinorhizal symbiosis (e.g. Jeong *et al.*, 1999; Roy and Bousquet, 1996; Swensen, 1996; Swensen and Mullin, 1997). In addition, the phylogenetic utility of other markers for resolving relationships among families of angiosperms has been investigated over the last decade. These markers include the 18S ribosomal RNA gene of the nuclear ribosomal DNA repeat and the chloroplast-encoded ATP synthase beta subunit (*atpB*) gene (Soltis and Soltis, 2000). These additional molecular markers have allowed independent assessments of the phylogenetic relationships of the actinorhizal families as well as simultaneous analysis of data from multiple genes representing two cellular compartments.

A maximum parsimony tree of combined data from all three loci is presented in *Figure 6.2*. This treatment places the actinorhizal taxa into three well-supported subClades within Eurosids I. These three subClades have been recognised taxonomically. They are designated as Rosales, Fagales and Cucurbitales (APG, 1998). They contain, respectively, the actinorhizal members of the Rosaceae,

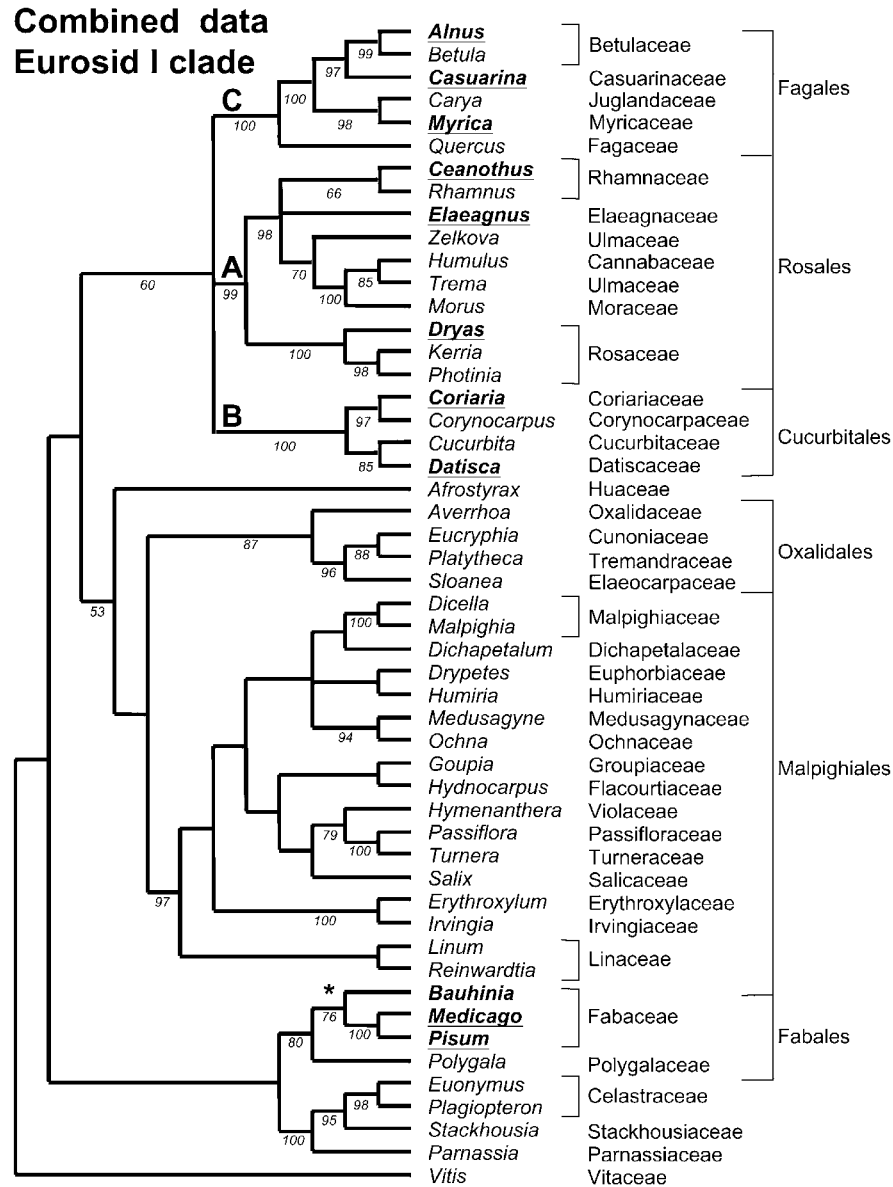


Figure 6.2. A strict consensus of the eight most parsimonious trees based on the combined data from the *rbcL* (1394 aligned basepairs), *atpB* (1520 aligned basepairs), and nuclear ribosomal 18S small subunit (1828 aligned basepairs) for 51 taxa representing all major families in the Rosid I Clade (see Figure 6.1). The numbers found below the branches represent bootstrap support from 100 replications. Taxon labels in bold and underlined represent the actinorhizal and rhizobial taxa. The family and order for each taxon in the tree can be seen to the right based on the Angiosperm Phylogeny group (1998) designations. Clades A, B and C highlight the three major Clades that include actinorhizal taxa. The asterisk denotes the rhizobial Clade. Clades A and C correspond to the same Clades identified by Soltis *et al.* (1995) while Clade B was identified as Clade D in their publication.

Rhamnaceae and Elaeagnaceae; those of the Betulaceae, Casuarinaceae and Myricaceae; and those of the Datisceae and Coriariaceae (Table 6.1, Figure 6.2). Maximum parsimony trees based on data from both chloroplast loci yield the same three Clades but trees based on the 18S data alone provide weak resolution within Eurosid I and place the actinorhizal taxa in four Clades, resulting in polyphyletic Rosales and Cucurbitales. The discrepancy between topologies may reflect different evolutionary histories for the nuclear and plastid genomes due to past hybridisation or lineage sorting in some lineages, or it may be due to a lack of phylogenetically informative variation within the 18S data set.

Each of the three orders that includes actinorhizal taxa also contains taxa that do not form the symbiosis; indeed, that is also true of several of the actinorhizal families. In some families all members are nodulated (Coriariaceae, Elaeagnaceae, Datisceae and Casuarinaceae) whereas in others only a portion of the genera are nodulated (Betulaceae, Myricaceae, Rhamnaceae and the Rosaceae). In at least one case (*Dryas*), nodulation apparently does not extend to all members of a single genus (Kohls *et al.*, 1994). These observations have led to the conclusion that, while the predisposition, or *potential*, to form the nitrogen-fixing symbiosis may have evolved only once, the realization of that potential has occurred and/or been lost multiple times (Benson and Clawson, 2000; Swensen, 1996).

6.3.2 Phylogeny of *Frankia*

The phylogeny of the genus *Frankia* has been deduced by comparative sequence analysis of the 16S rRNA gene, the genes for nitrogen fixation (*nif* genes) and by other genes (Benson and Clawson, 2000). All analyses agree that the genus is comprised of three major groups or clusters (referred to here as Groups 1, 2 and 3), each having different and sometimes overlapping plant specificity, physiological properties and symbiotic interactions (Figure 6.3). Within each group are definable subgroups that constitute 'genospecies' as defined by DNA-DNA homology studies (An *et al.*, 1985; Benson and Clawson, 2000; Dobritsa and Stupar, 1989; Fernandez *et al.*, 1989; Normand *et al.*, 1996).

In general, Group 1 *Frankia* strains form nodules on members of the 'higher' Hamamelidae, now all classified in the order Fagales, including the Betulaceae, Myricaceae and Casuarinaceae. The 'casuarina strains' that primarily infect members of the Casuarinaceae form a subgroup within Group 1. The latter strains also infect members of the Myricaceae as well as *Casuarina* spp., although the extent of their ability to do so in the field is unclear (Simonet *et al.*, 1999). 'Alder strains' generally infect most species of alder tested in greenhouse experiments, with some variability in effectiveness depending on the plant-symbiont combination. They too are generally able to infect members of the Myricaceae.

Group 2 *Frankia* strains are limited to infecting members of the Coriariaceae, Datisceae, Rosaceae and *Ceanothus* of the Rhamnaceae. These strains have not been isolated in pure culture despite many attempts to do so by several investigators and may therefore be obligate symbionts. Cross inoculation studies using crushed nodules suggest that symbionts from *Dryas*, *Ceanothus*, *Datisca* and *Coriaria* are in the same cross inoculation group (Kohls, *et al.*, 1994; Mirza *et al.*, 1994(Q8); Torrey, 1990).

