SOURCES OF VARIATION IN THE SYMBIOTIC ASSOCIATION BETWEEN *ALNUS* AND *FRANKIA* IN INTERIOR ALASKA

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for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

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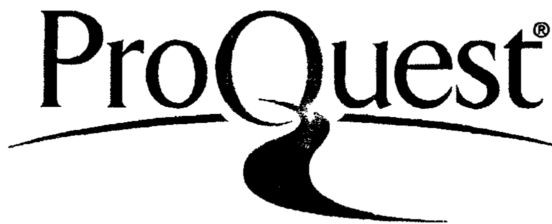

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by

Michael D. Anderson

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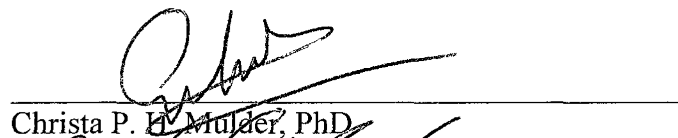

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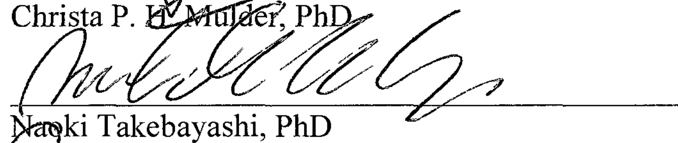

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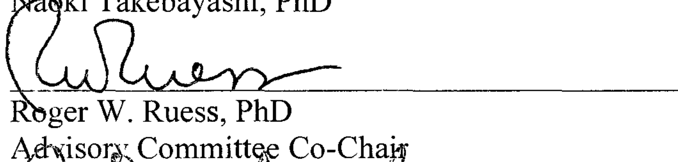

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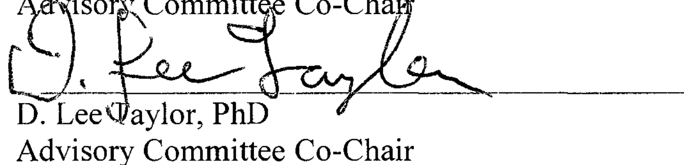

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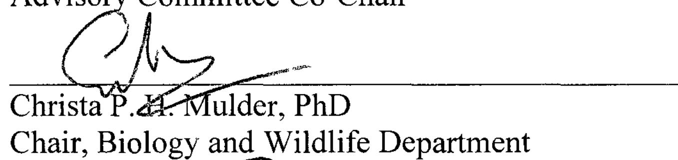

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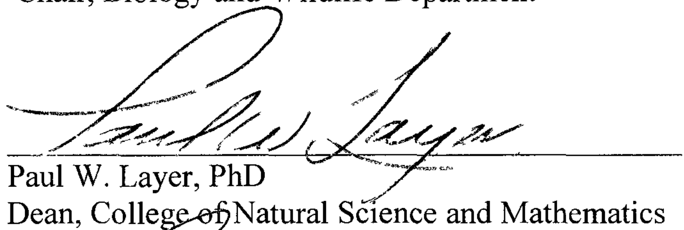

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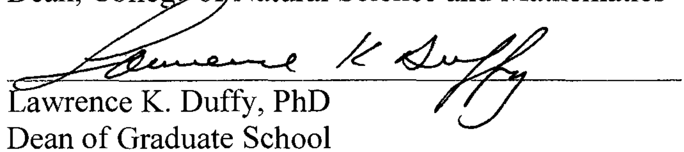

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ABSTRACT

Symbioses between plants and nitrogen (N) fixing bacteria are ecologically and economically important interactions with complex evolution and ecology. Theoretical and experimental studies suggest that host specificity and environmental variation are important determinants of both evolutionary and ecological patterns in such interactions, but detailed descriptions of these parameters in natural habitats are lacking for most N-fixing systems. The aim of this set of studies was to provide such information for the symbiosis between alder (*Alnus* spp.) plants and *Frankia* bacteria in interior Alaska. Major objectives were to determine whether: 1) different *Alnus* species (*A. tenuifolia* and *A. viridis*) associate with different *Frankia* genotypes in the field, 2) genetic composition and distribution of *Frankia* associated with the two hosts differ among successional habitats, 3) differences in *Frankia* are paralleled by differences in host physiology at plant (leaf N) or nodule (N-fixation rate) scales, and 4) occurrence of *Frankia* genotypes is correlated with specific soil variables. The two hosts were found to associate with *Frankia* representing largely different clades, even in sites in which the two hosts co-occurred. Genetic composition and spatial distribution of *Frankia* in *A. tenuifolia* nodules differed between successional habitats, but were largely consistent among replicate sites representing each habitat. Habitat-related differences were negligible in *A. viridis*. Leaf N differed among habitats for both hosts, but evidence for differences in N-fixation rate among *Frankia* genotypes was equivocal. Occurrence of the dominant genotype in early succession sites was strongly correlated with carbon : nitrogen ratio of

the mineral soil fraction, while in late succession the most common genotypes were correlated with carbon and nitrogen content of the organic soil fraction. These results demonstrate the importance of both host specificity and environmental variation in determining patterns of symbiont distribution in natural populations of *Alnus*. While these field results cannot distinguish between mechanisms impacting soil populations of *Frankia* directly and those involving selection of *Frankia* genotypes by host plants, it is hoped that the information provided will aid in the development of hypotheses and experiments most relevant to actual populations of these interacting species.

Dedicated to

Patty, Ian and Miles

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GENERAL INTRODUCTION

Background and purpose

Root nodule-based symbioses between plants and nitrogen (N) fixing bacteria are ecologically and economically important interactions with a complex evolutionary ecology. These interactions, which involve 13 bacterial genera from α and β proteobacterial and gram positive actinobacterial lineages and plants from ten families within the broad Eurosid I clade (Soltis et al. 1995; Sawada et al. 2003; Swensen and Benson 2008), collectively provide the bulk of natural inputs to the global N cycle and include many species important in agriculture, forestry and bioremediation. A wealth of descriptive studies along three lines: 1) host-symbiont specificity, 2) effects of genetic variation in bacteria on plant fitness, and 3) distribution of plant and bacterial genotypes in natural habitats, collectively suggest a complex evolutionary ecology in these interactions. Cross-inoculation studies have found that the specificity of associations ranges across a broad spectrum for both plant and bacterial partners (Young and Johnston 1989; Swensen and Benson 2008), and measurements of plant growth, N-fixation, and reproduction in some such studies suggest that bacterial partners in many N-fixing systems range from highly effective to relatively ineffective mutualists or even parasites (e.g., Parker 1995; Markham 2008; Heath 2010). In the field, distribution studies report wide variation in genetic structure of natural host populations among taxa, with some species showing strong structure (e.g., Parker 1996), while others are apparently panmictic (Bousquet and Lalonde 1990). On the bacterial side, genetic variation among

locales is commonly described in host nodules (e.g., Navarro et al. 1999; Kennedy et al. 2010), as well as in the soil-dwelling populations from which the host plants derive their symbionts (McInnes et al. 2004; Chaia et al. 2010). Such variation generally correlates with variation in both host distribution and environmental factors (Navarro et al. 1999; Lipus and Kennedy 2011), each of which may independently affect bacterial assemblages due to the facultative nature of the symbiosis for most bacterial genotypes and the independent existence of bacteria in soil (Benson and Silvester 1993; Mateos et al. 2011). These complex patterns in host-symbiont specificity, symbiotic behavior, and distribution of genetic variation suggest a complexity of underlying ecological and evolutionary causal mechanisms. Relatively recent theoretical developments in both root-nodule symbioses and interspecific interactions in general are providing an increasing diversity of hypothetical mechanisms which collectively hold much promise for explaining these patterns. Experimental tests of some of these mechanisms currently represent a leading edge in the understanding of these interactions. While such tests can demonstrate the potential importance of specific mechanisms, assessing their importance to natural populations requires, in addition, appropriate field methods and more detailed field observations than are usually collected. It is the purpose of this thesis to provide such tools for the symbiotic partnership between *Alnus* and *Frankia* in interior Alaska.

Evolutionary ecology of symbiotic mutualisms

N-fixing symbioses are generally considered to be mutualisms, based largely on the fact that each partner organism possesses physiological capabilities that potentially

benefit the other that the other does not possess: N-fixing bacteria are able to reduce atmospheric N_2 to plant-available NH_4^+ using plant-derived photosynthate as material and energy within a protected environment provided by the plant (the nodule) in which bacterial reproduction can also be significantly enhanced (West et al. 2002). Mutualisms have presented numerous difficulties to theoreticians for several decades. The major difficulties have been explaining: 1) ecological and evolutionary stability of mutualism, 2) maintenance of diversity in interacting partner species, and 3) specificity of associations. The question of stability dates at least to May's (1976) attempts to model mutualisms using population-based approaches that had been successfully applied to other interactions such as competition. May's models produced biologically unrealistic runaway positive feedbacks, leading him to famously characterize mutualism as an 'orgy of mutual benefaction'. Two broad solutions to this problem have been successfully implemented: 1) including mutualistic benefits that saturate, rather than boundlessly increase, as partner populations grow (e.g., Dean 1983), and 2) including not only the benefits but also the inherent costs of mutualism in theoretical models; i.e., each participant pays a cost of participation in the form of material and energy allocated to the partner organism that could otherwise be used for the participants' own growth and reproduction (Bronstein 2001). The latter solution, however, produced a further problem: the inherent cost of mutualistic behavior creates a strong potential for positive selection on genotypes that eschew investment in mutualistic behavior while retaining the ability to reap the rewards offered by the partner organism. The selective advantage possessed by such 'cheaters', if unchecked by other processes, could eventually undermine the

mutualism. In symbiotic interactions with large size disparity between partner organisms and horizontal transmission of symbionts between host generations (which include both N-fixing and mycorrhizal symbioses) the prevailing hypothesis for what checks this tendency is the ability of the host to selectively reward beneficial symbiont genotypes ('host-choice' (Simms and Taylor 2002)), and/or punish uncooperative or less cooperative genotypes ('host-sanctions' (Denison 2000)). While the interpretation of mechanisms proposed to represent such sanctions/choice has been recently challenged (Weyl et al. 2010), the choice/sanctions hypothesis may be considered the leading contender with respect to the question of stability-against-cheating in N-fixing symbioses, generating numerous theoretical (e.g., West et al. 2002; Foster and Kokko 2006; Marco et al. 2009a,b) and several experimental investigations (e.g., Kiers et al. 2003, 2006; Simms et al. 2006; Heath and Tiffin 2009). The empirical support it has received has been mixed, with some studies reporting supporting results (Kiers et al. 2003, 2006; Simms et al. 2006), others failing to find support (Marco et al. 2009a,b) and still others suggesting different mechanisms (Heath and Tiffin 2009). Other mechanisms proposed to prevent the takeover of cheating genotypes in symbiont populations include honest signaling of bacterial symbionts (Heath and Tiffin 2009), spatially targeted rewards to mutualistic genotypes in soil-dwelling populations (Simms and Bever 1998), and context-dependence of the non-cooperator phenotype (Heath and Tiffin 2007; Heath 2010); i.e., the symbiotic phenotype of a given symbiont genotype can vary, even flip between mutualism and parasitism, with variation in the genetic composition of the host and/or environmental conditions. Each of these proposed mechanisms has received some

empirical support in N-fixing (Heath and Tiffin 2007, 2009; Heath 2010) or mycorrhizal (Bever et al. 2009) systems.

Whatever the mechanism by which it is achieved, the possibility of selection against cheating genotypes creates another puzzle: if effective mechanisms for curtailing cheating exist, then why do cheating phenotypes demonstrably persist? Taken to the extreme, such selection mechanisms might even be expected to remove all but the most mutualistic genotypes from the symbiont population, paradoxically removing the need for a selection mechanism (Foster and Kokko 2006). Under such scenarios, both the persistence of cheaters and the relatively high level of genetic diversity commonly observed in natural N-fixing symbiotic bacteria (e.g., Benson and Dawson 2007) are puzzling. This, the second problem in theories of mutualism, has received an enormous amount of theoretical attention investigating both the factors that lead to stable coexistence, and the conditions under which such factors are likely to evolve. Theoretical solutions most applicable to N-fixing systems generally invoke spatial structure in host and/or symbiont distribution (e.g., Yu et al. 2001; Bronstein et al. 2003), some form of context-dependence (e.g., Bever 1999; Neuhauser and Fargione 2004), or both (e.g., Parker 1999; Gomulkiewicz et al. 2003; Nuismer et al. 2003); although it has also been shown that specific cost structures alone can lead to disruptive selection that produces both mutualists and cheaters (Ferriere et al. 2002). Spatial structure can take many forms, including environmental heterogeneity (e.g., Bruns 1995), dispersal limitation (Bronstein et al. 2003) and/or differences in dispersal capability (e.g., Yu et al. 2001), genetic structure in host populations (Parker 1996), structure in intraspecific

competition (Doebeli and Knowlton 1998), and distribution of other interacting species (e.g., Bronstein et al. 2003). Likewise, context-dependence of symbiotic outcomes has been suggested to occur among host genotypes (Heath and Tiffin 2007; Heath 2010) or populations (Hoeksema and Thompson 2007), across different nutrient regimes (Heath et al. 2010) and under the influence of other interactions (Piculell et al. 2008; Heath and Lau 2011), and all of these specific factors have received recent empirical support in N-fixing or mycorrhizal systems. Both spatial structure (e.g., differences in strength and/or direction of selection; genetic structure in interacting organisms) and context-dependence of interaction outcomes are fundamental components of the increasingly influential Geographic Mosaic Theory of Coevolution (Thompson 1994; 2005). The interaction of these two components in this theory, which also includes metapopulation dynamics (e.g., extinction/colonization regimes; gene flow patterns) as a third fundamental component, are proposed to account for a large portion of the ongoing evolution of interacting species, and to form a bridge between micro- and macro-evolutionary processes (e.g., Eldredge et al. 2005). The predictions of this theory have received considerable support for antagonistic interactions (Thompson 2005), and are beginning to be applied to root-microbe mutualistic systems (e.g., Heath 2010; Hoeksema 2010). Finally, it should be noted here that at least one mechanism for maintenance of diversity has been suggested by observational and experimental approaches – that a diversity of symbiont genotypes, rather than a single optimal one, might provide synergistic advantages to the host (Prat 1989; Martin et al. 2003) – that runs counter to theoretical considerations, which suggest that mixing of symbiont lineages in single host individuals should favor the evolution of

virulence in symbionts (Frank 1994; 1996), potentially undermining the mutualism. The resolution of this puzzle is currently an open question.