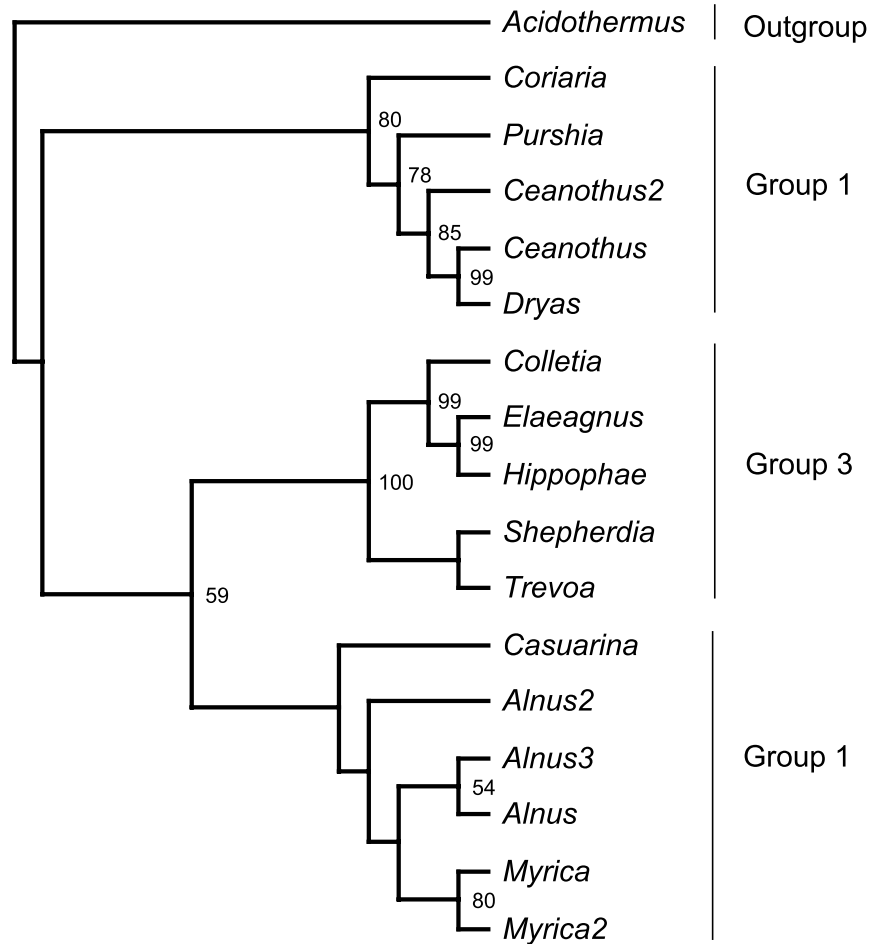


Figure 6.3. Phylogenetic relationships among *Frankia* strains. The major groups of *Frankia* strains are indicated along with the plant families they are known to infect. *Ceanothus* and *Gymnostoma* are listed separately as they are the only members of their families to be infected by the indicated Group of strains. Bootstrap support out of 1000 bootstrap samples is indicated at nodes where it occurred above 50%. Sequences and their accession numbers include: *Acidothermus*, X70635; *Coriaria* nodule, AF063641; *Purshia* nodule AF034776; *Ceanothus1* nodule AF063639; *Ceanothus2* nodule, U69265; *Dryas* nodule, L40616; *Colletia* nodule, AF063640; *Elaeagnus* strain Ea1-2, L40618; *Hippophae* strain HR27-14, L40617; *Shepherdia* strain SCN10a, L40619; *Trevoa* nodule, AF063642; *Casuarina* strain CeD, M55343; *Alnus*, strain AcoN24d, L40610, *Alnus2*, strain AVN17s, L40613; *Alnus3*, strain ACN14a, M88466; *Myrica*, nodule L40622; *Myrica2*, nodule, AF158687.

Group 3 strains form effective nodules on members of the Myricaceae, Rhamnaceae, Elaeagnaceae and *Gymnostoma* of the Casuarinaceae and are sometimes isolated as poorly effective, or non-effective, strains from nodules of the Betulaceae, Rosaceae, other members of the Casuarinaceae and *Ceanothus* of the Rhamnaceae.

Of these three major Clades, most ecological information, including distribution and diversity measurements, is available for Group 1 strains that are commonly known as alder and casuarina strains but that also infect *Myrica* spp. Less is known about members of Group 3 and even less about members of Group 2. Some studies have investigated members of these other groups and these will be mentioned below in the context of their plant families.

6.4 Biogeographic distribution of actinorhizal plants and *Frankia* strains

Actinorhizal plants have a global distribution. They are present on every continent except Antarctica, where their predecessors probably did exist for a time during the late Cretaceous. Each of the eight actinorhizal families has a distinctive native range that varies from very limited to global. The *Frankia* strains that infect these various groups of plants co-exist with the plants and some apparently have an independent life in the soil without the plant.

6.4.1 The Betulaceae

The Betulaceae is composed of six genera and about 130 species (Mabberly, 1988). The family is mostly distributed throughout the temperate regions of the northern hemisphere, with the exception of *Alnus glutinosa* (L.) Gaertn., which is found in Africa, and *A. acuminata* HBK, found throughout Central America south to Argentina. The genus *Alnus* is the only actinorhizal genus within the Betulaceae (Table 6.1). The family is well-defined, being held together by the synapomorphies of male and female compound catkins and pollen morphology (Chen *et al.*, 1999). The angiosperm *rbcL* phylogeny of Chase *et al.* (1993) strongly supported the classification of Betulaceae within Fagales (a relationship long recognized based on morphology). More recent studies of this group (Manos and Steele, 1997) have placed the family in a subClade with Casuarinaceae and Ticodendraceae.

Recent molecular phylogenies for the Betulaceae suggest two lineages (Figure 6.4C) (Chen *et al.*, 1999). One lineage contains the genera *Corylus*, *Ostryopsis*, *Carpinus* and *Ostrya*, and the other includes *Alnus* and *Betula*. The early divergence of *Alnus* agrees with previous morphological and fossil evidence (Chen *et al.*, 1999). The oldest known fossil infructescence for *Alnus* dates to the mid-Eocene (33–55 MYA), but *Alnus*-like pollen has been reported from much earlier, in the late Cretaceous (83–85 MYA), earlier than any fossils for the other genera in the family (Miki, 1977).

Given the distribution of known fossils and the recent molecular phylogeny, it appears the Betulaceae first originated in a Mediterranean climate in Laurasia during the late Cretaceous (89–65 MYA) (Laurasia was the northern supercontinent formed after Pangaea broke up during the Jurassic and included what are now North America, Europe, Asia, Greenland, and Iceland). Fossil evidence suggests that all six genera, including *Alnus*, were differentiated by the early Eocene (55 MYA) (Chen *et al.*, 1999). This observation suggests that if the ability to nodulate was ancestral in the Betulaceae, loss of that ability occurred very early on in the evolution of the family.

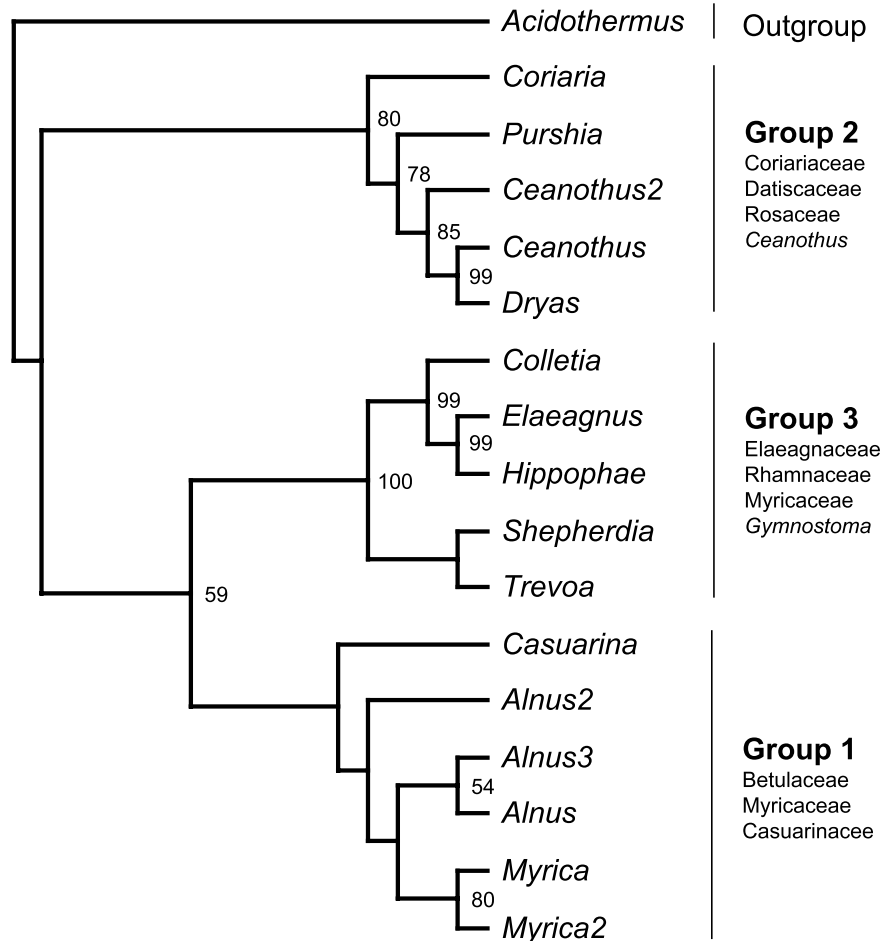


Figure 6.4. Phylogenetic relationships among members of actinorhizal families.