The third major challenge in mutualism theory concerns specificity of associations between host and symbiont taxa. While host-symbiont associations in antagonistic interactions are often highly specific, particularly in symbiotic parasitisms, mutualisms generally display much lower levels of specificity (Thompson 1994). This makes intuitive sense, given the prevailing hypothesis that specificity in parasitic interactions results primarily from an arms race in which parasite populations must continually adapt to host defenses, which must continually adapt to the adaptations of the parasite. This iterative process, together with the fact that parasite survival and reproduction in association with a given host generally requires very close matching between host and parasite in multiple traits (Thompson 1994), should produce very specific associations in parasitic interactions. By contrast, specificity in mutualistic interactions is thought to be limited by several factors which include the attraction of multiple species to resources offered by a host, dilution of mutualistic effects of each individual partner species by other partners, physiological difficulties of 'filtering out' disfavored partners, unpredictability in locating specialist partners, and swamping of selective pressures for increased specialization by other sources of selection (Boucher et al. 1982; Howe 1984; Thompson 1994). Despite this generally lower specificity compared with antagonistic interactions, specificity in mutualisms appears to increase with increasing intimacy of an association (e.g., obligate vs. facultative; symbiotic vs. non-symbiotic, vertical vs. horizontal transmission) (Borowicz and Juliano 1991;

Thompson 1994, 2005; Ollerton 2006), with some intimate mutualisms displaying striking specificity (e.g., Visik and McFall-Ngai 2000; Pellmyr 2003). In mutualisms, such specificity may be favored by the increasing benefits provided to both partners by the evolution of more closely matched phenotypic traits (Ollerton 2006). While this process is theoretically facilitated by vertical transmission of symbionts and/or obligate associations for at least one partner (Ollerton 2006), there is some evidence to suggest fitness gains associated with host specificity in some N-fixing associations (Wilkinson and Parker 1996; Simonet et al. 1999), which are generally facultative (Benson and Silvester 1993; Mateos et al. 2011) and transmit symbionts horizontally. However, given the constraints on mutualistic specialization listed above, it is not obvious how selection for such specialization might begin. One very popular hypothesis is that such specialized mutualisms begin as antagonistic interactions that subsequently evolve into mutualisms (Roughgarden 1975; Ewald 1987; Thompson 1994). This idea has garnered some support in obligate pollination systems such as the yucca-yucca moth association (Pellmyr and Thompson 1992), but the generality of this phenomenon remains an open question even among these well-studied intimate mutualisms (Kawakita et al. 2010). Under some circumstances, host-symbiont specificity may be evolutionarily maintained through 'phylogenetic tracking', the strict co-speciation of host and symbiont over evolutionary timescales, but this process is countered by several factors including: 1) incomplete specialization between host and symbiont taxa, 2) 'host-switching', in which host or symbiont evolves the ability to associate with a relatively distantly related partner,

possibly through pre-adaptation for physically or biochemically similar hosts, and 3) limited co-speciation in one of the partner lineages (Thompson 1994).

Theoretical progress and the importance of field studies

The foregoing review of mutualism theory was not meant to be exhaustive; rather, it was intended primarily to provide some historical context and, more importantly, an appreciation for the recent burst of theoretical developments and experimental activity relevant to the evolutionary ecology of root nodule symbioses. While further theoretical solutions to the three interrelated puzzles discussed above continue to be explored (e.g., Holland et al. 2004; Foster and Kokko 2006; Weyl et al. 2010), the diversity of ideas developed over the last 10-20 years seems rich enough to begin to explain the complex patterns of host specificity, symbiotic outcomes, and geographic distribution described in studies of root-nodule symbioses. Experimental tests of some of these processes (e.g., the host choice/sanctions hypothesis, the Geographic Mosaic of Coevolution) have been applied to plant root symbioses only recently and, together with continuing theoretical developments, may be considered the leading edge in the understanding of these interactions. Such experiments serve to demonstrate whether or not specific theoretical mechanisms can operate under ideal conditions, but the applicability and relative importance of such mechanisms to natural populations require additional field manipulations and detailed field surveys in addition to controlled lab experiments. Such multilateral approaches have not often been conducted. In perhaps the most comprehensive set of studies by a single research group, Parker and others investigated

the symbiosis between the wild annual legume *Amphicarpaea bracteata* and *Bradyrhizobium* spp. using a combination of field surveys of host nodules, cross-inoculation studies with performance measures, and genetic characterizations of both host and symbiont. These authors demonstrated intra-specific specialization of host plants on particular bacterial taxa (intra-generic within *Bradyrhizobium* spp.), as well as specialization of bacterial taxa on particular host genotypes, the latter of which appeared to favor both plant fitness (dry biomass) and bacterial fitness (nodule number) (Wilkinson et al. 1996; Wilkinson and Parker 1996; Parker and Spoerke 1998). These patterns of host-symbiont specialization were the most important factor in explaining distribution of symbiont diversity across spatial scales ranging from 50 m to 1000 km (Spoerke et al. 1996; Wilkinson et al. 1996; Parker and Spoerke 1998), and inoculation of plants drawn from different populations with bacteria obtained from the same field population significantly enhanced reproductive fitness compared with non-native crossings (Parker 1995).

The relatively simple picture suggested by the work of Parker and others, in which host-symbiont specificity appears to be driven by selection for mutualistic behavior on both partners and also underlies distribution of symbiont genotypes in natural populations, may be limited in applicability to systems which share important characteristics of the authors' study system, particularly annual habit, high degree of self-fertilization, and limited dispersal. The latter two factors, especially, are likely to be important in generating the patchy genetic structure the authors observed in plant populations, which created parallel structure in the bacterial populations via host-

symbiont specificity. Many ecologically important N-fixing plants, however, are long-lived perennial shrubs or trees with highly outcrossing reproductive modes. This is particularly true of the actinorhizal plants, which share the feature of forming root nodules with N-fixing bacteria of the actinomycete genus *Frankia* (Dawson 2008). All of the 25 plant genera (from eight disparate families) that make up this group are perennial (Dawson 2008; Swensen and Benson 2008), and most are quite long-lived. Wind and insect pollination are the most common pollination modes in this group, and dispersal of seeds by wind or animals is also very common (Dawson 2008). In such systems populations may be panmictic over large areas (Bousquet and Lalonde 1990), and selection on symbiotic traits is likely to vary with time over the course of the plants lifespan (Thompson 1994). Further, because significant environmental change can also occur over this time period, and bacterial symbionts are generally capable of independent existence in soil, environmental variation can generate differential selection regimes across environments. Such regimes may: 1) act independently on either or both partners, 2) act interactively on the symbiosis, or 3) simply correlate with variation in selection across the lifespan of the plant. Finally, these complex environmental effects can both be constrained by inherited patterns of host-symbiont specificity, and contribute to the further evolution of specificity. Disentangling these effects and assessing the relative importance of various hypothetical mechanisms to the evolutionary ecology of specific systems obviously requires a multifaceted approach. A straightforward place to begin is with detailed descriptions of the patterns of host-symbiont associations in natural systems, yet such characterizations are surprisingly uncommon. The primary goal of this

thesis is to provide such data for the *Alnus-Frankia* symbiosis in interior Alaska. I have focused on describing four aspects of this symbiosis in natural habitats, based on their importance in theoretical treatments: 1) host specificity, 2) environmental variation, 3) spatial patterns, and 4) physiological variation.

This dissertation is divided into four chapters. Chapter one provides a description of what is known about the evolution and ecology of the *Alnus-Frankia* symbiosis, with a focus on the utility of this system for investigating theoretical questions in symbiotic mutualisms. Chapter two is a study investigating the utility of the commonly-used acetylene-reduction assay (ARA) for quantifying physiological N-fixation rates across environments in the two *Alnus* species I studied, in order to determine whether the ARA could be used to investigate differences among *Frankia* strains in N-fixation rate in host nodules. Chapters three and four describe patterns of association between *Alnus* and *Frankia* in natural habitats in the vicinity of the Tanana River floodplain near Fairbanks, Alaska. Chapter three is a broad study comparing the effects of environmental variation and host specificity on the genetic composition of *Frankia* symbionts in root nodules of *Alnus incana* ssp. *tenuifolia* (syn. *A. tenuifolia*) and *A. viridis* ssp. *fruticosa* (syn. *A. crispa*, hereafter *A. viridis*), and on host physiology (N-fixation rate and leaf N). Chapter four is a deeper investigation of the environmental patterns in symbiont composition in *A. tenuifolia* nodules discovered in the chapter three study, which includes much larger sample sizes and a characterization of the spatial patterns within and among host plants in the sites included for this host in the previous study.

REFERENCES

Benson DR, Dawson JO (2007) Recent advances in the biogeography and genecology of symbiotic *Frankia* and its host plants. *Physiologia Plantarum,* **130**, 318-330.

Benson DR, Silvester WB (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiological Reviews,* **57**, 293-319.

Bever JD (1999) Dynamics within mutualism and the maintenance of diversity: inference from a model of interguild frequency dependence. *Ecology Letters,* **2**, 52-61.

Bever JD, Richardson SC, Lawrence BM, Holmes J, Watson M (2009) Preferential allocation to beneficial symbiont with spatial structure maintains mycorrhizal mutualism. *Ecology Letters,* **12**, 13-21.

Borowicz VA, Juliano SA (1991) Specificity in host-fungus associations: do mutualists differ from antagonists? *Evolutionary Ecology,* **5**, 385-392.

Boucher DH, James S, Keeler KH (1982) The ecology of mutualism. *Annual Review of Ecology and Systematics,* **13**, 315-347.

Bousquet J, Lalonde M (1990) The genetics of actinorhizal Betulaceae. In *The Biology of* Frankia *and Actinorhizal Plants* (eds. Schwintzer CR, Tjepkema JD). pp. 239-261, Academic Press, Inc., San Diego, CA.

Bronstein JL (2001) The costs of mutualism. *American Zoologist,* **41**, 825-839.

Bronstein JL, Wilson WG, Morris WF (2003) Ecological dynamics of mutualist/antagonist communities. *The American Naturalist,* **162S**, S25-S39.

Bruns TD (1995) Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant and Soil,* **170**, 63-73.

Chaia EE, Wall LG, Huss-Danell K (2010) Life in soil by the actinorhizal root nodule endophyte *Frankia*: a review. *Symbiosis,* **51**, 201-226.

Dawson JO (2008) Ecology of actinorhizal plants. In: *Nitrogen Fixation: Origins, Applications, and Research Progress, Vol. 6 Nitrogen-fixing Actinorhizal Symbioses.* (eds Pawlowski K, NewtonWE) Springer, Dordrecht, The Netherlands.

Dean AM (1983) A simple model of mutualism. *The American Naturalist,* **121**, 409-417.

Denison RF (2000) Legume sanctions and the evolution of symbiotic cooperation by rhizobia. *The American Naturalist,* **156**, 567-576.

Doebeli M, Knowlton N (1998) The evolution of interspecific mutualism. *Proceedings of the National Academy of Sciences USA,* **95**, 8676-8680.

Eldredge N, Thompson JN, Brakefield PM, Gavrilets S, Jablonski D, Jackson JBC, Lenski RE, Lieberman BS, McPeek MA, Miller III W (2005) The dynamics of evolutionary stasis. *Paleobiology,* **31**, 133-145.

Ewald PW (1987) Transmission modes and evolution of the parasitism-mutualism continuum. *Annals of New York Academy of Sciences*, **503**, 295-306.

Ferriere R, Bronstein JL, Rinaldi S, Law R, Gauduchon M (2002) Cheating and the evolutionary stability of mutualisms. *Proceedings of the Royal Society of London B,* **269**, 773-780.

Foster KR, Kokko H (2006) Cheating can stabilize cooperation in mutualisms. *Proceedings of the Royal Society B,* **273**, 2233-2239.

Frank SA (1994) Kin selection and virulence in the evolution of protocells and parasites. *Proceedings of the Royal Society of London B*, **258**, 153-161.

Frank SA (1996) Host-symbiont conflict over the mixing of symbiotic lineages. *Proceedings of the Royal Society of London B,* **263**, 339-344.

Gomulkiewicz R, Nuismer SL, Thompson JN (2003) Coevolution in variable mutualisms. *The American Naturalist* **162S**, S80-S93.

Heath KD (2010) Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. *Evolution,* **64**, 1446-1458.

Heath KD, Lau JA (2011) Herbivores alter the fitness benefits of a plant-rhizobium mutualism. *Acta Oecologica,* **37**, 87-92.

Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial mutualists. *Proceedings of the Royal Society B,* **274**, 1905-1912.

Heath KD, Tiffin P (2009) Stabilizing mechanisms in a legume-rhizobium mutualism. *Evolution,* **63**, 652-662.

Heath KD, Stock AJ, Stinchcombe JR (2010) Mutualism variation in the nodulation response to nitrate. *Journal of Evolutionary Biology,* **23**, 2494-2500.

Hoeksema JD (2010) Ongoing evolution in mycorrhizal interactions. *New Phytologist,* **187**, 286-300.

Hoeksema JD, Thompson JN (2007) Geographic structure in a widespread plant-mycorrhizal interaction: pines and false truffles. *Journal of Evolutionary Biology,* **20**, 1148-1163.

Holland JN, DeAngelis DL, Schultz ST (2004) Evolutionary stability of mutualism: interspecific population regulation as an evolutionary stable strategy. *Proceedings of the Royal Society of London B,* **271**, 1807-1814.

Howe HF (1984) Constraints on the evolution of mutualism. *The American Naturalist,* **123**, 764-777.

Kawakita A, Okamoto T, Goto R, Kato M (2010) Mutualism favors higher host specificity than does antagonism in plant-herbivore interaction. *Proceedings of the Royal Society of London B,* **277**, 2765-2774.

Kennedy PG, Weber MG, Bluhm AA (2010) *Frankia* bacteria in *Alnus rubra* forests: genetic diversity and determinants of assemblage structure. *Plant and Soil,* **335**, 479-492.

Kiers ET, Rousseau RA, Denison RF (2006) Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly. *Evolutionary Ecology Research,* **8**, 1077-1086.

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. *Nature,* **425**, 78-81.

Lipus A, Kennedy PG (2011) *Frankia* assemblages associated with *Alnus rubra* and *Alnus viridis* are strongly influenced by host species identity. *International Journal of Plant Sciences,* **172**, 403-410.

McInnes A, Thies JE, Abbott LK, Howieson JG (2004) Structure and diversity among rhizobial strains, populations and communities – a review. *Soil Biology and Biochemistry,* **36**, 1295-1308.

Marco DE, Carbajal JP, Cannas S, Pérez-Arnedo R, Hidalgo-Perea A, Olivares J, Ruiz-Sainz J, Sanjuán J (2009a) An experimental and modelling exploration of the host-sanctions hypothesis in legume-rhizobia mutualism. *Journal of Theoretical Biology,* **259**, 423-433.

Marco DE, Pérez-Arnedo R, Hidalgo-Perea A, Olivares J, Ruiz-Sainz JE, Sanjuán J (2009b) A mechanistic molecular test of the plant-sanction hypothesis in legume-rhizobia mutualism. *Acta Oecologica,* **35**, 664-667.

Markham JH (2008) Variability of nitrogen-fixing *Frankia* on *Alnus* species. *Botany,* **86**, 501-510.

Martin KJ, Tanaka Y, Myrold DD (2003) Dual inoculation increases plant growth with *Frankia* on red alder (*Alnus rubra* Bong.) in fumigated nursery beds. *Symbiosis,* **34**, 253-260.

Mateos PF, Rivas R, Robledo M, Velásquez E, Martínez-Molina E, Emerich DW (2011) The path of rhizobia: from free-living soil bacterium to root nodulation. In:*Ecological Aspects of Nitrogen Metabolism in Plants* (eds Polacco JC, Todd CD). pp. 167-194, Wiley & Sons, West Sussex UK.

May RM (1976) Models for two interacting populations. In: *Theoretical Ecology* (ed. May RM), pp.49-70 Saunders, Philadelphia.

Navarro E, Jaffre T., Gauthier D, Gourbiere F, Rinaudo G, Simonet P, Normand P (1999) Distribution of *Gymnostoma* spp. microsymbiotic *Frankia* strains in New Caledonia is related to soil type and to host-plant species. *Molecular Ecology,* **8**, 1781-1788.

Neuhauser C, Fargione JE (2004) A mutualism-parasitism continuum model and its application to plant mycorrhizae interactions. *Ecological Modelling,* **177**, 337-352.

Nuismer SL, Gomulkiewicz R, Morgan MT (2003) Coevolution in temporally variable environments. *The American Naturalist,* **162**, 195-204.

Ollerton J (2006) "Biological barter": patterns of specialization compared across different mutualisms. In *Plant-pollinator interactions: from specialization to generalization.* pp. 411-435 (eds. Waser NW, Ollerton J) University of Chicago Press.

Parker MA (1995) Plant fitness variation caused by different mutualist genotypes. *Ecology,* **76**, 1525-1535.

Parker MA (1996) Cryptic species within *Amphicarpaea bracteata* (Leguminosae): evidence from isozymes, morphology, and pathogen specificity. *Canadian Journal of Botany,* **74**, 1640-1650.

Parker MA (1999) Mutualism in metapopulations of legumes and rhizobia. *The American Naturalist,* **153 Suppl**, S48-S60.

Parker MA, Spoerke JM (1998) Geographic structure of lineage associations in a plant-bacterial mutualism. *Journal of Evolutionary Biology,* **11**, 549-562.

Pellmyr O (2003) Yuccas, yucca moths, and coevolution: a review. *Annals of the Missouri Botanical Garden,* **90**, 35-55.

Pellmyr O, Thompson JN (1992) Multiple occurrence of mutualism in the yucca moth lineage. *Proceedings of the National Academy of Sciences USA,* **89**, 2927-2929.

Piculell BJ, Hoeksema JD, Thompson JN (2008) Interactions of biotic and abiotic environmental factors in an ectomycorrhizal symbiosis, and the potential for selection mosaics. *BMC Biology*, **6**, 23.

Prat D (1989) Effects of some pure and mixed *Frankia* strains on seedling growth in different *Alnus* species. *Plant and Soil,* **113**, 31-38.

Roughgarden J (1975) Evolution of marine symbiosis — a simple cost-benefit model. *Ecology,* **56**, 1201-1208.

Sawada H, Kuykendall LD, Young JM (2003) Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. *Journal of General and Applied Microbiology,* **49**, 155-179.

Simms EL, Bever JD (1998) Evolutionary dynamics of rhizopine within spatially structured rhizobium populations. *Proceedings of the Royal Society of London B,* **265**, 1713-1719.

Simms EL, Taylor DL (2002) Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and Comparative Biology,* **42**, 369-380.

Simms EL, Taylor DL, Povich J, Shefferson RP, Sachs JL, Urbina M, Tausczic Y (2006) An empirical test of partner-choice mechanisms in a wild legume-rhizobium interaction. *Proceedings of the Royal Society B,* **273**, 77-81.

Simonet P, Navarro E, Rouvier C, Reddell P, Zimpfer J, Dommergues Y, Bardin R, Combarro P, Hamelin J, Domenach A-M, Gourbiére F, Prin Y, Dawson JO, Normand P (1999) Coevolution between *Frankia* population and host plants in the family Casuarinaceae and consequent patterns of global dispersal. *Environmental Microbiology,* **1**, 525-533.

Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. *Proceedings of the National Academy of Sciences USA,* **92**, 2647-2651.

Spoerke JM, Wilkinson HH, Parker MA (1996) Nonrandom genotypic associations in a legume-*Bradyrhizobium* mutualism. *Evolution,* **50**, 146-154.

Swensen SM, Benson DR (2008) Evolution of actinorhizal host plants and *Frankia* endosymbionts. In:*Nitrogen Fixation: Origin, Applications, and Research Progress, Vol. 6 Nitrogen-Fixing Actinorhizal Symbioses* (eds Pawlowski K, Newton WE) Springer, Dordrecht, The Netherlands.

Thompson JN (1994) *The Coevolutionary Process*. University of Chicago Press, Chicago, Il.

Thompson JN (2005) *The Geographic Mosaic of Coevolution*. University of Chicago Press, Chicago Il.

Visik KL, McFall-Ngai MJ (2000) An exclusive contract: specificity in the *Vibrio fisceri-Euprymna scolopes* partnership. *Journal of Bacteriology,* **182**, 1779-1787.

West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proceedings of the Royal Society B,* **269**, 685-694.

Weyl EG, Frederickson ME, Yu DW, Pierce NE (2010) Economic contract theory tests models of mutualism. *Proceedings of the National Academy of Sciences USA,* **107**, 15712-15716.

Wilkinson HH, Parker MA (1996) Symbiotic specialization and the potential for genotypic coexistence in a plant-bacterial mutualism. *Oecologia,* **108**, 361-367.

Wilkinson HH, Spoerke JM, Parker MA (1996) Divergence in symbiotic compatibility in a legume-*Bradyrhizobium* mutualism. *Evolution,* **50**, 1470-1477.

Young JPW, Johnston AWB (1989) The evolution of specificity in the legume-rhizobium symbiosis. *Trends in Ecology and Evolution,* **4**, 341-349.

Yu DW, Wilson HB, Pierce NE (2001) An empirical model of species coexistence in a spatially structured environment. *Ecology,* **82**, 1761-1771.

Chapter 1:

Two in the Far North: The Alder-*Frankia* Symbiosis, with an Alaskan Case Study[1]

INTRODUCTION

Root-nodule symbioses, in which plants house nitrogen (N) fixing bacteria in specialized organs derived from fine roots (the nodules), occur between 10 families of angiosperms and at least 13 genera of bacteria (Sawada *et al.* 2003). For plants capable of supporting such a symbiosis, the availability of atmospheric N it provides can be a significant ecological benefit, allowing colonization of N-poor soils or alternative N-utilization strategies. Such plants also frequently act as keystone organisms, providing the majority of N entering the N cycle in the ecosystems in which they occur.

As for any interaction between organisms, the outcome (mutualism vs. parasitism) and evolutionary trajectory of root-nodule symbioses are subject to modulation both by factors intrinsic to the interaction (*e.g.*, host-symbiont specificity, dispersal mechanisms, etc.) and a variety of extrinsic factors (*e.g.*, availability of light or nutrients, interactions with other organisms, etc.). Variation in such factors can produce very different evolutionary dynamics among local populations, which in turn interact at regional and ultimately global levels to determine macroevolutionary patterns of the interaction (Thompson 1994, 2005). Because N availability in soils commonly limits plant

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productivity, factors modulating N-fixing interactions at the local scale can affect local ecosystem function, and the upward cascade of evolutionary effects just described can have broad implications for landscape and regional-scale processes. Many root-nodule-based symbiotic systems are also important crops (*e.g.*, soybean, alfalfa), or have applications in forestry and/or bioremediation (*e.g.*, many species of alder). Detailed characterization of geographic variation in N-fixing symbioses can thus provide insights into the evolutionary ecology of inter-specific interactions, into controls over key ecosystem processes, and may have important economic and environmental applications as well.

The symbiosis that occurs between alder (genus *Alnus* Mill. (Betulaceae)) and N-fixing *Frankia* bacteria in Alaskan ecosystems is an excellent subject for such studies, for several reasons. First, the alder-*Frankia* system is widespread and diverse, occurring circum-polarly in the northern hemisphere in widely varied environmental contexts and involving several plant species as well as bacterial strains that vary widely in physiology, interaction strategy, and effects on host plants. Alaska provides a microcosm of this global diversity in which alder occurs in habitats ranging from coastal rain forests to arctic tundra at densities ranging from sporadic to abundant. Second, complementing the diversity of regional habitats available in Alaska are: 1) wide differences among local habitats due to variation in landscape position and successional dynamics, and 2) a high degree of habitat redundancy that results from low regional plant diversity and relatively predictable successional pathways following deposition or disturbance (Chapin *et al.* 2006). Such a combination makes for relatively straightforward habitat replication,

allowing methodologically robust testing of ecological hypotheses. Third, the ecological impacts of alder in the region are significant and, for some species, are among the best-characterized of any naturally-occurring N-fixing system.

This chapter presents recent work conducted across several ecosystems in both arctic and boreal biomes characterizing variation in the alder-*Frankia* interaction, and modulation of N-fixation rates and inputs by some biotic and abiotic factors in boreal forests. Appropriate background information including biology of *Alnus* and *Frankia* genera, evolutionary ecology of the *Alnus-Frankia* interaction, and the ecology of alder in Alaska precedes this discussion.

Background

Alder

Taxonomy, distribution and general biology

Depending on the taxonomic treatment, alder encompasses between 29 and 47 species of trees and shrubs with global distribution throughout the northern hemisphere and south into the Andes (Baker and Schwintzer 1990; Chen and Li 2004). Furlow (1979) grouped *Alnus* species into three subgenera – *Alnus*, *Alnobetula*, and *Clethropsis* – a treatment which is largely supported by DNA sequence data (Chen and Li 2004)(Figure 1.1). The three subgenera have distinct global distribution patterns, with subg. *Alnus* occupying most of Europe and N. America and a portion of Andean S. America, subg. *Alnobetula* distributed over most of Siberia, NE Asia and N. America, and subg. *Clethropsis* highly disjunct in southern Asia and the United States. Within these ranges,

individual species distributions vary from highly cosmopolitan to highly restricted. In the former case is *A. viridis*, various subspecies of which occur continuously across the North American arctic from Alaska to Greenland, south as far as California in the western US and North Carolina in the eastern US, and throughout most of Europe and parts of Asia, as well. An example of a restricted species is *A. maritima*, which consists of small, disjunct populations each representing a distinct subspecies in Delaware, Maryland, and Oklahoma (Furlow 1979; Schrader and Graves 2004). Different alder species also vary to a large degree in preferred habitat, with some species such as *A. incana* ssp. *rugosa* restricted to wet habitats (Furlow 1979), and others such as *A. jorullensis* and *A. viridis* able to occupy relatively dry habitats, including mid-elevation intermittent streams in central Mexico (Furlow 1979) and rocky slopes in subalpine tundra of boreal Alaska, respectively (personal observation).

Alder species can also vary widely in growth form, both within and among species. *A. viridis*, for example, can occur as a dwarf shrub in arctic tundra habitats, a large shrub in boreal forests, and a small tree in boreal and temperate forests (Viereck and Little 2007). Several species (*e.g.*, *A. incana*) occur as large (~4 m height) shrubs in boreal and temperate regions, and some species (*e.g.*, *A. glutinosa*, *A. rubra*) are moderately sized trees (McVean 1953; Viereck and Little 2007). Both tree and shrub forms can reproduce clonally through formation of multiple stems, and in shrub forms multiple clumps of stems can be formed by genetically individual plants via lateral stem growth and adventitious rooting (McVean 1953; Wilson *et al.* 1985; Harrington 2006). With respect to sexual reproduction, alders are monoecious, mostly self-incompatible,

and wind-pollinated. The small seeds are also wind-dispersed, and bear small wings in some species to aid in dispersal (Furlow 1979).

N-fixation

All known *Alnus* species form root-nodule symbioses with N-fixing bacteria belonging to the genus *Frankia* (Dawson 2008). While such associations can be energetically costly (Lundquist 2005), the ability to fix atmospheric N also provides several ecological advantages. For example, alders are often among the first plants to colonize N-poor primary seres following glacial retreat (Chapin *et al.* 1994), floodplain deposition (Walker and Chapin 1986), or volcanic eruption (Heilman 1990), and are also important secondary colonists following disturbance such as fire and tree harvest (Harrington 2006; Mitchell and Ruess 2009a). The ability of alder to exploit such N-limited habitats is almost certainly related to its relationship with *Frankia*. Alder can persist in the understory throughout succession, and can colonize mineral soil exposed by animal activity or windthrow in mature forests (Wurtz 1995). The ability of alder to compete in these habitats may be enhanced by an N source independent of soil supplies, which can allow *Alnus* species to supplement available soil N (Markham and Zekveld 2007) or, in some species, to drop leaves without N resorption, extending their seasonal growth period relative to non-N-fixing plants (Tateno 2003). The N-rich leaf litter that results, together with root and nodule turnover in the soil, can enhance N availability in alder-associated soils and plants (*e.g.*, Walker and Chapin 1986; Chapin *et al.* 1994), which can have significant ecosystem effects.

Regulation of N-fixation by Alder

The ecological advantages of N-fixation come at a cost to the host plant. N fixation is metabolically expensive in terms of respiration (CO_2 evolution) (Tjepkema and Winship 1980; Lundquist 2005), and phosphorous (P) demand (Gentili and Huss-Danell 2003), and the symbiosis requires tight control to avoid over-investment in nodules (Simms and Taylor 2002). Presumably to minimize such costs, *Alnus* species regulate both nodulation and N-fixation rate under varying environmental conditions.

Nodule investment in *Alnus* is regulated at both local and systemic levels; induction of a nodule on a developing root inhibits further nodulation on that root and contributes to a systemic suppression of nodulation over the entire root system (Wall and Huss-Danell 1997; Wall and Berry 2008). Both processes are linked to the N and P status of the plant; nodule inhibition in response to N fertilization is well-known in *Alnus* (MacConnell and Bond 1957; Gentili and Huss-Danell 2003; Laws and Graves 2005), but systemic inhibition can be countered by addition of P (Gentili and Huss-Danell 2003). In greenhouse-grown *A. incana* seedlings the level of nodulation is sensitive to the N:P ratio of the growth medium (Wall 2000), and in mature *A. incana* ssp. *tenuifolia* plants in Alaskan field sites addition of P results in increased nodulation (Uliassi and Ruess 2002).

Regulation of symbiotic N fixation in *Alnus* also occurs at the level of N-fixation rate. Availability of soil N has been observed to inhibit N-fixation rate in *Alnus* at both the whole-plant level (*e.g.*, Stewart and Bond 1961), and per unit nodule biomass ('specific-N-fixation', SNF) (*e.g.*, Gentili and Huss-Danell 2003; Laws and Graves

2005). In contrast to its nodulation effects, P does not appear to counteract N inhibition of SNF, which appears to act systemically across the root system (Gentili and Huss-Danell 2003).

Frankia

Phylogenetics and general biology

Frankia is an actinomycete, belonging to the high G+C group of Gram-positive bacteria. *Frankia* are mostly filamentous in morphology but, unusually for bacteria, form several distinct cell morphologies based on a division of labor among cell types. In culture most of the *Frankia* cell mass consists of filamentous cells ('hyphae'), while N-fixation is restricted to specialized cells called vesicles, which are generally spherical and have thickened cell membranes for protection of oxygen-labile nitrogenase -- the enzyme complex responsible for N-fixation. Reproductive spores, localized in sporangia, are formed freely in culture and occasionally in symbiosis (Benson and Silvester 1993), from which they appear to enter the soil during nodule senescence (Holman and Schwintzer 1987).

There is a great deal of evidence to suggest that most symbiotic *Frankia* strains are also capable of independent growth in soil and thus are only facultatively symbiotic with plants. Circumstantial evidence for this includes the ready isolability of numerous strains from nodules on non-specialized media (Lechevalier and Lechevalier 1990), the frequent occurrence of symbiotic *Frankia* in soils devoid of any known hosts (*e.g.*, Batzli *et al.* 2004; Maunuksela *et al.* 1999), the presence of *Frankia* in decaying wood (Li *et al.*

1997), and the observation that population size of soil *Frankia* estimated by 'trapping' with *Alnus* seedlings is not correlated with estimates based on *Frankia*-specific PCR of DNA from the same soils (Myrold and Huss-Danell, 1994). Direct evidence for saprophytic growth has been obtained using two *Alnus*-infective strains: one isolated from *A. glutinosa* and one from *A. rubra*. Mirza *et al.* (2007) inoculated both strains into two types of media: non-sterile soil with very low organic matter, and sterile mineral medium with no source of C or N. Both strains showed no growth in either medium. However, when each medium was amended with ground leaf litter from *A. glutinosa* the *A. glutinosa* isolate showed significant temporal increases in filament length, cell number per filament, and RNA content. Interestingly, the *A. rubra* isolate showed significant decreases in the same parameters over the experiment, indicating the effect of C source on *Frankia* growth can be host-strain specific. Subsequent work by these authors has also recently demonstrated growth of several *Frankia* strains on root exudates from birch, and a small group of related strains appear to be capable of growth on birch litter (Mirza *et al.* 2009a).