A – Phylogeny of the Casuarinaceae adapted from Sogo *et al.* (2001). The arrow denotes the possible origin of the actinorhizal symbiosis within the phylogeny, and the thickened branches lead to actinorhizal taxa. Taxon labels in bold and underlined denote the actinorhizal taxa. **B** – A phylogeny for the genus *Coriaria* adapted from Yokoyama *et al.* (2000) based on *rbcL* and *matK* DNA regions. Yokoyama identified two Clades, labelled I and II in the figure. The geographic locations of the taxa in the phylogeny can be found in the parentheses at the end of the taxon name. **C** – A phylogeny for the Betulaceae adapted from Chen *et al.* (1999) based on nuclear ribosomal ITS data and morphology. The taxon labels in bold and italics are actinorhizal. The arrow denotes the possible origin of the actinorhizal symbiosis within the Betulaceae. The thickened branches lead to actinorhizal taxa. **D** – A hypothetical representation of relationships within the Myricaceae adapted from a discussion of morphology and fossil history found in MacDonald (1989). **E** – A representation of a molecular phylogeny of the Rhamnaceae based on *rbcL* and *trnL-F* DNA sequences adapted from Richardson *et al.* (2000). The taxon labels are tribes within the Rhamnaceae. Those taxa with names in bold and underlined are actinorhizal. Arrows denote the two possible origins of the actinorhizal symbiosis. **F** – A representation of a phylogeny for the Rosaceae based on plastid *trnL-F* intergenic spacer and the *matK* DNA regions adapted from Potter *et al.* (2002). Taxon names in both bold and italics are actinorhizal. The arrow denotes the possible origin of the actinorhizal symbiosis within the Rosaceae. (Q6)

By the early Tertiary (65 MYA), movement between Eurasia and North America was possible, and the range of *Alnus* probably increased. The distribution of *Alnus* to Africa and to Taiwan probably occurred later, during the Pleistocene (1.8 MYA–11 000 years ago) when sea levels were lower (Chen *et al.*, 1999). Beyond the extensive geographic distribution, *Alnus* also grows in a wide range of habitats, from glacial till, sand hills, and bogs to dry volcanic lava, ash alluvium, and water courses (Schwencke and Caru, 2001; Silvester, 1977). To date, all species of *Alnus* examined have been shown to nodulate.

Alnus associates with *Frankia* strains that are similar to those that nodulate the other actinorhizal families in the Fagales, the Casuarinaceae and Myricaceae. These 'alder strains' belong to the diverse Group 1 frankiae. A few members of Group 3 have also been shown to nodulate alders but do so only rarely and are poorly effective (Bosco *et al.*, 1992; Lumini and Bosco, 1996).

Most studies that have focused on the distribution of frankiae in soil have used *Alnus* spp. as the trapping plant, largely because alder seeds are readily available and easily germinated. Except for a few environments such as at the foot of retreating glaciers in Alaska (Kohls, *et al.*, 1994) alder *Frankia* strains are cosmopolitan and seem to persist independently of host plants.

Population estimates of alder frankiae vary from a few per gram to several thousands per gram in soils both with and without actinorhizal plants (Markham and Chanway, 1996; Maunuksela *et al.*, 1999; Myrold *et al.*, 1994; Smolander, 1990; Smolander and Sarsa, 1990; Smolander and Sundman, 1987; Van Dijk, 1979, 1984(Q9)). Alder strains are commonly detected outside the geographic ranges of their compatible hosts and they persist long after compatible hosts have disappeared from a site (Arveby and Huss-Danell, 1988; Huss-Danell and Frej, 1986; Maunuksela, *et al.*, 1999; Paschke and Dawson, 1992a, 1992b; Smolander and Sundman, 1987; Wollum II *et al.*, 1968). In New Zealand, *Alnus* species nodulate at every site where they are grown at elevations from sea level up to 1700 m, even though the genus is a recent arrival to the islands (Benecke, 1969). Molecular work has shown that the nodules of these 'exotic' plants contain 'typical' *Frankia* strains, that is, those from phylogenetic Group 1 normally associated with the genera (Clawson *et al.*, 1997). Indeed, the diversity of strains in the alder nodules from New Zealand is greater than the diversity of strains infecting the native *Coriaria* sp.

As might be predicted, the nodulation capacity of soils for alders is affected by season (Myrold and Huss-Danell, 1994), acidity (Crannell *et al.*, 1994; Griffiths and McCormick, 1984; Smolander and Sundman, 1987; Zitzer and Dawson, 1992), fertility (Kohls and Baker, 1989; Myrold and Huss-Danell, 1994; Myrold *et al.*, 1994; Sanginga *et al.*, 1989; Thomas and Berry, 1989; Yang, 1995), water availability (Dawson *et al.*, 1986; Nickel *et al.*, 1999; Schwintzer, 1985), the physiological status of *Frankia* strains (Myrold and Huss-Danell, 1994) and by the type of plant cover (Huss-Danell and Frej, 1986; Markham and Chanway, 1996; Myrold and Huss-Danell, 1994; Smolander, 1990; Smolander and Sarsa, 1990; Smolander and Sundman, 1987; Smolander *et al.*, 1988; Zimpfer *et al.*, 1999).

In the case of plant cover, the number of alder nodulation units (NUs) seems to be as high or higher in soil beneath *Betula* (birch) stands than in soils beneath alder (Paschke and Dawson, 1992(Q10); Smolander and Sarsa, 1990; Smolander and Sundman, 1987; Van Dijk *et al.*, 1988) perhaps indicating a rhizosphere relationship

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between alder frankiae and other members of the Betulaceae. On the other hand, a study on the nodulation capacity of soils beneath birch, pine and spruce indicated similar alder frankiae populations of 3160, 2267 and 2747 NUs g⁻¹, respectively, suggesting that factors other than plant genotype can sustain populations in soil lacking actinorhizal plants.

Although many studies have been done on enumerating alder frankiae in soil, relatively few have directly examined the correlation between the diversity of the strains trapped and the environmental parameters of the soil examined. Some morphological work has indicated sorting of strains by soil type in alder. For example, the character of sporangium formation in a nodule seems to be stable among strains, thereby enabling geographical studies on sp(+) (containing sporangia) or sp(-) (devoid of sporangia) nodules (Schwintzer, 1990). Thus, in British Columbia, sp(-) nodules of *A. rubra* dominate in subarctic zones with no sp(+) nodules observed. The proportion of sp(+) nodules increased moving inland, up to 53% of the total (Markham and Chanway, 1996). In some studies, more acidic soils appear to select for *Frankia* strains with the sp(+) phenotype (Holman and Schwintzer, 1987; Kashanski and Schwintzer, 1987; Weber, 1986). Other studies have related the development of sp(+) or sp(-) nodules to the age of the stands, to plant selection or to moisture content of the soil (reviewed in (Schwintzer, 1990) and (Markham and Chanway, 1996)).

Alder frankiae can be concluded to be cosmopolitan and quite diverse within the limits of Group 1 strains. Their ubiquity implies that many alder frankiae are soil organisms with an independent existence not requiring continuous symbiotic interaction. It is likely that their wide distribution is related to the ranges of their hosts, both *Alnus* and *Myrica* spp., that extend throughout the northern hemisphere and into South America and Africa. The explanation for their abundance in exotic environments such as New Zealand may lie in their ability to grow as saprophytes in the absence of actinorhizal hosts, although their rapid spread once introduced cannot be ruled out.

6.4.2 The Myricaceae

The Myricaceae is composed of three genera, *Myrica* L., *Comptonia* L'Herit., and *Canacomyrica* Guillaumin. The family is classified in the Higher Hamamelidae, now included in the order Fagales (APG, 1998) and molecular phylogenetic studies have not provided strong support for its relationships within the order (Manos and Steele, 1997). *Myrica* is by far the largest genus, having about 50 described species with a wide distribution in North America, Europe, Africa, and Asia. *Myrica* spp. have been transported to new sites. For example, *M. faya* has been introduced to Hawaii where it has become an invasive exotic pest (Mabberly, 1988). The other two genera are monotypic. *Comptonia peregrina* L. is native to North America and *Canacomyrica monticola* Guillaumin is endemic to New Caledonia. Nodulation has been observed on all species of *Myrica* and *Comptonia*, but has yet to be documented on *Canacomyrica* (Navarro *et al.*, 1999).