In addition to *Alnus*, *Frankia* also forms root-nodule based symbioses with 24 other plant genera from eight families (Benson and Silvester 1993). Collectively these associations are referred to as 'actinorhizal' to distinguish them from the 'rhizobial' associations which occur between legume plants (and *Parasponia* of the Ulmaceae) and several genera of N-fixing proteobacteria. Molecular phylogenetic studies indicate that relationships among *Frankia* strains broadly follow host infection patterns. In a broad study using two loci from isolates and nodule DNA extracts from a total of 17 host plant

genera, Clawson *et al.* (2004) discerned three clusters within *Frankia* with mostly non-overlapping host infection ranges: Clade I, containing strains symbiotic with plants in the Rosaceae, Datiscaceae and *Ceanothus* spp. (Rhamnnaceae), Clade II, symbiotic with the 'higher' Hamamelididae (Myricaceae, Casuarinaceae and *Alnus* spp.), and Clade III, symbiotic with members of the Elaeagnaceae and most of the actinorhizal Rhamnaceae. Although Clade II and Clade III appear to be sister groups, very little overlap in infection ranges occurs with respect to *Alnus*-infective strains (*e.g.*, Normand *et al.* 1996; Welsh *et al.* 2009). Exceptions include a few related strains from Clade III that are able to infect alder to a limited degree (Bosco *et al.* 1992; Lumini *et al.* 1996), and a recent report of a Clade II sequence derived from an *Elaeagnus angustifolia* nodule (Mirza *et al.* 2009b).

The alder-*Frankia* symbiosis

Frankia phylogenetics and host specificity

Phylogenetic studies of *Frankia* have primarily focused on examining relationships among strains belonging to different host infection groups, so little information has historically been available on relationships within the *Alnus*-infective group. However, recent studies examining large sample collections from *Alnus* species suggest two patterns especially relevant to the symbiosis between alder and *Frankia*: 1) the possible paraphyly of this group and, 2) variability in host-specificity among clusters of related sequences.

Phylogenies of the entire *Frankia* genus consistently resolve a single apparent clade containing strains infective on *Alnus*, *Myrica*, and *Casuarina* species (*e.g.*,

Normand *et al.* 1996; Clawson *et al.* 2004), but such studies typically include only a few representatives from each host genus. Studies of multiple *Alnus*-infective strains occasionally resolve a second group which is outgroup to both Clade II and Clade III *Frankia* (Hahn 2008; Welsh *et al.* 2009; Mirza *et al.* 2009b). Among such studies there is a trend toward better resolution of this group with larger samples both in terms of sequence number and sequence length (Hahn *et al.* 1999; Hahn 2008), and in two recent large datasets the branch defining this group is statistically significant (Welsh *et al.* 2009; Mirza *et al.* 2009b).

Phylograms in such studies also suggest that host specificity among smaller clades within both apparent groups of *Alnus*-infective *Frankia* are highly variable, with all members of some groups derived from nodules of a single host species or subgenus, and other groups made up of samples from plants in multiple subgenera or even different families (Hahn 2008; Welsh *et al.* 2009). Such a pattern is somewhat at odds with cross-inoculation studies characterizing infectivity of *Frankia* isolates on a range of alder species. Table 1.1 summarizes the results from 10 such studies. Collectively, they seem to indicate low levels of host specificity among alder species and *Frankia* strains; while some strains appear to be infective primarily on hosts closely related to their host of origin (*e.g.* AvsI2), for the most part strains derived from most host species appear to be able to form nodules across a broad range without regard to host phylogeny (*e.g.*, strains derived from both host subgenera are mostly infective on plants of the other subgenus) or geography (*e.g.*, strains derived from *A. glutinosa* and *A. rubra* are mostly compatible with both hosts, despite the former's native Eurasian range and the latter's restricted

range within North America). While such studies may overestimate the breadth of host-symbiont associations in natural environments (Simonet *et al.* 1999; Huguet *et al.* 2005), they provide an idealized range of potential associations between both organisms that is likely to be limited mainly by relatively 'hard' genetic barriers in each partner. In natural habitats such potential ranges are further subject to a variety of softer barriers.

The data in Table 1.1 are based on host compatibility in very broad terms – the ability of strains to form nodules on a small sample of plants for each host species under greenhouse conditions. Strains with the same host range by this criterion can vary widely in finer-scale compatibility-related traits such as the time required for nodulation of a given host (Nesme *et al.* 1985), number and/or biomass of nodules formed on a host (Hooker and Wheeler 1987; Prat 1989; Weber *et al.* 1989) and even the ability to nodulate different plant genotypes within a host species (Hahn *et al.* 1988; van Dijk and Sluimer 1994). Variation in such traits seems likely to contribute to the restricted realized range of associations in natural vs. artificial habitats (others are discussed in the following three sections), and reflects the complex evolutionary interplay at work between host and symbiont.

Variation in host interactions

The interaction between alder and *Frankia* is generally considered a mutualism which, by definition, means that it is beneficial to both the plant and the bacterium. The plant receives access to atmospheric N which can provide a competitive edge in some environments, and the bacterium receives access to an exclusive environment, the nodule

interior, and a source of C, photosynthate provided by the plant. Under this simple conceptual model one might expect selection pressure on each partner to increase or at least maintain a constant level of benefit to the other, since this also benefits itself by maintaining or increasing access to the resource provided by the other organism. However, the interaction also carries an inherent cost to each partner in the form of allocation of materials and energy to the symbiotic partner that could otherwise be used to directly support its own growth and reproduction (Bronstein 2001). This cost results in much more complex evolutionary dynamics in at least two ways: 1) the production of evolutionary pressure on each partner toward 'cheating' behavior; *i.e.*, the development of traits that allow it to reap the benefits of the interaction while minimizing or even eliminating the costs, and 2) variation in the cost:benefit ratio of the interaction for both partners with varying biotic and abiotic environmental conditions (Thompson 1982; Egger and Hibbett 2004). Such complexity is evident in several respects in the alder-*Frankia* symbiosis, including: 1) the spectrum of symbiotic behaviors exhibited by different bacterial strains, 2) varying compatibility of host genotypes within a species with particular bacterial strains, and 3) interacting effects of bacterial strain, host species and environmental conditions on traits related to host fitness.

Variation in symbiotic behavior

Rather than distinct categories of interaction, mutualism and parasitism are now generally thought of as representing opposite ends of a spectrum of symbiotic outcomes mediated by the cost of the interaction to each partner (Bronstein 1994). A mutualistic outcome occurs when the benefit of the interaction exceeds the cost for both partners; when this occurs for only one partner the result is a parasitic interaction. One result of the inherent cost of symbiosis is the production of evolutionary pressure to 'cheat'--*i.e.*, to utilize resources normally devoted to maintenance of the partnership for an organisms own growth and reproduction. Because a single plant can simultaneously associate with multiple microbial genotypes but not vice-versa and bacteria can complete several generations in a single plant lifetime, this pressure is probably most intense on the microbe (Denison and Kiers 2004; Kiers and Denison 2008). In the alder-*Frankia* symbiosis, *Frankia* strains appear to exhibit the full spectrum of symbiotic behaviors, from mutualism to parasitism.

The mutualistic nature of most strains is suggested both by field observations--*e.g.*, the universal occurrence of nodules on alders in the field (Dawson 2008), the ability of nodulated alders to colonize and maintain high N levels on low N soils, and the high proportion of N derived from fixation of alder species investigated using stable isotope tracer methods (Domenach *et al.* 1989; Markham and Chanway 1999)--and greenhouse experiments, which collectively indicate the ability of alder species to meet their entire N requirement in association with a wide range of *Frankia* strains. Studies which have attempted to provide chemical N sources to non-nodulated *A. incana* at the same rate that N was fixed in nodulated plants have found that, despite the C cost to the host plant,

nodulated plants with no other N source grew either as well as (in the case of NO_3) or better than (in the case of NH_4) fertilized non-nodulated plants (Sellstedt 1986; Sellstedt and Huss-Danell 1986). In the latter case, nodulated alders also had higher N content, and the authors suggest that the plant may be better adapted to deriving N symbiotically than via direct uptake. However, N-fixation rates in both studies were measured as acetylene reduction, which may not be quantitatively reliable under all assay conditions (Anderson *et al.* 2004).

At the other end of the mutualism-parasitism spectrum are the so-called 'ineffective' strains, which induce nodule formation on host roots but do not fix N. This habit appears to occur in at least two ways: 1) an inability of some strains to fix N in symbiosis due to a lack of N fixation genes and/or an inability to form vesicles in symbiosis, and 2) lack of symbiotic compatibility with a particular host species in strains otherwise capable of symbiotic N fixation.

Strains in the former category isolated from *Alnus* nodules that are ineffective on *Alnus* hosts occur in at least two distinct phylogenetic groups: one which clusters with the 'atypical' *Frankia* strains (non-N-fixing strains incapable of reinfecting their host of origin), and one which clusters near the group of effective *Frankia* infective on alder (Normand *et al.* 1996; Wolters *et al.* 1997). Both groups form nodules with similar phenotypes, which are distinct from those of effective nodules. Ineffective nodules are typically very small, with bacteria lacking vesicles and producing narrower hyphae than in effective nodules (Lechevalier *et al.* 1983; Hahn *et al.* 1988; Wolters *et al.* 1997). Small nodule size may result from slow nodule growth rather than arrested development;

other than the lack of vesicles, bacterial growth in most of these strains appears to be similar to that of effective strains in terms of proportion of nodule cells infected and hyphal density in infected cells (Lechevalier *et al.* 1983; van Dijk and Sluimer-Stolk 1990, but see Hahn *et al.* 1988). Most of these strains appear to lack at least one of the genes necessary for nitrogen fixation (Wolters *et al.* 1997), and one strain isolated from *A. incana* ssp. *rugosa* exhibited very different physiological characteristics from an effective comparison strain, including higher oxygen tolerance and differences in the ability to utilize various C sources (Lechevalier *et al.* 1983).

Ineffectivity can also result from incompatibility with particular host species. This has been observed both across host infection groups--*e.g.*, a few strains isolated from *Elaeagnus* spp. nodules form ineffective nodules on some *Alnus* spp.--and also within the *Alnus*-infective group--both crushed nodule inoculum from some *A. incana* nodules and soil from some *A. incana* stands in Finland consistently induce ineffective symbioses with *A. glutinosa* but effective symbioses with *A. incana* (van Dijk *et al.* 1988; Weber 1990).

Host intraspecific variation in *Frankia* compatibility

Intraspecific variation among host genotypes in nodulation with different *Frankia* strains has not been extensively examined within *Alnus*, but studies that have been performed suggest that it may be an important component of the evolutionary dynamics of the symbiosis in some host species. For example, in *A. glutinosa*, the most extensively studied host in this respect, genetic variation in both ability to nodulate and level of

nodulation for both effective and ineffective *Frankia* strains has been observed among host seed source families, ecotypes and clones (Hall *et al.* 1979; Maynard 1980; Hahn *et al.* 1988; van Dijk and Sluimer 1994). In *A. crispa* a non-nodulating genotype has also been reported (Tremblay *et al.* 1984). Intraspecific variation in nodulation with different *Frankia* strains has been examined in *A. rubra*, but no evidence for it was found (Monaco *et al.* 1982).

Effects on host fitness

Even among non-cheating bacterial genotypes able to nodulate a particular host genotype considerable differences in mutualistic behavior can occur. Differences among strains in level of nodulation or benefit provided on a given host can interact with similar effects of host genetic variation (both intra- and inter-specific) and environmental variation, resulting in considerable ecological and evolutionary complexity in a given interaction, including shifting interaction outcomes among different habitats (Bronstein 1994), spatial mosaics of host colonization success (Parker 1999), and increased host-symbiont specificity (Egger and Hibbett 2004). Because of the importance of fixed N to community structure and ecosystem function, these effects can also ripple out to larger ecological scales.

Numerous studies have investigated the effects of host and strain variation on host performance for different suites of alder species and *Frankia* inocula (both pure isolates and crushed nodules). For a given host species, variation in *Frankia* strain has been consistently observed to affect host growth and N-fixation (Hall *et al.*, 1979; Dawson and

Sun, 1981; Dillon and Baker 1982; Monaco *et al.* 1982; Sellstedt *et al.* 1986; Hooker and Wheeler 1987; Sheppard *et al.* 1988). Such strain effects can be complex and are not predictable from prior knowledge of host-symbiont associations. For example, the highest host performance is not always obtained with strains originating from a given host species or even genus (Dawson and Sun 1981; Dillon and Baker 1982; Prat 1989; Weber *et al.* 1989), and there is some evidence that simultaneous inoculation of a single plant with multiple strains can have a synergistic effect on host performance, regardless of symbiont origin relative to the host or even colonization of host nodules by all of the strains (Prat 1989; Martin *et al.* 2003). In Martin *et al.* (2003) dual inoculation significantly increased biomass of *A. rubra* seedlings over single inoculation with either strain, but only one of the two strains was detectable in nodules from the dual inoculation treatment. The authors suggest that the undetected strain may have enhanced the speed of nodulation by the detected strain, resulting in more rapid growth in dual inoculated plants over the course of the experiment. Adding further complexity, such strain effects on host performance can also interact with effects of intra-specific host genetic variation (Hall *et al.* 1979) and environmental variation (Sheppard *et al.* 1988; Kurdali *et al.* 1990). Overall, such studies suggest that *Frankia* strains symbiotic with alder define a broad and dynamic mutualism-parasitism spectrum, and that the position along this spectrum for a given strain can be modified by variation in both host genetics and environment.

A special case of these phenomena is provided by the so-called 'spore-positive' (Sp+) strains. While most *Frankia* strains sporulate freely in culture (Benson and Silvester 1993), Sp+ strains are characterized by the ability to form numerous spores

within the host nodule, an ability which appears to be generally suppressed in host nodules (Schwintzer 1990). Considerable circumstantial evidence suggests that this phenotype is a genetically-determined property of the bacterium (Schwintzer 1990; Wheeler *et al.* 2008). Effects of Sp+ strains on host performance have been well-studied and suggest that Sp+ strains generally occupy an intermediate position along the mutualism-parasitism spectrum. Unlike ineffective strains, Sp+ strains retain the ability to fix N, but appear to minimize the cost of mutualism by reallocation of resources from maintenance of the host interaction to their own reproduction (Schwintzer 1990). Compared to strains that do not sporulate in symbiosis ('spore-negative', or Sp-), Sp+ strains generally result in less growth and lower N-content and fixation rates in host plants (*reviewed in* Schwintzer 1990). Sp+ strains also appear to be much more infective on alder host plants than Sp- strains, (van Dijk 1984; Weber 1990; Markham 2008), suggesting the Sp+ condition may be a multi-trait alternative strategy based on superior competitivity of Sp+ strains for nodule sites on host roots rather than, and at the expense of, mutualistic behavior. Interestingly however, some evidence suggests that the mutualistic behavior of Sp+ strains may be modulated by both host species and environmental conditions. Markham (2008) found a detectable negative effect on host biomass of Sp+ strains compared to Sp- strains in *A. rubra*, but not in *A. incana* ssp. *rugosa* or *A. viridis* ssp. *crispa*. Kurdali *et al.* (1990) found that when *A. incana* was grown in artificial soil Sp- strains maintained consistently higher N-fixation rates per unit nodule mass than Sp+ strains across a range of treatments, but that the situation was reversed when plants were grown in natural soil.