To date, there has been no family-wide molecular phylogeny generated for the Myricaceae, yet the fossil history of the family has been well-discussed (see (Macdonald, 1989)). Briefly, there are two different opinions concerning the first appearance of the Myricaceae in the fossil record. One view holds that the

Myricaceae appeared early, during the Santonian period (83–85 MYA), based on *Myrica*-like pollen. The other view holds that the Myricaceae is instead a much later lineage, originating during the Eocene where there is more fossil evidence, and that the previous fossilised pollen was misidentified (Macdonald, 1989).

The geographic origin of the family is also in dispute. Both a Southeast Asian origin during the early Cretaceous (146–65 MYA) and a northern Tethyan origin during the late Cretaceous have been postulated. The enigmatic genus *Canacomyrica* appears to have many morphological similarities to the fossil, ancestral Juglandaceae and may represent the extant relictual ancestor to the Myricaceae (Macdonald, 1989). If *Canacomyrica* does indeed represent the basal lineage to the Myricaceae, then it would appear that the ability to nodulate occurred after the family had diverged or that the lineage leading to *Canacomyrica* lost the ability to nodulate (Figure 6.4D). A phylogeny and detailed biogeographic study is needed to further explore this issue.

Myrica species represent an interesting subject for studying the diversity of *Frankia* strains since they are considered to be promiscuous hosts based on the results of greenhouse cross inoculation studies and on ecological evidence (Baker, 1987; Clawson *et al.*, 1998; Torrey, 1990). It has been known for some time that *Myrica* species are effectively nodulated by frankiae from phylogenetic Groups 1 and 3 (Baker, 1987). Group 2 strains have not been reported in *Myrica*.

The natural diversity of *Frankia* strains in the nodules of *Myrica* spp. native to northeast North America has been examined using the variability between 16S rDNA sequences or PCR-RFLP of 16S rDNA as measures of diversity (Clawson and Benson, 1999(Q11); Huguet *et al.*, 2001). In one study, root nodules were collected from 30 sites with *Comptonia peregrina*, 29 with *Myrica pensylvanica* and 37 with *M. gale*, 37 unique sequences were found in 97 nodules analysed. Only two were present in all three plant species and two more were found in both *C. peregrina* and *M. pensylvanica*.

Interestingly, the richness and evenness components of diversity differed markedly between plant species. Nine Group 1 sequences were obtained from 37 *M. gale* nodules but only three dominated, accounting for 81% of the total. *C. peregrina* nodules had 15 Group 1 sequences in 30 nodules with four accounting for 60% of the total. Bayberry (*M. pensylvanica*) nodules yielded the highest diversity with 20 *Frankia* strain sequences in 29 nodules. Of the 20 sequences, 13 from Group 1 were found in 20 nodules, six Group 3 sequences were found in eight nodules, and one nodule yielded a sequence like that of Nod-/Fix- actinomycetes isolated from a variety of actinorhizal plants (Clawson *et al.*, 1998). Identical sequences were commonly found in plants growing at widely dispersed sites indicating that some *Frankia* strains are cosmopolitan. These results should be viewed with the understanding that strains in nodules with identical 16S sequences are not necessarily identical, only very similar.

The northern circumboreal species, *M. gale*, has historically been considered to be a 'promiscuous host' because it nodulated with most *Frankia* isolates in greenhouse trials (Torrey, 1990). It is not, however, overtly promiscuous in nature. This observation has been further confirmed in a separate study using PCR-RFLP of 16S rDNA PCR amplified from root nodules collected from *M. gale* nodules in Canada (Huguet *et al.*, 2001). Low diversity in *M. gale* nodules may be attributed to its preference for growing in water-saturated soils near lakes, swamps or bogs.

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Such locations are typically acidic and low in oxygen; such conditions may limit the selection of *Frankia* to those strains capable of tolerating them.

In older studies, *M. gale* exhibited distinctive patterns of distribution of sp(+) and sp(-) (indicating the presence or absence of *Frankia* sporangia in nodules) root nodules with sp(+) strains more common in nodules collected at southern interior and coastal regions and the sp(-) strains more common in northern and western Maine (Schwintzer, 1990). The presence of sp(+) nodules positively correlated with the average number of frostfree days per year, and with the percentage organic matter in the soil, and negatively correlated with pH, with more sp(+) nodules found in more acidic soils. Although the sporulation phenotype has low resolution, its significance is enhanced by the finding that the diversity of frankiae in these nodules is low. A similar sorting by environment has been observed in sp(+) and sp(-) nodules growing on alders (see above). Thus, local edaphic factors are clearly important in determining which *Frankia* strains get into nodules and most likely how strains distribute among environments.

Since many *Myrica* spp. and *C. peregrina* can be nodulated by alder strains, the environmental distribution of Group 1 strains is potentially very wide. The same holds true for the elaeagnus strains from Group 3 that can infect *Myrica* spp. The degree of overlap is not known since the degree of individual strain specificity for different plant species has not been well documented. However, the wide geographical distribution of the plants from these groups most likely accounts for, and reinforces, the similarly wide distribution of homologous symbionts. The distribution of casuarina *Frankia* strains provides a contrast to this ubiquity.

6.4.3 The Casuarinaceae

The Casuarinaceae is a well-characterised family of four genera and roughly 96 species. Based on DNA sequencing, the Casuarinaceae has been placed in the Fagales (APG, 1998; Chase *et al.*, 1993). The family is easily recognised by its slender, wiry branches and highly reduced leaves. It is geographically restricted to Australia and the Melanesian region of the Pacific (Johnson and Wilson, 1989) but *Casuarina* spp. have been naturalised in islands and coastal regions of the Indian Ocean, Africa and the Americas. Recent molecular phylogenies found *Gymnostoma* L.A.S. Johnson sister to the rest of the family and *Allocasuarina* L.A.S. Johnson most derived (Maggia and Bousquet, 1994; Sogo *et al.*, 2001). This topology agrees with the traditional, morphological view based on branchlet and infructescence structure (Figure 6.4A) (Sogo *et al.*, 2001). Fossil evidence for the family dates back to the Eocene (55–39 MYA). The family had a much wider distribution in the past, with macrofossils of *Gymnostoma* and *Ceuthostoma* L.A.S. Johnson discovered outside of its present range in New Zealand and South America. The absence of both macrofossils and undisputed fossil pollen in the northern hemisphere has led most researchers to postulate that the family had its origins in Gondwanaland (Johnson and Wilson, 1989).

Members of all four genera nodulate, although the genera differ with respect to the type and diversity of *Frankia* strains with which they associate. *Gymnostoma* spp. interact with diverse Group 3 *Frankia* strains, while more derived members of the family, *Casuarina* L. and *Allocasuarina*, interact with a more reduced set of strains from Group 1. This observation has led authors to postulate that perhaps

the family is evolving towards strain-specificity (Maggia and Bousquet, 1994), or the specificity of *Casuarina* and *Allocasuarina* is simply due to the drier habitats of Australia that these genera inhabit, where a smaller subset of *Frankia* strains are able to survive.

The ubiquity of alder and myrica frankiae in soils does not extend to the casuarina frankiae (Diem and Dommergues, 1990), even though both sets of strains belong to phylogenetic Group 1 and the Casuarinaceae is sister to the Betulaceae and Myricaceae among the higher hamamelids. A study done in Jamaica serves to illustrate the status of casuarina strains outside of the native range (Zimpfer *et al.*, 1997). A most probable number approach was used to estimate the relative abundance of frankiae capable of nodulating the native *Myrica cerifera* versus the exotic *Casuarina cunninghamiana* in soils collected at sites lacking actinorhizal plants. Myrica strains occurred in variable abundance at all sites sampled whereas no *C. cunninghamiana* strains were detected. As found elsewhere, the occurrence of myrica strains in Jamaican soils is independent of the host plant whereas casuarina strains seem to depend on the presence of the host. On the other hand, *C. cunninghamiana* is nodulated where it has been established in Jamaica suggesting that a compatible strain had been introduced along with the plant but has not spread. Indeed, in soil collected along transects leading away from *C. cunninghamiana* trees, NUs of casuarina frankiae diminished to undetectable levels at about 20 m away from the trees. Myrica frankiae NUs were present at all distances along the transects despite the nearest host being 25 km distant (Zimpfer *et al.*, 1999). This observation plus the fact that *Casuarina* plants must be deliberately inoculated in areas of the world where they are first planted (Diem and Dommergues, 1990; Simonet *et al.*, 1999) indicates that the presence of the host is critical for maintaining soil populations of casuarina frankiae.