Field associations between alder and *Frankia*

As in most symbioses between plant roots and soil microbes, transmission of microbial symbionts from alder parents to offspring is strictly horizontal; germinating alder seedlings form symbiotic associations *de novo* with *Frankia* genotypes encountered in the soil. This is also the case for annual cohorts of nodules produced by perennial alder plants and, in wind-dispersed species such as alder, seedlings are not likely to encounter symbiont genotypes associated with parents. Under this type of transmission parents have no direct influence over the mutualistic quality of the symbionts utilized by their offspring, and it is thought that evolutionary counter-pressure against the pressure on microbes to cheat is brought about instead by a heritable ability of the plant to withhold resources from 'bad' mutualists (host sanctions) and/or selectively allocate resources to 'good' mutualists (host choice)(Simms and Taylor 2002; West *et al.* 2002). Sanctioning of cheaters is suggested by the small size of ineffective nodules on alder (Hahn *et al.* 1988; van Dijk and Sluimer 1994), but the possibility of choice among non-cheating *Frankia* strains does not appear to have been examined. Such choice has been observed in N-fixing symbiosis between *Bradyrhizobium* genotypes and the wild legume *Lupinus arboreus* (Simms *et al.* 2006). Since bacterial densities in nodules can be several orders of magnitude higher than in soil (West *et al.* 2002), any choice exercised by alder hosts has a strong potential to feed back to soil bacterial populations after nodule senescence.

Field surveys of *Frankia* diversity in alder nodules suggest a degree of influence of the host plant over symbiont populations at the inter-specific level; variation in alder

host species is generally correlated with genetic structure of *Frankia* assemblages in host nodules (van Dijk *et al.* 1988; Weber 1990; Dai *et al.* 2005; Anderson *et al.* 2009). However, since different alder species tend to occupy different habitats in most studies, even when they occur in the same region, such structure also correlates with environmental factors in most studies. A few studies have examined sites in which more than one host species occur, and these indicate that different host species can associate with widely different *Frankia* assemblages even in the same soil (Weber 1986; Anderson *et al.* 2009).

Within a host species, genetic structure among host populations could result in symbiont structure for alder species that vary intra-specifically in compatibility with specific *Frankia* strains (see above section, 'host intraspecific variation in *Frankia* compatibility'). Geographic population structure has been reported in such hosts (Bousquet *et al.* 1987; King and Ferris 1998), but no studies of whether host structure correlates with structure of *Frankia* assemblages in the field appear to have been conducted.

Field studies of single alder species occurring in different environments commonly report variation with sampling locale in symbiotic *Frankia* structure, whether based on molecular genetic tools (Dai *et al.* 2004; Huguet *et al.* 2004; Igual *et al.* 2006), or proportion of Sp+ nodules (*e.g.*, Weber 1986; Holman and Schwintzer 1987; Markham and Chanway 1998a). Regional-scale studies which have included replicated levels of broad environmental variables have found consistent effects of elevation (Khan *et al.* 2007) and habitat type (Holman and Schwintzer 1987) on symbiont structure in *Alnus*

spp., and recently such structure was shown to be consistent among replicate sites representing different habitats at the local scale (Anderson *et al.* 2009).

Alder in Alaska

Distribution, habits and habitats of Alaskan alder

Three species of *Alnus* are native to Alaska: *A. incana* ssp. *tenuifolia* (a.k.a. *A. tenuifolia*, thinleaf alder), *A. rubra* (red alder), and two subspecies of *A. viridis*--ssp. *sinuata* (a.k.a. *A. sinuata*, Sitka alder) and ssp. *fruticosa* (Siberian alder) (Viereck and Little 2007). For each, Alaska is the northern and/or westernmost extent of a larger North American range. *A. tenuifolia* is widespread in North America, occurring throughout Canada, the Yukon and the Pacific Northwest, south nearly to Mexico and east to the Rockies (eFloras 2008). In Alaska, *A. tenuifolia* occurs across the southern 2/3 of the state, south of the Brooks Range, with the exception of narrow portions of the southeast Alaskan panhandle, the Alaska peninsula and most offshore islands (see Figure 1.2) (Viereck and Little 2007). *A. rubra* is mainly a coastal species, occurring along the Pacific coast from central California to the northern extent of the Alaskan panhandle, which is the only portion of the state in which it occurs (Viereck and Little 2007). Within *A. viridis*, the two Alaskan subspecies have fairly distinct distributions. Subsp. *fruticosa* is primarily northern, occupying arctic portions of western Canada, as well as the entire Yukon and nearly all of Alaska, but occurring only sporadically farther south in the Pacific Northwest (eFloras 2008). Subsp. *sinuata*, by contrast, occupies large portions of

the Pacific Northwest and the southern half of Alaska, but is nearly absent north of the Alaska Range (Viereck and Little 2007; eFloras 2008).

All Alaskan alders reach tree size except for Siberian alder, which occurs as a small to large multi-stemmed shrub (Viereck and Little 2007). However, *A. sinuata* and *A. tenuifolia* only rarely reach tree size in Alaska, and occur more commonly as multi-stemmed shrubs similar to Siberian alder. *A. rubra* occurs as a tree across its range.

All three Alaskan alder species have ruderal growth strategies, with rapid growth and abundant seed production (Viereck and Little 2007). In *A. rubra*, at least, this strategy also includes a fairly short lifespan (~100 y)(Harrington 2006), but in shrubby species such as *A. viridis* and *A. tenuifolia* the lifespan of the plant is difficult to determine due to the continuous production of new stems, which may each be much younger than the parent plant (Wilson *et al.* 1985). Within its range each species generally reaches its greatest densities in early succession habitats such as fluvial deposits, glacial outwash, or areas disturbed by fire or logging (Harrington 2006; Viereck and Little 2007). Despite this early-succession peak, alder presence throughout succession can be significant. On the Tanana River floodplain near Fairbanks, for example, maximum stem density of *A. tenuifolia* occurs 5-10 years after colonization of new substrate (Van Cleve *et al.* 1971), but this species can continue to make up a significant portion of the shrub layer throughout the successional sequence of canopy dominance by balsam poplar (~50-100 years post-substrate deposition) and white spruce (~200 years post-substrate)(Van Cleve and Viereck 1981). In *A. viridis* ssp. *fruticosa*, Mitchell and Ruess (2009a) even report an increased stem density across an upland

secondary sere from post-burn through birch dominance to mature white spruce forest. All Alaskan species of alder are also common components of disturbed areas and riparian communities, often forming dense bands along rivers, around lakes, and adjacent to roads and trails (Viereck and Little 2007). The widespread occurrence of alder in Alaska, together with its symbiotic N-fixing capability, make it an important organism in Alaskan ecosystems.

Alder in Alaskan ecosystems

The state of Alaska is large and ecologically diverse, occupying an area of $\sim 1.6 \times 10^6$ km^2, or greater than 1/5 that of the contiguous United States, and containing 20 of the 104 ecoregions described for the United States by the US Geological Survey and Environmental Protection Agency (US EPA 2007), distributed in three major biomes: arctic, boreal, and temperate. Alders are common components of Alaskan ecosystems, occurring at important frequency in 15 of the 20 Alaskan ecoregions (Gallant *et al.* 1995) in all three biomes: *A. rubra* in temperate coastal forests of southeast Alaska, *A. tenuifolia* and *A. sinuata* in boreal and southern coastal regions, and *A. viridis* ssp. *fruticosa* in coastal, boreal and arctic regions (Viereck and Little 2007). In arctic regions *A. viridis* ssp. *fruticosa* is currently undergoing a range expansion concurrent with an expansion of shrub tundra into historically herbaceous tundra that appears to be related to global climate change (Sturm *et al.* 2001; Tape *et al.* 2006).

The effects of alder presence on ecosystem structure and function have been studied to varying degrees for each species of *Alnus* (and for each subspecies of *A.*

viridis) in all three Alaskan biomes, and in all regions the effects of alder appear to be significant. In arctic northwest Alaska, presence of *A. crispa* (syn. *A. viridis* ssp. *fruticosa*) is associated with enhanced N in both soils and plants across multiple ecosystems in habitats that include floodplain terraces, tussock tundra, and valley slopes (Binkley *et al.* 1994; Rhoades *et al.* 2001), and in subarctic southwest Alaska both subspecies of *A. viridis* enhance the nutrient concentrations and productivity of alder-associated lakes (Devotta 2008). Ecosystem effects of *A. rubra* have not been intensively studied in the Alaskan portion of its range (Hanley *et al.* 2006), but the information that exists suggests that the presence of this species enhances understory productivity, diversity, and suitability as wildlife habitat in regional conifer forests (Wipfli *et al.* 2003; Hanley *et al.* 2006). Enhancement of soil and plant N has been reported for *A. sinuata* in coastal post-glacial areas (Chapin *et al.* 1994; Kohls *et al.* 2003) and *A. incana* ssp. *tenuifolia* on river floodplains in interior Alaska (Van Cleve *et al.* 1971; Walker and Chapin 1986; Uliassi and Ruess 2002), in which alder presence is also associated with high N mineralization and turnover rates (Clein and Schimel 1995). Work with these species, however, suggests that the effects of alder on associated ecosystems are more complex than simple facilitation via enhancement of N availability.

In a series of elegant studies using field observations, field manipulations and greenhouse experiments, Walker and Chapin (1986) and Chapin *et al.* (1994) demonstrated a range of effects of *A. incana* ssp. *tenuifolia* and *A. sinuata*, respectively, that included both facilitation and inhibition of seedlings of plant species commonly associated with alder in each study area. Both studies demonstrated positive effects of

alder on N-status of associated plant species, but in both studies these facilitative effects on N-status were counterbalanced by inhibitory effects of alder which included shading and root competition. The net balance of effects appeared to differ between the two systems investigated--in the Chapin *et al.* (1994) study in Glacier Bay on the Alaskan panhandle the authors conclude that the overall effect of *A. sinuata* on white spruce (the successional climax species in both areas) seedlings is facilitative, while the opposite conclusion is reached by Walker and Chapin (1986) for *A. incana* ssp. *tenuifolia* on the Tanana River floodplain in interior Alaska. The authors suggest this difference may be due to differences in N availability between the two systems, with competition outweighing facilitation where N is less limiting.

Ecology of the alder-*Frankia* interaction in Alaska

This section presents recent and ongoing work investigating sources of variation in three broad aspects of the *Alnus-Frankia* interaction in Alaska: 1) host-endophyte interactions, 2) host physiology, and 3) ecosystem effects. Our ongoing work centers on the hypothesis that alder exercises choice among *Frankia* genotypes, and that this choice contributes to the facilitative component of alder effects in some Alaskan ecosystems.

Field associations between alder and *Frankia* in Alaska

Distribution of *Frankia* endophytes has been examined at both local (<15 km) and regional scales (several hundred km) in two of the four host species--*A. tenuifolia* and *A. viridis* ssp. *fruticosa*--in ecosystems from boreal and arctic biomes. As in other geographic areas, composition of symbiotic *Frankia* assemblages in Alaska is correlated with variation in host species and environmental conditions. In Alaska it has been possible to disentangle these effects to an extent, and independent effects of both factors have been observed.

The primary tool used to characterize *Frankia* in these studies is PCR-RFLP of the non-coding bacterial *nif*D-K spacer locus performed on surface-sterilized nodules. We have found that digestion with two restriction enzymes (*CfoI* and *HaeIII*) detects >95% of the sequence variation present in this locus, that this locus is more variable than the 16S-23S IGS spacer, and that both loci yield congruent phylogenies. Across both species and all sampling sites, sixteen *nif*D-K RFLP genotypes of *Frankia* have been detected. The following discussion will refer to these genotypes (termed RF1-RF16) when discussing distribution patterns of endophytic *Frankia*.

Frankia assemblages on the two host species differ in composition and phylogeny. In three sites on the Tanana River floodplain with very similar environmental conditions (Table 1.2) and both hosts in close proximity (≤5 m), very little overlap in composition of symbiont assemblages is evident (Figure 1.3b,c), suggesting a relatively high level of host-symbiont specificity. Such specificity is further suggested by DNA sequence-based phylogenies (both *nif*D-K and IGS loci), in which the RF types most common on *A. tenuifolia* form a single cluster distinct from a second well-defined cluster

containing RF types most common on *A. viridis* ssp. *fruticosa* (Anderson, Taylor and Ruess, unpublished data). The specificity apparent in the two hosts may result from different mechanisms, however; the fact that no *A. viridis* ssp. *fruticosa* nodule has been found to yield any of the dominant RF patterns from *A. tenuifolia* nodules suggests the presence of 'hard' genetic barriers, while the detection of both RF8 and RF9 in the latter host, albeit at very low frequency, suggests specificity in this host is mediated by 'softer' mechanisms.

Environmental effects on *Frankia* structure also differ between the two hosts. In *A. tenuifolia* large differences occur between nodule assemblages of plants occupying early (alder canopy) and late (white spruce canopy with alder understory) succession habitats at relatively small (1.5-13.5 km) spatial scales on the Tanana floodplain. *Frankia* structure is consistent among replicate sites representing each habitat, and appears to be largely consistent year-to-year, particularly in early succession (Figure 1.3a). Late succession sites dominated by white spruce (*Picea glauca* (Moench) Voss) support higher richness and evenness of RF types at both the site and individual plant level in this host (Anderson, Taylor and Ruess unpublished data). In *A. viridis* ssp. *fruticosa*, by contrast, two genotypes differing by a single base pair in the *nif*D-K spacer (RF8 and RF9) occupy >95% of the nodules examined from sites ranging from the Seward Peninsula to the Brooks Range to the Tanana floodplain (Anderson *et al.* 2009; Taylor, MacFarland and Ruess, unpublished data).

The large and consistent environmental effect in *A. tenuifolia* may be due to one or many of the environmental factors differing between these habitats (Table 1.2), and

may act at several points in the development of the symbiosis, including dispersal of *Frankia* genotypes to a particular site, survival of *Frankia* in the soil, and/or differential host interactions (*e.g.*, host choice) among bacterial genotypes. For host choice to be important would require, at a minimum, variation in supply and/or demand of plant nutrients among habitats, a host mechanism capable of choosing symbionts based on the plant's varying physiological needs, and variation in relevant parameters among symbionts (*e.g.*, N-fixation rate or unit cost). Two of these three factors have been observed in Alaskan alder.

Host physiology

Variation among host species and habitats

Environmental variation in host physiological parameters such as nutrient demand, resource allocation among nutrient acquisition options (N-fixation vs. root uptake vs. mycorrhizae) or nutrient use strategies (*e.g.*, nutrient resorption prior to leaf drop) can modify the outcome of the alder-*Frankia* interaction among plant species and/or habitats by modifying the 'value' to the plant of atmospheric N as well as the 'cost' of fixed N in terms of other nutrients such as C or P. Such parameters would be expected to interact with any selection mechanism possessed by the plant. Both nutrient demand and use appear to differ between hosts and among habitats in *A. tenuifolia* and *A. viridis* ssp. *fruticosa* in the region of the Tanana River.

Both alder species maintain relatively high leaf N content across succession (Table 1.3), which is similar in the two species by mass, but differ when expressed by

area, largely due to differences in leaf thickness (Table 1.3). In both species the highest leaf N concentrations occur in environments with the greatest light availability, likely in order to maximize photosynthetic rates under high light. In *A. tenuifolia* this high N demand occurs in an environment with very low soil N, so a large proportion of this N must come from N fixation. Any host choice that occurs would be expected to be most stringent under such conditions, and nodules in these environments are consistently dominated by one *Frankia* genotype - RF7.