Surprisingly, several lines of evidence indicate that only one group of closely related or identical strains is responsible for nodulating *Casuarina* and *Allocasuarina* spp. outside their normal geographic range (Fernandez *et al.*, 1989; Honerlage *et al.*, 1994; Maggia *et al.*, 1992; Nazaret *et al.*, 1989, 1991; Rouvier *et al.*, 1990, 1996; Simonet *et al.*, 1999). For example, except for one nodule harvested in Kenya, 160 nodules from five *Casuarina* and *Allocasuarina* species sampled outside of Australia from several countries yielded the same PCR-RFLP group that dominated culture collections. The same group was identified in other work using DNA-DNA reassociation (Fernandez *et al.*, 1989), and by PCR-RFLP of the intergenic regions between the *rrs* and *rnl* genes and between the *nifH* and *nifD* genes (Honerlage *et al.*, 1994; Maggia *et al.*, 1992; Rouvier *et al.*, 1992; Simonet, *et al.*, 1999). That group may be the one best adapted to a saprophytic lifestyle in an exotic environment and is the one most likely to be cultured from root nodules (Simonet *et al.*, 1999).

More diversity prevails within the native range of the plants. Twenty-two nodules collected from two *Casuarina* spp. and two *Allocasuarina* spp. in Australia yielded a total of five *rrn* and *nif* PCR-RFLP groups (Rouvier *et al.*, 1996). Nodules from *C. equisetifolia* contained one group in six of eight nodules and another group was present in the remaining two nodules. The dominant strain belonged to the same PCR-RFLP group found in nodules collected from regions of the world where *Casuarina* has been introduced. An additional group was found in six *C. cunninghamiana* nodules, another was found in four *Allocasuarina lehmanniana*

nodules, and the final group was found in four *A. torulosa* nodules. Despite the relatively small number of nodules used in the study some degree of plant species–*Frankia* strain specificity was noted. In another study using a similar approach, seven groups were found among 110 nodules sampled from five Casuarinaceae species in Australia (Simonet *et al.*, 1999). Each IGS group was found in only one or two of the plant species. This specificity could be due to the host plant selecting specific strains in the rhizosphere from among a population of strains, or to environmental selection limiting the types of frankiae that are available in a particular soil.

Another member of the Casuarinaceae, *Gymnostoma*, provides a contrast to its nearest relatives. *Gymnostoma* spp. are nodulated by members of phylogenetic Group 3 *Frankia* strains rather than by the casuarina strains from Group 1. The reason for this symbiont shift is not obvious but may be related to an early allopatric distribution of the plants and presumably symbionts (*Gymnostoma* in the north and east of Gondwana islands; *Casuarina/Allocasuarina* in the drier Australia) (Simonet *et al.*, 1999). Consistent with this hypothesis is the observation that the only *Casuarina* species present in New Caledonia, *C. collina*, commonly contains both Group 1 casuarina strains and Group 3 strains; the latter are similar or identical to those nodulating *Gymnostoma* (Gauthier *et al.*, 1999).

Also in contrast to other members of the Casuarinaceae is a relatively higher diversity of *Frankia* strains found in *Gymnostoma* nodules. A study in New Caledonia using PCR-RFLP of the ribosomal *rrs-rrl* intergenic spacer as a probe detected 17 different RFLP patterns in 358 nodules from eight *Gymnostoma* species (Navarro *et al.*, 1999). No sharp species specificity was noted among the 17 patterns but a correlation was found between soil type, host species and RFLP pattern. One pattern predominated and accounted for 56% of the total from all species. It was, however, absent from 45 nodules obtained from two *Gymnostoma* species growing at acidic sites and was thus deemed specific for ultramafic soils. Another pattern belonged to a more cosmopolitan strain group found in all species on all soils and was represented in 14.5% of the total. Strains that nodulate *Gymnostoma* are widespread in New Caledonia with some indication, based on trapping experiments, that populations are amplified in soils beneath *Gymnostoma* but also persist without a covering host plant (Gauthier *et al.*, 2000). Populations are also amplified in the rhizosphere of a member of a non-nodulating member of the Rhamnaceae (Gauthier *et al.*, 2000).

The conclusion from these studies and others (Jaffre *et al.*, 2001) is that different populations of Group 3 frankiae colonize different *Gymnostoma* spp. more as a function of soil type rather than species specificity. This work, together with the *M. gale* work noted above, provide the strongest examples of strain sorting by environment.

6.4.4 The Elaeagnaceae

The Elaeagnaceae is a well-defined family of three genera *Elaeagnus* L., *Hippophae* L. and *Shepherdia* Nutt. *Elaeagnus* is distributed across North America and Eurasia, although the range of this genus has greatly increased due to cultivation and use in land reclamation (Baker and Schwintzer, 1990). In Australia and western North America, *Elaeagnus* is often considered an invasive exotic

(Mabberly, 1988). *Elaeagnus* has about 45 described species, most of which have been shown to nodulate (Table 6.1). *Hippophae* is native to central Asia, distributed from the North Sea to the Black Sea and east to the Himalayas. The number of species within *Hippophae* has been in dispute, with numbers ranging from one to seven with numerous subspecies. This discrepancy in classification is primarily due to hybridisation and intergradation of morphology (Bartish *et al.*, 2002). *Shepherdia* is composed of three species, restricted to North America (Mabberly, 1988).

The phylogenetic placement of the Elaeagnaceae within angiosperm diversity has been in dispute. The *rbcL* angiosperm phylogeny placed the family close to the Rhamnaceae within the Rosales (Chase *et al.*, 1993), the order in which it is currently classified (APG, 1998). Further phylogenetic studies have placed the Elaeagnaceae sister to the Rhamnaceae, within the Rhamnaceae, or in a loose alliance with the Barbeyaceae, Ulmaceae and Cannabaceae (Richardson *et al.*, 2000). A comprehensive phylogeny for the Elaeagnaceae has not been constructed, yet members of all three genera were included in an *rbcL* phylogeny constructed by Swensen (1996). In that tree, *Hippophae* was sister to *Elaeagnus* and *Shepherdia*. The Elaeagnaceae has a late appearance in the fossil record (Oligocene 22–39 MYA), and based on present distribution, the family most likely originated in Laurasia before the breakup of the continents in the northern hemisphere (Bartish *et al.*, 2002).

Elaeagnus, *Shepherdia* and *Hippophae* spp. are generally well nodulated even in geographical regions where they are not native or where alternate hosts are absent. The *Frankia* strains present in root nodules seem to be shared among the three genera and all belong to Group 3 (Benecke, 1969; Clawson *et al.*, 1998; Huguet *et al.*, 2001; Jamann *et al.*, 1992). Thus, elaeagnus strains from Group 3 can be considered to be cosmopolitan. In part, their wide distribution may stem from their roles as potentially effective symbionts in four of the actinorhizal plant families (Elaeagnaceae, Rhamnaceae, Casuarinaceae (*Gymnostoma*), Myricaceae) and their less well-characterized roles as occasional symbionts in the Betulaceae or as associative strains not clearly involved in nitrogen fixation in the Rosaceae, *Ceanothus* and members of the Casuarinaceae other than *Gymnostoma* (Benson and Clawson, 2000). Like alder strains, the specificity exhibited by individual Group 3 strains is not well characterized.

In Europe, *Elaeagnus* spp. have been recently introduced but *Hippophae rhamnoides* was present throughout Europe during all stages of the late glaciation and probably helped maintain the populations of Group 3 frankiae (Jamann *et al.*, 1992). The same situation holds for North America where most *Elaeagnus* species are introduced and universally nodulated. The native *Shepherdia* is infected by a wide variety of Group 3 frankiae that can also be presumed to infect introduced *Elaeagnus* spp. (Huguet *et al.*, 2001).

Few reports have directly addressed the diversity and distribution of *Frankia* strains that infect members of the Elaeagnaceae. However, all species in the family examined thus far are effectively nodulated only by a set of *Frankia* strains belonging to phylogenetic Group 3. Molecular studies using sequencing of 16S rRNA genes and DNA–DNA hybridisation have indicated that isolated strains are diverse within the confines of Group 3 (Fernandez *et al.*, 1989; Huguet *et al.*, 2001; Nazaret *et al.*, 1989). However, a survey of published Clade 3

sequences from the field reveals that some are cosmopolitan (Clawson *et al.*, 1998; Nalin *et al.*, 1997). For example, an identical partial 16S rDNA sequence has been reported from an *E. angustifolia* and *Myrica pensylvanica* growing in Connecticut, an *E. pungens* in Hamilton, New Zealand, and *Discaria trinervis*, *Talguenea quinquenervia*, *Trevoa trinervis* and an unidentified *Elaeagnus* in Chile (Clawson *et al.*, 1998). The same sequence was also reported as belonging to a major group of strains in France (Ea1-2, HR27-14) (Jamann *et al.*, 1992). While this finding may partly reflect the DNA region that was sequenced, it does support the notion that a group of elaeagnus strains (Genomic group 4 (Fernandez *et al.*, 1989)) is widely distributed in nature. A study focused on the distribution of elaeagnus strains through a soil column collected from an area lacking host plants yielded seven PCR-RFLP profiles for DNA obtained from nodules induced by trapping experiments. Six of the profiles corresponded to previously identified genomic species in France and the seventh, collected from the deepest layers, was unique. Thus, a relatively high diversity was found in the samples but it was within the bounds of the diversity of strains known to infect the plants (Nalin *et al.*, 1997).

It would be interesting to determine the patterns of richness and evenness of elaeagnus strains across the native distribution zones of the various species. For example, Russian olive, *E. angustifolia* L., has been widely transplanted as a wind-break or ornamental throughout the world. A useful study might be to compare the diversity of strains found in root nodules within its native range to that of strains found outside its native ranges where it has been transplanted.

6.4.5 The Rhamnaceae

The Rhamnaceae is distributed worldwide, containing 50 genera and about 900 species (Richardson *et al.*, 2000). Traditional taxonomic treatments have placed the Rhamnaceae with the Vitaceae based on shared floral characters (Takhtajan, 1980) or with the Elaeagnaceae based on shared vegetative features (Thorne, 1992). The angiosperm *rbcL* phylogeny placed the Rhamnaceae within the Eurosoid I Clade and indicated a close relationship between the Rhamnaceae and the Elaeagnaceae in the Rosales. Quite surprisingly, in past *rbcL* reconstructions, the Rhamnaceae is paraphyletic with Barbeyaceae, Dirachmaceae, and the Elaeagnaceae (Richardson *et al.*, 2000). Further molecular data have not supported this topology, but instead favour a monophyletic Rhamnaceae (Richardson *et al.*, 2000).

Six genera within the Rhamnaceae have been identified as nodulating with *Frankia* strains. Except for *Ceanothus* L., all belong to the tribe Colletieae Reis. Ex. Endl. These genera are: *Colletia* Comm. ex Juss. (17 species found in South America), *Discaria* Hook. (15 described species found in South America, Australia, and New Zealand), *Kentrothamnus* Susseng. and Overk. (one species restricted to Bolivia), *Retanilla* (DC) Brongn. (four species found in Peru and Chile), and *Trevoa* Mires ex. Hook. (one species found in South America). *Trevoa* was recently revised to include the previously separate, actinorhizal genus *Talguenea* (Tortosa, 1992). The one member of the tribe Colletieae whose actinorhizal nature is unconfirmed is *Adolphia* Meisner., located in southwestern North America (Cruz-Cisneros and Valdés, 1991). The other actinorhizal genus in the Rhamnaceae is *Ceanothus* L., a strictly North American genus of approxi-

mately 55 species (Mabberly, 1988). Most nodulated members of the Rhamnaceae grow in dry matorral or chaparral regions.

The recent molecular phylogenies constructed for the Rhamnaceae by Richardson *et al.* (2000) found that the five genera within the tribe Colletieae were indeed monophyletic (Figure 6.4E). However, the genus *Ceanothus* did not cluster with the Colletieae, giving rise to the possibility that the actinorrhizal symbiosis may have evolved twice within the Rhamnaceae, although the authors indicate that the inclusion of more data may unite *Ceanothus* as sister to the Colletieae.

The Rhamnaceae appears to be a very old lineage, with a rhamnaceous fossilised flower and pollen dating to 94–96 MYA to give a minimum age for the family (Basinger and Dilcher, 1984). Both *Ceanothus* and the tribe Colletieae belong to a large Clade within the family termed the ziziphoid group, which is mostly distributed in the southern hemisphere, suggesting that this branch of the family may be of Gondwanan origin. The major exception to this distributional hypothesis is the genus *Ceanothus*, that Richardson *et al.* (2000) have suggested may have been part of the ziziphoid group with a Laurasian distribution before the Gondwanan split and has had a relictual distribution in North America, primarily California. This hypothesis requires that the genus *Ceanothus* be quite old (65 MYA). An ancient split between *Ceanothus* and the tribe Colletieae may explain why the two groups differ in the *Frankia* strains with which they associate.

Members of the tribe Colletieae in the southern hemisphere associate with ubiquitous Group 3 *Frankia* strains that potentially also associate with the Elaeagnaceae, Myricaceae and *Gymnostoma*. Although several *Frankia* strains have been isolated and characterized from the root nodules of the South American Colletieae (Carú, 1993; Schwencke and Caru, 2001), studies have not yet been done on the ecological diversity patterns of strains in the nodules from different species or environments.

The North American *Ceanothus* spp., on the other hand, associate primarily with Group 2 *Frankia* strains similar to those that nodulate *Datisca*, *Coriaria* and the actinorrhizal Rosaceae (see Figure 6.3) (Benson and Clawson, 2000). The approximately 55 species of this genus are limited to western parts of North America with the range of one, *C. americanus*, extending to the east coast. Some work has addressed the diversity of symbionts in North American *Ceanothus* root nodules.

An initial study on *C. americanus* found a relatively high level of diversity of *Frankia* strains in root nodules as assessed using RFLP of total DNAs probed with *nifDH* genes or with random probes (Baker and Mullin, 1994). In a separate study, repetitive extragenic palindromic PCR (Rep-PCR) was used as a measure of diversity in six *Ceanothus* spp. taken from seven sites in a 10 mile radius along coastal southern California (Murry *et al.*, 1997). Overall, 54 nodules yielded 11 different Rep-PCR patterns, some of which were very similar to others. Subsequent sequencing of a region of the 16S rRNA gene from a few nodules indicated habitation by Group 3 *Frankia* strains, that is, elaeagnus strains.

This finding is at odds with other studies that have detected Group 2 *Frankia* strains in *Ceanothus* nodules. The picture is further clouded by the finding that some isolates from *Ceanothus* nodules can infect *Elaeagnus* spp. while others belong to a group of Nod-/Fix- strains that sometimes occupy actinorrhizal nodules. None of the isolates, however, can reinfect *Ceanothus* plants (Lechevalier

and Ruan, 1984; Ramirez-Saad *et al.*, 1998; Torrey, 1990). California is considered to be the centre of *Ceanothus* distribution and might be expected to support a diverse population of *ceanothus frankiae*, by analogy with the situation for *casuarina* strains in their native Australia. On the other hand, cohabitation of *Ceanothus* nodules by more than one organism might explain some of the diversity observed by molecular techniques. Additional work needs to be done to sort out the relationship of the different lineages of bacteria that inhabit *Ceanothus* root nodules.

A study on *Ceanothus* in Oregon suggested a relationship between strains and the soil conditions from where nodules were harvested (Ritchie and Myrold, 1999). This work relied on a PCR-RFLP analysis of the ribosomal *rrs-rrl* region. Four RFLP groups were identified with one predominating in mountainous regions and two others limited to the Willamette Valley. The fourth group was limited to *C. americanus* collected from Tennessee. Overall, the diversity of strains reported was less than that reported using other methods. In a similar manner, sampling of nodules from co-populations of different *Ceanothus* species indicated that *Frankia* strain PCR-RFLP patterns were more likely to be related to the environment from which the nodules came than to the plant species infected (Jeong, 2001; Jeong and Myrold, 1999).

In their native range, *Ceanothus* strains have been enumerated by trapping experiments from soils with and without hosts. Populations have been found to be amplified beneath *Ceanothus* stands although sites lacking host plants retained a small population (Jeong, 2001; Wollum II *et al.*, 1968). Low levels of *Ceanothus* nodulation by soils beneath old-growth (300 years) Douglas Fir stands has been noted (Wollum II *et al.*, 1968). However, even in soil beneath host plants, the nodulation capacity is low; in one study nodulation units were estimated at 3.6 to 5.2 NUs g⁻¹ soil, which is at the low end of estimates for alder-type frankiae in soils lacking alders (Jeong, 2001). This low population density seems to be characteristic of *Ceanothus* strains and may reflect an actual low population or an inherent difficulty in nodulating *Ceanothus* plants in the greenhouse (Rojas *et al.*, 2001). *Frankia* strains in trapping experiments were found to have similar levels of diversity in both forest soil and *Ceanothus* stands albeit at different population densities. No strong correlation has yet been found with strain type (as determined by rep-PCR or PCR-RFLP) and *Ceanothus* species.