Nutrient resorption and interactions between nutrients differ between the two species, and between habitats for *A. tenuifolia*. In *A. viridis* ssp. *fruticosa* in early (alder canopy), mid (paper birch canopy/alder understory), and late succession (white spruce canopy/alder understory) in an upland secondary sere, Mitchell and Ruess (2009b) describe relatively high seasonal N resorption (site means of 19-37% by leaf area) that is on the same order as P resorption (16-33%) in these plants, suggesting a degree of N limitation in this species. By contrast, in *A. tenuifolia* in early (alder) and mid (balsam poplar with alder understory) succession sites on the Tanana floodplain, Uliassi and Ruess (2002) report much lower values of N resorption (7-14%), but much higher P-resorption values (39-51%), suggesting P-limitation rather than N-limitation in this species. Fertilization with P in this study demonstrated that N fixation at the plant level was limited by P availability in early succession, but not in mid-succession.

Differences among *Frankia* genotypes

For any host choice mechanism to be effective variation must exist in relevant physiological parameters among symbiont genotypes. Unfortunately, putatively relevant parameters such as specific N-fixation (SNF) rate or unit cost of N-fixation are highly variable and/or difficult to measure *in situ*. Preliminary evidence for such genotypic differences has nevertheless been observed in Alaskan alder. In the local-scale field survey described above (Anderson *et al.* 2009), SNF (^{15}N uptake) was measured on all nodules collected, and revealed an overall effect of genetic variation among *Frankia* at the seasonal peak of fixation activity. Recent preliminary data suggests that the unit cost of N fixation may also differ among *Frankia* strains. Simultaneous measurement of ^{15}N uptake and respiration in *A. tenuifolia* nodules examined from three early succession sites on the Tanana floodplain revealed significant correlations between respiration and N fixation rate for two of the three most common bacterial genotypes in the sites (Ruess, unpublished data). The regression slope of the most common genotype among all sites (RF7) was significantly lower than for the other type for which the relationship was significant, suggesting both that genotypes differ in unit cost and that alder preferentially associates with the less expensive type.

Ecosystem effects of the alder-*Frankia* symbiosis

Factors affecting the alder-*Frankia* symbiosis are likely to modify both the inhibitory ecosystem effects of alder, by altering alder competitivity, and its facilitative effects, chiefly by altering N-inputs. However, for the sake of simplicity, the following discussion will focus on the latter.

N-inputs by alder at the ecosystem level depend on the balance between N-fixation and N-uptake in individual alders, and are a product of processes operating at multiple scales--SNF at the scale of individual nodules, total nodule biomass at the plant scale, and plant density and N-release rates from nodules and litter at the ecosystem scale. The variation in the *Alnus-Frankia* symbiosis discussed in the previous sections may affect such inputs directly, or through interactions with other variables. Combined with phylogenetic and/or geographic limitations on the suite of strains available to a host, variation among strains in SNF may directly constrain N-input rates, while similar variation in unit cost of fixation may alter the relationship between alder productivity and N inputs. The latter would also be affected by the ability of alder to minimize the C cost of N-fixation through *Frankia* choice. N availability alters both nodule production and SNF, and other environmental variables such as light, water, and P modify N demand among habitats and, in the case of P, also specifically affect nodule biomass (Uliassi and Ruess 2002). More complex modulation of N inputs can occur through interactions with other organisms. This section ends with two detailed examples.

Mycorrhizae

In addition to symbiosis with *Frankia*, alder forms both arbuscular-mycorrhizal (AM) and ecto-mycorrhizal (EM) interactions with fungi. Considerable variation exists in these interactions in terms of taxonomic and environmental patterns of association and impacts on host performance. Such variation can interact with variation in the alder-

Frankia symbiosis to alter selection pressures on both partners, and may also modify the ecosystem effects of alder.

While alder is capable of associating with several AM fungal genera (Gardner 1986), such associations may not occur at high densities in the field (Helm *et al.* 1996; Pritsch *et al.* 1997), or may be limited to early developmental stages of the plant (Arveby and Granhall 1998). EM associations with alder occur in ~50 distantly related fungal species, many of which are specific to *Alnus* (Bruns *et al.* 2002; Tedersoo *et al.* 2009), and a few of which show apparent specificity for particular alder species (Gardner 1986). Despite this comparatively low overall diversity, EM communities on alder in the field can be quite variable among habitats, particularly across successional seres (Helm *et al.* 1996; Arveby and Granhall 1998).

Due to the wide variety of services attributable to mycorrhizal infection--*e.g.*, acquisition of water, nitrogen, phosphorous, and various micronutrients, and/or alteration of pathogen susceptibility (Koide 1991)--such interactions have the potential to modify both the evolutionary dynamics and the ecosystem effects of the alder-*Frankia* association in a given habitat in several opposing ways. For instance, under conditions in which availability of water, P, or other nutrients limits N-fixation, mycorrhizal associations capable of enhancing plant access to such resources could lower the relative cost of N-fixation to the plant and possibly lessen the stringency of any host choice process. Synergistic positive effects of mycorrhizal symbiosis in concert with *Frankia* have been observed in both AM and EM fungal symbionts in greenhouse studies (*e.g.*, Russo 1989; Yamanaka *et al.* 2003, 2005), and probably act via enhanced P availability

(Mejstrik and Benecke 1969). Release of limitations on N-fixation by P or other factors would, of course, result in greater N-inputs to associated ecosystems, tipping the balance of alder effects toward facilitation.

On the other hand, mycorrhizal associations with N-mobilizing fungi could provide alders with access to organic soil N. If mycorrhizal-derived N is metabolically cheaper in a given environment than atmospheric N, and alder is capable of selecting between *Frankia* and mycorrhizae, such a process could result either in selection for more efficient *Frankia* strains or in a local breakdown of the coevolutionary process between plant and bacterium. At the point where N-uptake by alder exceeds N-fixation, such a process would also represent a threshold in the ecosystem role of alder between N-source and N-sink.

Herbivores and pathogens

Alders are eaten by a wide variety of herbivores and are host to numerous pathogens. Interactions between alder and both guilds can vary across host species, with abiotic and biotic environmental factors, and across multiple spatial and temporal scales (*e.g.*, Hendrickson *et al.* 1991; Gange 1995; Markham and Chanway 1998b; Mulder *et al.* 2008). Such variation can be complex and unpredictable; *e.g.*, in a survey of leaf pathogens and five arthropod herbivore guilds on *A. viridis* ssp. *fruticosa* in interior Alaska, Mulder *et al.* (2008) found that damage levels by different guilds were differentially correlated with: 1) leaf position along the twig or, 2) distance to the nearest alder or other deciduous tree, and that many of these correlations were significant in one

year of the study but not the next (several correlations with leaf position), or were significant in both years but the direction of the relationship changed (correlation with tree distance). Additional complexity appears when one considers the interaction of such variation with the alder-*Frankia* symbiosis. For example, increases in foliar N levels in field-grown alders in response to inoculation with *Frankia* can both increase attack rates by insect herbivores and facilitate defenses against them, and such effects differ among herbivores and alder species (Hendrickson *et al.* 1991; Hendrickson *et al.* 1993).

The effects of alder herbivory on associated ecosystems may be significant. In *A. tenuifolia*, artificial defoliation of greenhouse grown seedlings ranging from 15-40% leaf area removal resulted in significant reductions of SNF from 33-68%, and one-time removal of 40% leaf area resulted in 73% reduction in SNF after a month of regrowth despite the recovery of biomass to levels indistinguishable from control plants (Ruess *et al.* 2006). While no such effect on SNF was observed in a recent field study of a current outbreak of stem canker (putatively *Valsa melanodiscus* [anamorph *Cytospera umbrina*]) in *A. tenuifolia* in Alaska, reductions of nodule biomass measured in three Alaskan stands of this species resulted in an estimated 31-38% reduction in N inputs (Ruess *et al.* 2009).

Conclusion

An emerging paradigm in evolutionary ecology holds that many inter-specific interactions shift along a continuum from mutualism to parasitism under varying biotic and abiotic environmental contexts (Bronstein 1994) and that such shifting outcomes among local populations, in addition to genetic variation in each partner, provide the raw

material for coevolution of symbiotic partners at the macroevolutionary level (Thompson 1994, 2005). In the case of N-fixing symbioses, such intrinsic and extrinsic modulators of symbiotic outcomes also have the strong potential to impact ecosystem structure and function, providing an opportunity to study links between evolutionary and ecosystem ecology. The importance of the alder-*Frankia* symbiosis in Alaskan ecosystems, together with the relative simplicity and repetition of such systems across a large geographic area, provides the researcher with an excellent system for such studies and a convenient excuse for doing large amounts of biology in one of the world's wildest and most wondrous areas.

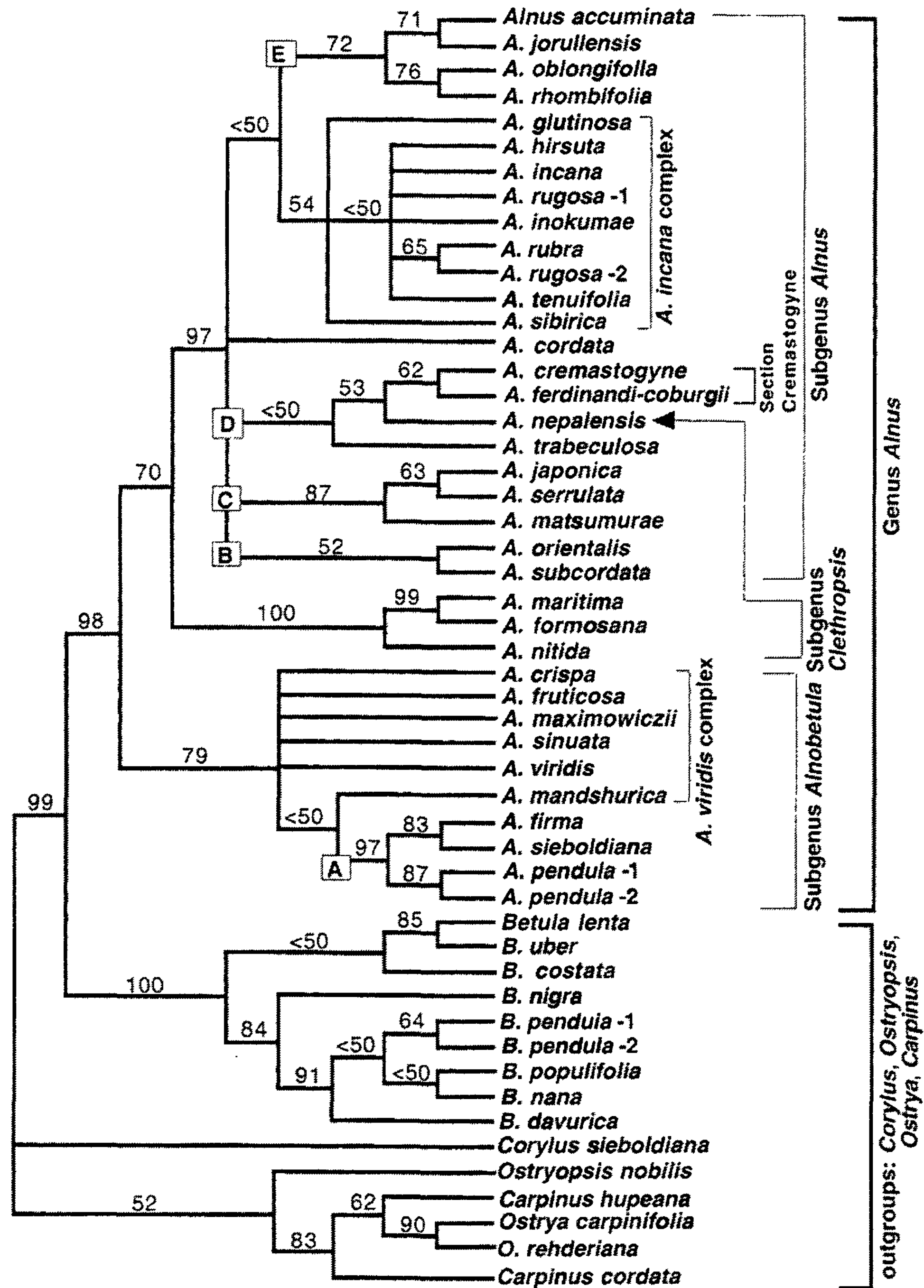

Figure 1.1. Phylogeny of *Alnus*. Strict consensus of 18 most parsimonious trees of 34 *Alnus* species based on DNA sequence comparison of the ribosomal internally transcribed spacer (ITS) region of the *Alnus* nuclear genome. Numbers above branches are bootstrap percentages. Traditional classifications are shown on the right. Taken from Chen and Li (2004) and reprinted by permission from the University of Chicago Press.

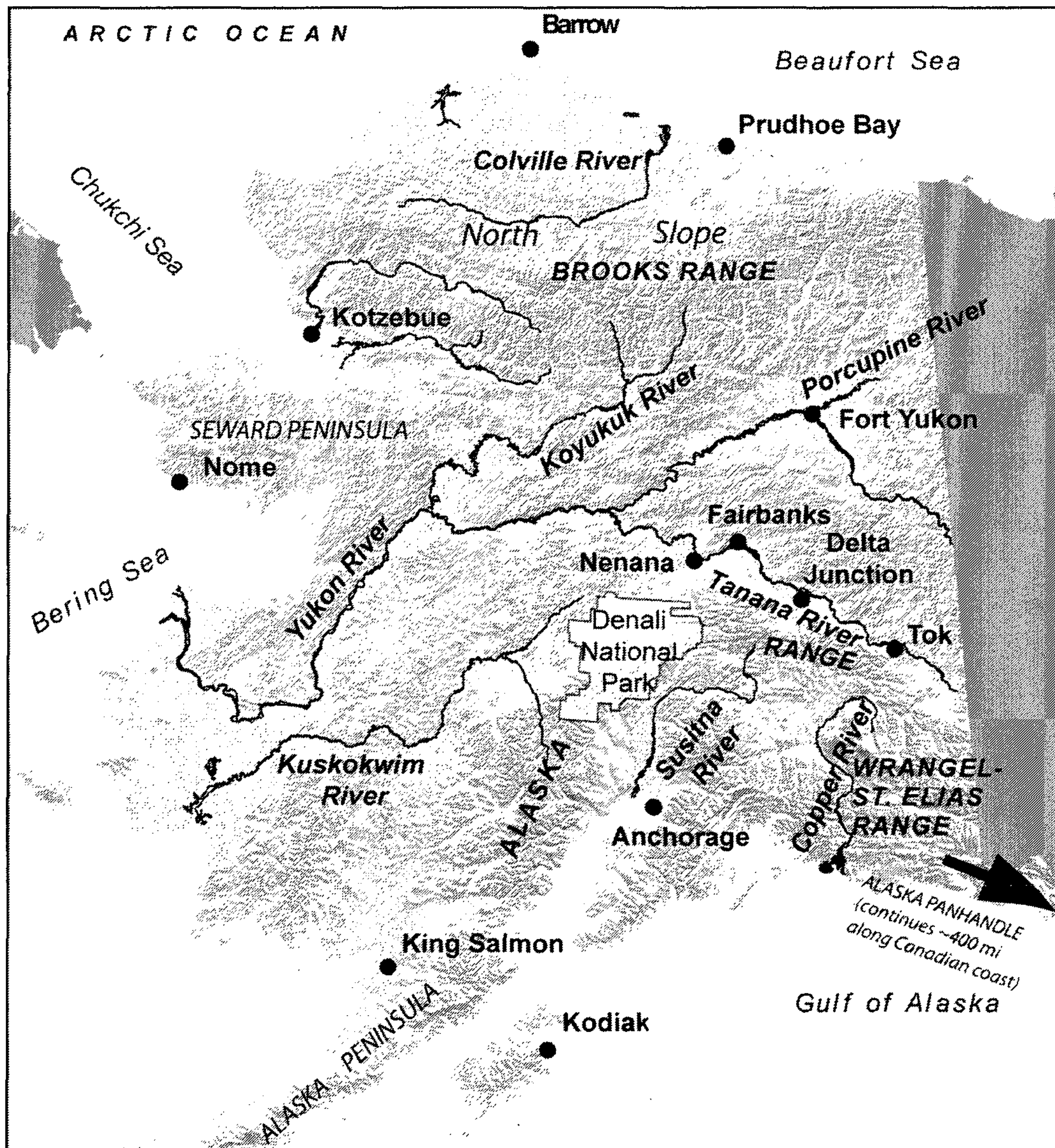

Figure 1.2. Map of Alaska. Reprinted from Chapin *et al.* (2006) by permission from Oxford University Press. Landmarks mentioned in the text have been added to the original.