Ribosomal RNA gene sequences amplified from *Ceanothus* nodules are generally very similar (99–100%) to each other and to some amplified from nodules in the Rosaceae, Datisceae and Coriariaceae, suggesting that some Group 2 strains are globally dominant (Benson *et al.*, 1996; Ramirez-Saad *et al.*, 1998; Ritchie and Myrold, 1999). This low diversity may also reflect the fact that relatively few 16S rDNA sequences have been obtained from Group 2 *Frankia* strains. Plants from these families share an overlapping range in western North America although *Coriaria* and *Datisca* are more widespread with disjunct populations in several parts of the world (Benson and Clawson, 2000). It is possible that *Ceanothus* became geographically isolated from other Rhamnaceae and subsequently specialised in the Clade 2 *Frankia* strains that may have been more adapted to the environment or simply more numerous because of their proximity to other actinorrhizal plants.

6.4.6 The Coriariaceae

The Coriariaceae is a monotypic family whose taxonomic placement has varied considerably in different past treatments; molecular data firmly place it within the Cucurbitales (APG, 1998). The only genus, *Coriaria*, consists of between five and 20 species. Such a wide range in the number of described species, depending on the particular classification, is due to the large, shared morphological variation displayed by members of this genus (Yokoyama *et al.*, 2000). *Coriaria* L. has one of most spectacular native geographic distributions of any genus of its size, being found in four areas worldwide, the Mediterranean, Southeast Asia, Central and South America, and the Pacific islands of New Zealand and Papua New Guinea (Skog, 1972). Such a conspicuous geographic disjunction has attracted many previous authors to hypothesise about the origin and diversification of *Coriaria* (see review in Yokoyama *et al.* (2000)). In a recent molecular phylogeny, Yokoyama *et al.* (2000) tested these previous hypotheses and found that the most basal diverging members of the genus are present in Asia and Central America, leading to the conclusion that the genus originated in either Eurasia or North America. In addition, application of a molecular clock hypothesis led the authors to suggest that the genus had an origin some 60 MYA, far older than an estimate of 5–11 MYA based on fossil evidence (Yokoyama *et al.*, 2000). Based on the present distribution of *Coriaria*, an older date for the origin and diversification of the family may indeed be correct.

The molecular phylogeny for *Coriaria* produced two main Clades. Clade I consisted of taxa from the Mediterranean and Asia and Clade II consisted of taxa from Central and South America. The authors concluded that simple vicariance and dispersal caused by glaciation and drying during the Cenozoic may account for the distribution of *Coriaria* in Clade I, but could not be used to explain the distribution of the *Coriaria* diversity present in Clade II. The topology presented in Clade II favours the interesting hypothesis of long-distance dispersal from Central America to the Pacific islands, followed by another migration back to South America (Chile) (Figure 6.4B).

Nodules have been observed on *Coriaria* species from New Zealand (*C. arborea*, *C. plumosa*), from Central America (*C. microphylla*), Europe (*C. myrtifolia*) and Central Asia (*C. nepalensis*) (Mirza *et al.*, 1994(Q14); Nick *et al.*, 1992; Silvester, 1977). The total number of *Coriaria* spp. able to nodulate has yet to be determined. However, at least one species from all four major zones of diversity has been shown to nodulate, and known nodulating species are present in both Clade I and Clade II, indicating that the association with *Frankia* strains appears to be widespread throughout the genus. The distribution of *Coriaria* strains in soils devoid of *Coriaria* hosts has not been addressed. Some studies in New Zealand indicate that *Coriaria arborea* plants are nodulated wherever planted and will readily nodulate in new volcanic soils.

The *Frankia* strains associating with *Coriaria* are closely related to the unisolated Group 2 strains that associate with *Ceanothus*, to strains that associate with members of the actinorrhizal Rosaceae and to strains associating with *Datisca*, (Benson and Clawson, 2000). Available information suggests that the richness of strains is low in the Rosaceae, Datisceae and Coriariaceae. For example, *Coriaria arborea* nodules in New Zealand yielded only two 16S rRNA gene sequences,

differing by a single nucleotide, from 12 nodules collected at distant locales on the North Island (Clawson *et al.*, 1997). Additional sequences from a total of 30 nodules from *C. arborea* and four more from *C. plumosa* collected in New Zealand yielded the same sequences (DRB, unpublished).

Similarly, a collection of short 16S rDNA sequences spanning another 16S region (V6) PCR-amplified from *Coriaria* nodules collected in New Zealand, France and Mexico had only one mismatch in 274 bp analysed (Nick *et al.*, 1992). A further study in Pakistan used the V2 16S rDNA region and found some diversity in both *Coriaria nepalensis* and *Datisca cannabina* that would have been missed using the region analysed by Nick *et al.* (1992). Nevertheless, the number of differences among the sequences was still low suggesting low overall diversity of frankiae within the Coriariaceae and Datisceae. No studies have been done to date on the distribution of these strains in soils from areas that lack *Coriaria* spp., so their ubiquity remains unknown.

6.4.7 The Datisceae

As traditionally circumscribed, the Datisceae *sensu lato* includes three genera, *Datisca* L. (including two species), *Tetrameles* R. Br. (one species), and *Octomeles* Miq. (one species). The family is classified in the order Cucurbitales (APG, 1998). Recent molecular phylogenetic work within the family has shown the Datisceae *sensu lato* to be paraphyletic with respect to the Begoniaceae. This result has supported the classification, as originally proposed by Airy Shaw (1964) based on morphology, of *Tetrameles* and *Octomeles* in Tetramelaceae, leaving only the genus *Datisca* in the Datisceae. Therefore, the revised Datisceae no longer contains non-nodulating genera (Swensen *et al.*, 1994, 1998).

The two species of *Datisca*, *D. cannabina* L. and *D. glomerata* (Presl.) Baill., are adapted to Mediterranean climates and have an interesting distribution. *D. cannabina* is found in the Mediterranean basin and *D. glomerata* is found on the western slope of the Sierra Nevada from northern California to Baja California (Swensen *et al.*, 1994). Plants in California and the Mediterranean basin are known to have some taxonomic affinities (North America and Europe were only separated since the Tertiary) (Solbrig *et al.*, 1977). Detailed phylogenies for *Datisca* indicate that geographic subdivision rather than long-distance dispersal accounts for the present day distribution (Swensen *et al.*, 1998). Since the Mediterranean climate is relatively new, established only since the Pleistocene, it is more likely that after the vicariance both species converged on the Mediterranean climate instead of an ancestor to the two species being preadapted to the Mediterranean climate (Solbrig *et al.*, 1977). Fossil wood from India suggests that the Datisceae may have arisen in the Eocene (55–39 MYA), although it is important to point out that there is some question whether the fossil remains are correctly identified as Datisceae (Cronquist, 1981). Both species of *Datisca* are actinorhizal (Swensen *et al.*, 1994).

As noted above, *Frankia* strains that inhabit *Datisca* nodules appear to be closely related to those found in *Coriaria*, *Ceanothus* and the actinorhizal Rosaceae (Benson and Clawson, 2000; Benson *et al.*, 1996; Mirza *et al.*, 1994(Q15)). In fact, crushed nodule inoculations indicate that *Dryas*, *Ceanothus*, *Datisca* and *Coriaria* are in the same cross inoculation group (Kohls *et al.*, 1994; Mirza *et al.*,

1994(**Q16**); Torrey, 1990). The distribution of *Datisca frankiae* in soils has not been extensively studied. Some work indicates that the distribution of strains parallels the distribution of plants on a regional scale. For example, in Pakistan, all soils tested yielded nodules on *Datisca* except one from an eroded area (Mirza *et al.*, 1994(**Q17**)). Companion experiments testing for the nodulation of *Coriaria* with the same soils yielded less nodulation with some soils failing to nodulate, indicating that *Coriaria nepalensis* was more difficult to nodulate, in agreement with previous observations (Bond, 1962). The distribution of *Datisca Frankia* strains outside the native range of the plants is unknown.

6.4.8 The Rosaceae

The Rosaceae is a large, economically important family with roughly 122 genera and 3000 species (Heywood, 1993). The family is distributed worldwide, but is found especially in north temperate regions. The Rosaceae has traditionally been subdivided into four subfamilies; the Rosoideae, the Spiraeoideae, the Maloideae and the Amygdaloideae on the basis of fruit type (Schulze-Menz, 1964). Due to the family's economic importance, it has been subject to many evolutionary and phylogenetic studies (Evans *et al.*, 2000; Kalkman, 1988; Morgan *et al.*, 1994; Potter, 1997; Potter *et al.*, 2002; Rohrer *et al.*, 1991). The first *rbcL* phylogeny (Morgan *et al.*, 1994) for the Rosaceae found that the four traditional subfamilies were not natural, and instead Clades appeared to correspond to base chromosome number and not fruit type. The *rbcL* phylogeny and later phylogenetic studies using other molecular markers, have found a strongly supported Clade consisting of the four actinorhizal genera of the Rosaceae (Figure 6.4F). These genera include *Cercocarpus* HBK (six to ten species restricted to southwestern North America), *Purshia* (eight species also restricted to southwestern North America), *Chamaebatia* (two species found in California), and *Dryas* (two species found circumpolar in alpine and Arctic habitats) (Evans *et al.*, 2000; Morgan *et al.*, 1994; Potter, 1997; Potter *et al.*, 2002). *Cowania* was recently combined with *Purshia* under the name *Purshia* (Henrickson, 1986).

The relationships near the base of the Rosaceae phylogenetic tree have not been resolved, but studies based on the chloroplast *matK* and *trnL-F* regions suggest that there are three main lineages in the family: the traditional Rosoideae (with some modifications), the actinorhizal Clade, and the rest of the family (Potter *et al.*, 2002). This orientation suggests that either the ability to nodulate evolved once as the family was beginning to diverge, or that nodulation was present in the common ancestor of the family and was lost twice in its diversification.

Aside from a few sequences of 16S rDNA that have been obtained by PCR amplification from root nodules (Benson *et al.*, 1996; Bosco *et al.*, 1994), very little is known about the ecology or diversity of Clade 2 frankiae that inhabit nodules in the Rosaceae. As noted above, the actinorhizal Rosaceae appear to associate only with *Frankia* strains related to those that nodulate the genera *Ceanothus*, *Coriaria* and *Datisca*. These four groups of plants share, at least in part, a range in western North America, although *Coriaria* and *Datisca* are more widespread (see above). Interestingly, an identical partial 16S rDNA sequence has been reported in *Purshia tridentata*, *P. glandulosa*, *Cowania stansburiana*, *Chamaebatia foliosa*, *Ceanothus velutinus*, *C. griseus*, *C. ceruleus* and *Dryas dummondii* all originating in

North America (DRB, unpublished). It is tempting to speculate that the presence of Clade 2 *Frankia* strains in these plants is related to their overlapping biogeography during the breakup of Laurasia and Gondwana in the late Cretaceous.

Nodulation in the rosaceous actinorhizal plants is sporadic (Klemmedson, 1979). One study reported nodulation rates of 8.3–32.2% of field plants of *Cercocarpus*, *Cowania* and *Purshia* (Nelson, 1983). Some species of *Dryas* have not been observed to nodulate (Kohls *et al.*, 1994). Both *D. octapetala* and *D. integrifolia* have been reported to bear nodules in the older literature but the observations are in need of verification (Baker and Schwintzer, 1990). A putative hybrid between *D. drummondii* and *D. integrifolia* found in Glacier Bay National Park, *D. drummondii*, var. *eglandulosa*, apparently does not nodulate even when deliberately inoculated in the greenhouse (Kohls *et al.*, 1994). When *Dryas* or other actinorhizal rosaceous plants are inoculated in the greenhouse with either soil or crushed nodules, nodules develop beginning 6–8 weeks after inoculation. This slow development contrasts with the 2–3 weeks normally required for nodules to appear on inoculated *Alnus* or *Myrica*.

Few studies have focused on the presence of rosaceous-infective frankiae in soils. What little information is available seems to suggest that strains are distributed in areas where the plants grow but are not abundant outside those areas. Kohls *et al.* (1994) found that soils from Glacier Bay, Alaska, where *Dryas* is abundant, failed to induce nodules on *Cercocarpus betuloides* but did contain *Frankia* strains that nodulated *Dryas drummondii* and *Purshia tridentata*. Crushed nodules from *Dryas* also nodulated *Dryas* and *Purshia* but not *Cercocarpus* suggesting that the cercocarpus strains may differ in some manner from the dryas strains. In the same study, ineffective (unable to fix nitrogen) nodules were formed on *Cercocarpus ledifolius* by CcI3, Cms13 and EuI1b. These strains are from *Casuarina cunninghamiana*, *Cowania mexicana* and *Elaeagnus umbellata*, respectively, suggesting that these strains may participate in forming ineffective nodules in the field. Other work has shown that *Ceanothus*, *Cercocarpus*, *Cowania* (now *Purshia*), *Chamaebatia* and *Purshia* can be nodulated by crushed nodules or soil from beneath *Chamaebatia* and *Cowania* (Nelson and Lopez, 1989; D. Nelson, personal communication).

6.5 Summary

The present patterns of distribution of actinorhizal plants and *Frankia* strains have been formed by the evolutionary histories of the plants, the movement of continents and adaptation of both symbionts to new environments as they have emerged over the past 120 million years. The eight actinorhizal plant families have very different distributions, estimated times of origin and fossil histories. The Casuarinaceae and Rhamnaceae appear to have a Gondwanan origin and the remaining actinorhizal families appear to have originated in Laurasia. The oldest fossil evidence provides a minimum age for some actinorhizal lineages, the Rhamnaceae and Myricaceae, in the Cretaceous (94 MYA). Molecular evidence suggests that the various lineages that eventually gave rise to present day actinorhizal plants were established shortly after the Mid- to Late Cretaceous appearance of eudicots about 125 MYA (Crane *et al.*, 1995; Magallon *et al.*, 1999).

This was a time period dominated by the separation of Gondwana from Laurasia. The major Groups of *Frankia* strains may have emerged at about the same time (Benson and Clawson, 2000).

Beyond distributions and origin dates, the actinorrhizal families differ in the degree of nodulation within each family. In the Casuarinaceae, Coriariaceae, Datisceae and Elaeagnaceae, all genera nodulate. In the Betulaceae, Myricaceae, Rhamnaceae and Rosaceae, a variable number of the lineages nodulate ranging from three of four genera in the Myricaceae to five of 122 genera in the Rosaceae. Molecular phylogenies have demonstrated that the actinorrhizal plant families have a common ancestor that was predisposed to nodulation (Soltis *et al.*, 1995). The number of times this predisposition became reality will never be known with any certainty. It is clear however that the symbiosis has been lost on many occasions as illustrated by the sporadic distribution of nodulating plants between and within orders, families and genera (Benson and Clawson, 2000).

At the local level, patterns of *Frankia* strain distribution are generally characterized by dominance of one particular strain depending on edaphic factors present in the soil (Clawson and Benson, 1999(Q12); Huguet *et al.*, 2001; McEwan *et al.*, 1999). Soil conditions appear at least as important as, if not determinative, in the strain of *Frankia* that succeeds in nodulating appropriate hosts. This conclusion is supported by direct demonstrations of dominance in alder and myrica stands (Clawson and Benson, 1999a, 1999b; McEwan *et al.*, 1999; Van Dijk, 1984(Q13)), and the observation that casuarinas are necessary for the persistence of casuarina strains when the plants are introduced outside their native range. This dominance effect forms the local pieces of the greater geographical mosaic.

A broader view of the patterns of symbiont associations provides some interesting observations related to vicariance of plant distributions. For example, there exist at least two cases where geographic separation has apparently led to a sorting of frankiae within a plant family. The cases include the South American Rhamnaceae versus the North American *Ceanothus* which interact with Clade 3 and Clade 2 frankiae respectively, and the Australian *Casuarina* versus the Pacific island species of *Gymnostoma* that interact mainly with Clade 1 and Clade 3 frankiae, respectively. It is possible that ancestors of these genera were infected by a greater range of *Frankia* strains that narrowed as the plants radiated into new environments. The mechanism of specialisation is unknown but might include differing abilities of *Frankia* strains to adapt to particular soils or climates, co-speciation of the plant and symbionts, or bottleneck effects on bacterial and plant diversity during climate fluctuations.

Another pattern that emerges indicates that the more widely distributed plants, such as *Alnus* and *Myrica* are infected by strains that are also widely distributed in soil, whereas the geographically limited plants *Casuarina* and *Allocasuarina* are infected by strains that are also geographically limited. Similarly, *Elaeagnus* species are globally distributed and strains that infect (Group 3) them also appear to be cosmopolitan. Frankiae that infect *Elaeagnus* species are also capable of infecting most nodulated members of the Rhamnaceae, plus *Gymnostoma* of the Casuarinaceae, and, to a lesser degree, some alders and many myricas. In this regard, less is known about the distribution of Group 2 frankiae. Those strains form the basal group of *Frankia*, and seem to be, as far as is known, obligate symbionts, although some evidence suggests that they can persist without the

continued presence of a host plant (Jeong, 2001). At present they are considered to have less diversity than strains in Groups 1 and 3. This lack of diversity may be an artifact of the few sequences that have been obtained or it may reflect the lack of a soil existence and increased reliance on the host. For that reason, one might anticipate that their distribution in soil parallels the patchy distribution of their hosts. This hypothesis remains to be tested.

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