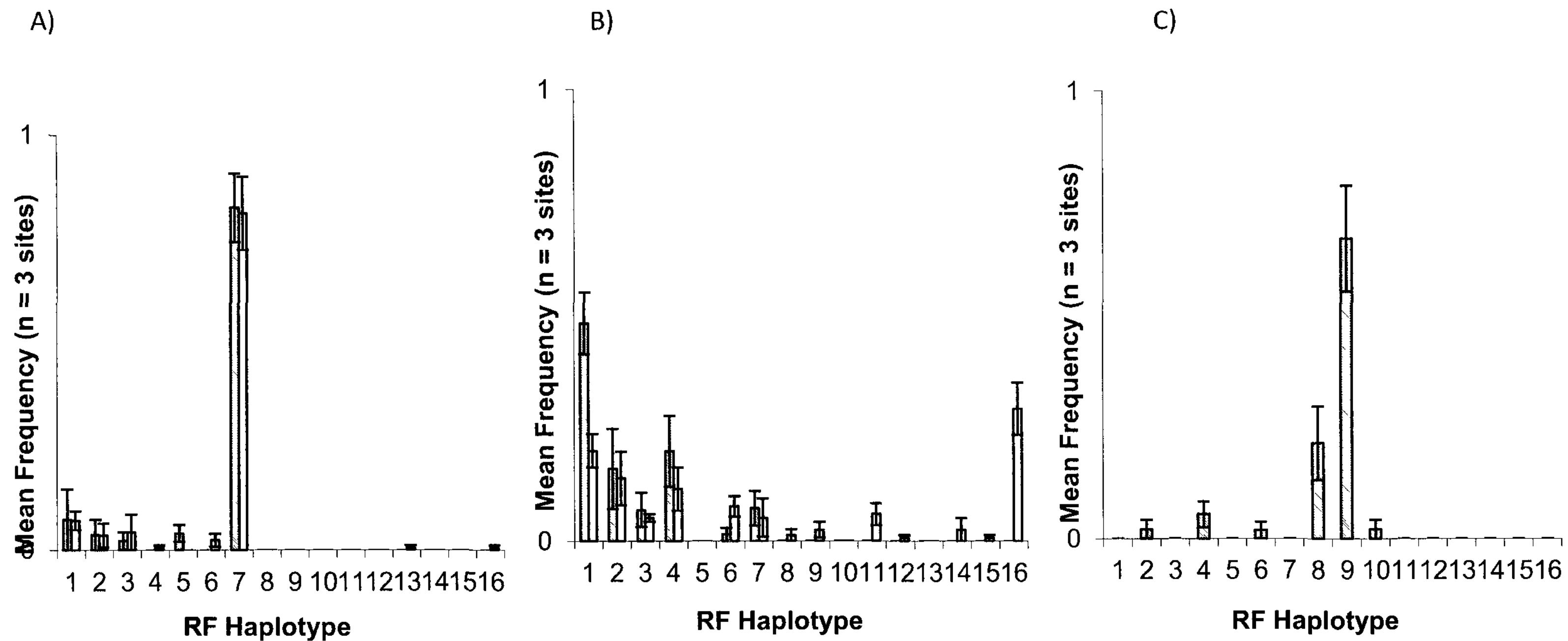

Figure 1.3. *Frankia* genotype frequencies. Average frequencies (± 1 standard error, n = 3 sites) of *Frankia nif*D-K restriction fragment (RF) genotypes occupying *Alnus tenuifolia* nodules in early (A) and late (B) succession sites, and *Alnus viridis* ssp. *fruticosa* in the same late succession sites (C) on the Tanana River floodplain, interior Alaska. Charcoal bars represent data from a survey conducted in 2002 and open bars represent a survey of the same sites conducted in 2005. 2002 data re-used by permission from Springer.

Table 1.1. Host specificity in *Alnus-Frankia*. Results of cross-inoculation studies between *Frankia*

- = negative nodulation, '*'= positive, but very low, nodulation. Superscripts indicate studies in which each interaction was observed, and are as follows: 1 Baker 1987, 2 Du and Baker 1992, 3 Jiabin *et al.* 1985, 4 Prat 1989, 5 Nesme *et al.* 1985, 6 Hooker and Wheeler 1987, 7 Weber *et al.* 1989, 8 Dawson and Sun 1981, 9 Dillon and Baker 1982, 10 Maynard 1980.

Subgenus				Alnus					Alnobetula
	Species	Subspp	Strain, informal	*A. glutinosa*	*A. rubra*	*A. incana*	*A. incana* ssp. *rugosa*	*A. cremastogyne*	*A. viridis* ssp. *crispa*
Alnobetula	*viridis*	.	AVP3n	+ [5]					
		crispa	AvcI1	+ [2]	+ [1,9]	+ [7]	+ [9]		+ [9]
		crispa	ACN1AG	+ [4,5,8,10]	+ [4,10]	* [8]		+ [4]	+ [4]
		sinuata	AvsI2		* [9]		- [9]		+ [9]
		sinuata	AvsI3	+ [2]	+ [1]				
		sinuata	AvsI6a	- [1]	- [1]				
		sinuata	AvsI6b	+ [1]					
		sinuata	54012	+ [1]	+ [1]				
		firma	Af2	+ [2]					

Table 1.1 cont'd

Subgenus			Alnus						Alnobetula
			Species						Subspp
Alnus	*incana*	*rugosa*	Strain, informal	*A. glutinosa*	*A. rubra*	*A. incana*	*A. incana* ssp. *rugosa*	*A. cremastogyne*	*A. viridis* ssp. *crispa*
		rugosa	AirI1	+ [1]					
	glutinosa	*tenuifolia*	AirI2		- [1]				
		tenuifolia	R52	- [1]	- [1]				
		.	ATP1d	+ [5]					
			54004	- [1]	- [1]				
	glutinosa		54005	- [1]	- [1]				
	glutinosa		Agc8204	+ [2]				+ [3]	
	glutinosa		AG10AI	+ [4]	+ [4]			+ [4]	+ [4]
	glutinosa		AGN1g	+ [5]					
	glutinosa			+ [6]	+ [6]				
	glutinosa			+ [6]	+ [6]				
	rubra			+ [6]	+ [6]				
	rubra		ArI4		+ [1,6]				
	rhombifolia		ArI5	+ [1]	+ [1,6,9]		+ [9]		+ [9]
	cremastogyne		ArhI2	- [1,2]	- [1]				
	cremastogyne		Acc8207	+ [2]				+ [3]	
	hirsuta		Acc13					+ [3]	
	japonica		Ahc8201	+ [2]				+ [3]	
	cordata		Ajc8206					+ [3]	

Table 1.2. Alaskan study site characteristics. Site characteristics of successional habitats and mean values (n = 3 representative sites) of selected soil properties in sites in which *Frankia* diversity has been most intensely investigated in Alaska. Soil moisture and temperature data are from Anderson et al. (2009), and the remaining soil variable means were calculated from publicly-available data on the Bonanza Creek Long-Term Ecological Research website (www.lter.uaf.edu, Oliver *et al.* unpublished data). Within a column common superscripts indicate homogeneous subsets (Tukey HSD, $P < 0.05$); no superscript indicates no statistical differences among rows in a column.

The largest differences in most parameters occurs between early and late succession floodplain habitats, which corresponds to the largest difference observed in *Frankia* genetic assemblage in nodules of *A. tenuifolia*, the only host species to occur in both of these habitats (see Figure 1.3).

SITE CHARACTERISTICS				SOIL VARIABLES					
Landscape	Stage/Sere	Species	Alder Crown Position	Moisture	Temperature	Organic Matter	%N by mass	N:P (mass)	pH
Floodplain	Early Primary	*A. tenuifolia*	Canopy	28.7 ± 0.5^{A}	9.5 ± 0.3^{A}	1.6 ± 0.3^{A}	0.04 ± 0.01^{A}	0.56 ± 0.11^{A}	7.4 ± 0.03^{A}
Floodplain	Late Primary	*A. tenuifolia* + *A. viridis*	Understory	32.7 ± 0.8^{B}	7.9 ± 0.2^{B}	11.5 ± 2.5^{B}	0.21 ± 0.04^{B}	3.00 ± 0.43^{BC}	5.6 ± 0.09^{B}
Upland	Early Secondary	*A. viridis*	Canopy	25.7 ± 0.7^{A}	9.3 ± 0.2^{A}	6.0 ± 1.1^{AB}	0.13 ± 0.02^{AB}	1.88 ± 0.33^{B}	5.6 ± 0.26^{B}
Upland	Late Secondary	*A. viridis*	Understory	26.5 ± 0.6^{A}	8.8 ± 0.2^{A}	8.1 ± 0.9^{B}	0.19 ± 0.02^{B}	3.96 ± 0.34^{C}	4.9 ± 0.13^{C}

Table 1.3. Physiological variables for alder in Alaskan study sites. Means (n = 3 sites, 30 plants per site) of physiological variables measured on two species of alder in Alaskan successional habitats. Data are from Anderson *et al.* (2009) and re-used by permission from Springer.

SNF values in field sites are highly variable and no difference can be detected among sampling blocks. Among habitats leaf N by area parallels light availability for both species (see Table 1.2), and leaf N by both mass and area parallels large differences in *Frankia* genetic assemblage structure in *A. tenuifolia* (see Figure 1.3).

SITE CHARACTERISTICS			PHYSIOLOGICAL VARIABLES			
Landscape	Stage	Species	SNF (μmol N_2 g noddwt^{-1}*h^{-1})	Leaf N (% by mass)	Leaf N by Area (g N/m^2)	SLW (gdwt leaf tissue/m^2)
Floodplain	Early	*A. tenuifolia*	28.3 ± 5.5	2.64 ± 0.05^A	1.59 ± 0.04^A	62.1 ± 1.9^A
	Late	*A. tenuifolia*	22.4 ± 3.2	2.46 ± 0.03^B	1.23 ± 0.03^B	50.6 ± 1.2^B
		A. viridis ssp. *fruticosa*	30.3 ± 3.3	2.26 ± 0.03^C	1.07 ± 0.03^C	47.9 ± 1.1^B
Upland	Early	*A. viridis* ssp. *fruticosa*	33.9 ± 4.5	2.50 ± 0.04^B	1.79 ± 0.03^D	71.7 ± 8.0^C
	Late	*A. viridis* ssp. *fruticosa*	34.0 ± 3.9	2.49 ± 0.03^B	1.05 ± 0.02^C	42.4 ± 0.9^D

REFERENCES

Anderson, M.D., Ruess, R.W., Uliassi, D.D., Mitchell, J.S. (2004) Estimating N_2 fixation in two species of *Alnus* in interior Alaska using acetylene reduction and $^{15}N_2$ uptake. *Ecoscience* 11, 102-112.

Anderson, M.D., Ruess, R.W, Myrold, D.D. *et al.* (2009) Host species and habitat affect nodulation by specific *Frankia* genotypes in two species of *Alnus* in interior Alaska. *Oecologia* 160, 619-630.

Arveby, A.S., Granhall, U. (1998) Occurrence and succession of mycorrhizas in *Alnus incana*. *Swedish Journal of Agricultural Research* 28, 117-127.

Baker, D.D. (1987) Relationships among pure-cultured strains of *Frankia* based on host specificity. *Physiologia Plantarum* 70, 245-248.

Baker, D.D., Schwintzer, C.R. (1990) Introduction. In: *The Biology of Frankia and Actinorhizal Plants*. (eds. C.R. Schwintzer and J.D. Tjepkema) Academic Press, Inc., San Diego.

Batzli, J.M., Zimpfer, J.F., Huguet, V. *et al.* (2004) Distribution and abundance of infective, soilborne *Frankia* and host symbionts *Shepherdia*, *Alnus*, and *Myrica* in a sand dune ecosystem. *Canadian Journal of Botany* 82, 700-709.

Benson, D.R., Silvester, W.B. (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiological Reviews* 57, 293-319.

Binkley, D., Stottlemyer, R., Suarez, F. *et al.* (1994) Soil nitrogen availability in some arctic ecosystems in northwest Alaska: responses to temperature and moisture. *Ecoscience* 1, 64-70.

Bosco, M., Fernandez, M.P., Simonet, P. *et al.* (1992) Evidence that some *Frankia* sp. strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. *Applied and Environmental Microbiology* 58, 1569-1576.

Bousquet, J., Cheliak, W.M., Lalonde, M. (1987) Genetic diversity within and among 11 juvenile populations of green alder (*Alnus crispa*) in Canada. *Physiologia Plantarum* 70, 311-318.

Bronstein, J.L. (1994) Conditional outcomes in mutualistic interactions. *Trends in Ecology and Evolution* 9, 214-217.

Bronstein, J.L. (2001) The costs of mutualism. *American Zoologist* 41, 825-839.

Bruns, T.D., Bidartondo, M.I., Taylor, D.L. (2002) Host specificity in ectomycorrhizal communities: what do the exceptions tell us? *Integrative and Comparative Biology* 42, 352-359.

Chapin III, F.S., Walker, L.R., Fastie, C.L. *et al.* (1994) Mechanisms of primary succession following deglaciation at Glacier Bay, Alaska. *Ecological Monographs* 64, 149-175.

Chapin III, F.S., Yarie, J., Van Cleve, K. *et al.* (2006) The conceptual basis of LTER studies in the Alaskan boreal forest. In: *Alaska's Changing Boreal Forest.* (eds. F.S. Chapin III, M.W. Osgood, K. Van Cleve, *et al.*) Oxford University Press, NY.

Chen, Z., Li, J. (2004) Phylogenetics and biogeography of *Alnus* (Betulaceae) inferred from sequences of nuclear ribosomal DNS ITS region. *International Journal of Plant Science* 165, 325-335.

Clawson, M.L., Bourret, A., Benson, D.R. (2004) Assessing the phylogeny of *Frankia*-actinorhizal plant nitrogen-fixing root nodule symbioses with *Frankia* 16S rRNA and glutamine synthetase gene sequences. *Molecular Phylogenetics and Evolution* 31, 131-138.

Clein, J.S., Schimel, J.P. (1995) Nitrogen turnover and availability during succession from alder to poplar in Alaskan taiga forests. *Soil Biology and Biochemistry* 27, 743-752.

Dai, Y., Zhang, C., Xiong, Z. *et al.* (2005) Correlations between the ages of *Alnus* host species and the genetic diversity of associated endosymbiotic *Frankia* strains from nodules. *Science in China* Ser. C Life Sciences 48 Suppl, 76-81.

Dai, Y., He, X.Y., Zhang, C.G. *et al.* (2004) Characterization of genetic diversity of *Frankia* strains in nodules of *Alnus nepalensis* (D. Don) from the Hengduan Mountains on the basis of PCR-RFLP analysis of the *nifD-nifK* IGS. *Plant and Soil* 267, 207-212.

Dawson, J.O., Sun, S-H. (1981) The effect of isolates from *Comptonia peregrina* and *Alnus crispa* on the growth of *Alnus glutinosa*, *A. cordata*, and *A. incana* clones. *Canadian Journal of Forest Research* 11, 758-762.

Dawson, J.O. 2008. Ecology of actinorhizal plants. In: *Nitrogen Fixation: Origins, Applications, and Research Progress, Vol. 6 Nitrogen-fixing Actinorhizal Symbioses.* (eds. K. Pawlowski and W.E. Newton) Springer, Dordrecht, The Netherlands.

Denison, R.F., Kiers, E.T. (2004) Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. *FEMS Microbiology Letters* 237, 187-193.

Devotta, D.A. (2008) The influence of *Alnus viridis* on the nutrient availability and productivity of sub-arctic lakes in southwestern Alaska. MS Thesis, University of Illinois at Urbana-Champaign.

Dillon, J.T., Baker D. (1982) Variations in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. *New Phytologist* 92, 215-219.

Domenach, A.M., Kurdali, F., Bardin, R. (1989) Estimation of symbiotic dinitrogen fixation in alder forest by the method based on natural ^{15}N abundance. *Plant and Soil* 118, 51-59.

Du, D., Baker, D.D. (1992) Actinorhizal host-specificity of Chinese *Frankia* strains. *Plant and Soil* 144, 113-116.

eFloras. 2008. Published on the Internet http://www.efloras.org [accessed 15 June 2009] Missouri Botanical Garden, St. Louis, MO and Harvard University Herbaria, Cambridge, MA

Egger, K.N., Hibbett, D.S. (2004) The evolutionary implications of exploitation in mycorrhizas. *Canadian Journal of Botany* 82, 1110-1121.

Furlow, J.J. (1979) The systematics of the American species of *Alnus* (Betulaceae). *Rhodora* 81, 1-121, 151-248.

Gallant, A.L., Binnian, E.F., Omernik, J.M., and Shasby, M.B., (1995) Ecoregions of Alaska: U.S. Geological Survey Professional Paper 1567, Washington D.C.

Gange, A.C. (1995) Aphid performance in an alder (*Alnus*) hybrid zone. *Ecology* 76, 2074-2083.

Gardner, I.C. (1986) Mycorrhizae of actinorhizal plants. *MIRCEN Jounal of Applied Microbiology and Biotechnology* 2, 147- 160.

Gentili, F., Huss-Danell, K. (2003) Local and systemic effects of phosphorous and nitrogen on nodulation and nodule function in *Alnus incana. Journal of Experimental Botany* 54, 2757-2767.

Hahn, D., Starrenburg, M.J.C., Akkermans, A.D.L. (1988) Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. *Plant and Soil* 107, 233-243.

Hahn, D., Nickel, A., Dawson, J. (1999) Assessing *Frankia* populations in plants and soil using molecular methods. *FEMS Microbiology Ecology* 29, 215-227.

Hahn, D. (2008) Polyphasic taxonomy of the genus *Frankia*. In: *Nitrogen Fixation: Origins, Applications, and Research Progress,* Vol. 6 *Nitrogen-fixing Actinorhizal Symbioses*. (eds. K. Pawlowski and W.E. Newton) Springer, Dordrecht, The Netherlands.

Hall, R.B., McNabb Jr., H.S., Maynard, C.A. *et al.* (1979) Toward development of optimal *Alnus glutinosa* symbiosis. *Botanical Gazette* 140 (Suppl), S120-S126.

Hanley, T.A., Deal, R.L., Orlikowska, E.H. (2006) Relations between red alder composition and understory vegetation in young mixed forests of southeast Alaska. *Canadian Journal of Forest Research* 36, 738-748.

Harrington, C.A. (2006) Biology and ecology of red alder. In: *Red Alder: A State of Knowledge.* General Technical Report PNW-GTR-669. Portland OR: U.S. Department of Agriculture, Pacific Northwest Research Station.

Heilman, P.E. (1990) Growth of Douglas-fir and red alder on coal spoils in western Washington. *Soil Science Society of America Journal* 54, 522-527.

Helm, D.J., Allen, E.B., Trappe, J.M. (1996) Mycorrhizal chronosequence near Exit Glacier, Alaska. *Canadian Journal of Botany* 74, 1496-1506.

Hendrickson, O.Q., Fogal, W.H., Burgess, D. (1991) Growth and resistance to herbivory in N_2-fixing alders. *Canadian Journal of Botany* 69, 1919-1926.

Hendrickson, O.Q., Burgess, D., Perinet, P. *et al.* (1993) Effects of *Frankia* on field performance of *Alnus* clones and seedlings. *Plant and Soil* 150, 295-302.

Holman, R.M., Schwintzer, C.R. (1987) Distribution of spore-positive and spore-negative nodules of *Alnus incana* ssp. *rugosa* in Maine, USA. *Plant and Soil* 104, 103-111.

Hooker, J.E., Wheeler, C.T. (1987) The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. *Physiologia Plantarum* 70, 333-341.

Huguet, V., Mergeay, M., Cervantes, E. *et al.* (2004) Diversity of *Frankia* strains associated to *Myrica gale* in Western Europe: impact of host plant (*Myrica* vs. *Alnus*) and of edaphic factors. *Environmental Microbiology* 6, 1032-1041.

Huguet, V., Gouy, M., Normand, P. *et al.* (2005) Molecular phylogeny of Myricaceae: a reexamination of host-symbiont specificity. *Molecular Phylogenetics and Evolution* 34, 557– 568.

Igual, J.M., Valverde, A., Valázquez, E. *et al.* (2006) Natural diversity of nodular microsymbionts of *Alnus glutinosa* in the Tormes River basin. *Plant and Soil* 280, 373-383.

Jiabin, H., Zheying, Z., Guanxiong, C. *et al.* (1985) Host range of *Frankia* endophytes. *Plant and Soil* 87, 61-65.

Khan, A., Myrold, D.D., Misra, A.K. (2007) Distribution of *Frankia* genotypes occupying *Alnus nepalensis* nodules with respect to altitude and soil characteristics in the Sikkim Himalayas. *Physiologia Plantarum* 130, 364-371.

Kiers, E.T., Denison, R.F. (2008) Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual Review of Ecology, Evolution, and Systematics* 39, 215-236.

King, R.A., Ferris, C. (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. *Molecular Ecology* 7, 1151- 1161.

Kohls, S.J., Baker, D.D., van Kessel, C. *et al.* (2003) An assessment of soil enrichment by actinorhizal N_2 fixation using $\delta^{15}N$ values in a chronosequence of deglaciation at Glacier Bay, Alaska. *Plant and Soil* 254, 11-17.

Koide, R.T. (1991) Tansley Review No. 29. Nutrient supply, nutrient demand and plant response to mycorrhizal infection. *New Phytologist* 117, 365-386.

Kurdali, F., Rinaudo, G., Moiroud, A. *et al.* (1990) Competition for nodulation and $^{15}N_2$-fixation between a Sp+ and a Sp- *Frankia* strain in *Alnus incana. Soil Biology and Biochemistry* 22, 57-64.

Laws, M.T., Graves, W.R. (2005) Nitrogen inhibits nodulation and reversibly suppresses nitrogen fixation in nodules of *Alnus maritime. Journal of the American Society of Horticultural Science* 130, 496-499.

Lechevalier, M.P., Baker, D., Horrière, F. (1983) Physiology, chemistry, serology, and infectivity of two *Frankia* isolates from *Alnus incana* ssp. *rugosa. Canadian Journal of Botany* 61, 2826-2833.

Lechevalier, M.P., Lechevalier, H.A. (1990) Systematics, isolation, and culture of *Frankia.* In: *The Biology of* Frankia *and Actinorhizal Plants* (eds. C.R. Schwintzer $ J.D. Tjepkema) Academic Press, Inc., San Diego.

Li, C-Y., Crawford, R.H., Chang, T-T. (1997) *Frankia* in decaying fallen trees devoid of actinorhizal hosts and soil. *Microbiological Research* 152, 167-169.

Lumini, E., Bosco, M., Fernandez, M.P. (1996) PCR-RFLP and total DNA homology revealed three related genomic species among broad-host-range *Frankia* strains. *FEMS Microbiology Ecology* 21, 303-311.

Lundquist, P-O. (2005) Carbon cost of nitrogenase activity in *Frankia-Alnus incana* root nodules. *Plant and Soil* 273, 235- 244.

MacConnell, J.T., Bond, G. (1957) A comparison of the effect of combined nitrogen on nodulation in non-legumes and legumes. *Plant and Soil* 8, 378-388.

Markham, J.H., Chanway, C.P. (1998a) *Alnus rubra* (Bong.) nodule spore type distribution in southwestern British Columbia. *Plant Ecology* 135, 197-205.

Markham, J.H., Chanway, C.P. (1998b) Response of red alder (*Alnus rubra*) seedlings to a woolly alder sawfly (*Eriocampa ovata*) outbreak. *Canadian Journal of Forest Research* 28, 591-595.

Markham, J.H., Chanway, C.P. (1999) Does past contact reduce the degree of mutualism in the *Alnus rubra* - *Frankia* symbiosis? *Canadian Journal of Botany* 77, 434-441.

Markham, J.H., Zekveld, C. (2007) Nitrogen fixation makes biomass allocation to roots independent of soil nitrogen supply. *Canadian Journal of Botany* 85, 787-793.

Markham, J.H. (2008) Variability of nitrogen-fixing *Frankia* on *Alnus* species. *Botany* 86, 501-510.

Martin, K.J., Tanaka, Y., Myrold, D.D. (2003) Dual inoculation increases plant growth with *Frankia* on red alder (*Alnus rubra* Bong.) in fumigated nursery beds. *Symbiosis* 34, 253- 260.

Maunuksela, L., Zepp, K., Koivula, T. *et al.* (1999) Analysis of *Frankia* populations in three soils devoid of actinorhizal plants. *FEMS Microbiology Ecology* 28, 11-21.

Maynard, C.A. (1980) Host-symbiont interactions among *Frankia* strains and *Alnus* open-pollinated families. PhD Thesis. Iowa State University, Ames, Iowa.

McVean, D.N. (1953) *Alnus glutinosa* (L.) Gaertn. *Journal of Ecology* 41, 447-466.

Mejstrik, V., Benecke, U. (1969) The ectotrophic mycorrhizas of *Alnus viridis* (Chaix) D.C. and their significance in respect to phosphorous uptake. *New Phytologist* 68, 141-149.

Mirza, B.S., Welsh, A., Hahn, D. (2009a) Growth of *Frankia* strains in leaf litter-amended soil and the rhizosphere of a nonactinorhizal plant. *FEMS Microbiology Ecology* 70, 132- 141.

Mirza, B.S., Welsh, A., Rasul, G. *et al.* (2009b) Variation in *Frankia* populations of the *Elaeagnus* host infection group in nodules of six host plant species after inoculation with soil. *Microbial Ecology* 58, 384-393.

Mirza, B.S., Welsh, A., Hahn, D. (2007) Saprophytic growth of inoculated *Frankia* sp. in soil microcosms. *FEMS Microbiology Ecology* 62, 280-289.

Mitchell, J.S., Ruess, R.W. (2009a) N_2 fixing alder (*Alnus viridis* ssp. *fruticosa*) effects on soil properties across a secondary successional chronosequence in interior Alaska. *Biogeochemistry* 95, 215-229.

Mitchell, J.S., Ruess, R.W. (2009b) Seasonal patterns of climate controls over nitrogen fixation by *Alnus viridis* ssp. *fruticosa* in a secondary successional chronosequence in interior Alaska. *Ecoscience* 16, 341-351.

Monaco, P.A., Ching, K.K., Ching, T. (1982) Host-endophyte effects on biomass production and nitrogen fixation in *Alnus rubra* actinorhizal symbiosis. *Botanical Gazette* 143, 298-303.

Mulder, C.P.H., Roy, B.A., Güsewell, S. (2008) Herbivores and pathogens on *Alnus viridis* subsp. *fruticosa* in interior Alaska: effects of leaf, tree, and neighbor characteristics on damage levels. *Botany* 86, 408-421.

Myrold, D.D., Huss-Danell, K. (1994) Population dynamics of *Alnus*-infective *Frankia* in a forest soil with and without host trees. *Soil Biology and Biochemistry* 26, 533-540.

Nesme, X., Normand, P., Tremblay, F.M. *et al.* (1985) Nodulation speed of *Frankia* sp. on *Alnus glutinosa*, *Alnus crispa*, and *Myrica gale*. *Canadian Journal of Botany* 63, 1292-1295.

Normand, P., Orso, S., Cournoyer, B. *et al.* (1996) Molecular phylogeny of the genus *Frankia* and emendation of the family *Frankiaceae*. *International Journal of Systematic Bacteriology* 46, 1-9.

Parker, M.A. (1999) Mutualism in metapopulations of legumes and rhizobia. *The American Naturalist* 153 (Suppl), S48-S60.

Prat, D. (1989) Effects of some pure and mixed *Frankia* strains on seedling growth in different *Alnus* species. *Plant and Soil* 113, 31-38.

Pritsch, K., Munch, J.C., Buscot, F. (1997) Morphological and anatomical characterization of black alder *Alnus glutinosa* (L.) Gaertn. ectomycorrhizas. *Mycorrhiza* 7, 201-216.

Rhoades, C., Oskarsson, H., Binkley, D. *et al.* (2001) Alder (*Alnus crispa*) effects on soils in ecosystems of the Agashashok River valley, northwest Alaska. *Ecoscience* 8, 89-95.

Ruess, R.W., Anderson, M.D., Mitchell, J.S. *et al.* (2006) Effects of defoliation on growth and N fixation in *Alnus tenuifolia*: consequences for changing disturbance regimes at high latitudes. *Ecoscience* 13, 404-412.

Ruess, R.W., McFarland, J.W., Trummer, L.M. *et al.* (2009) Disease-mediated declines in N-fixation inputs by *Alnus tenuifolia* to early-successional floodplains in interior and south-central Alaska. *Ecosystems* 12, 489-502.

Russo, R.O. (1989) Evaluating alder-endophyte (*Alnus acuminata- Frankia*-mycorrhizae) interactions I. Acetylene reduction in seedlings inoculated with *Frankia* strain ArI3 and *Glomus intra-radices*, under three phosphorous levels. *Plant and Soil* 118, 151-155.

Sawada, H., Kuykendall, L.D., Young, J.M. (2003) Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. *Journal of General and Applied Microbiology* 49, 155-179.

Schrader, J.A., Graves, W.R. (2004) Systematics of *Alnus maritima* (Seaside alder) resolved by ISSR polymorphisms and morphological characters. *Journal of the American Society for Horticultural Science* 129, 231-236.

Schwintzer, C.R. (1990) Spore-positive and spore-negative nodules. In: *The Biology of Frankia and Actinorhizal Plants*. (eds. C.R. Schwintzer and J.D. Tjepkema. Academic Press, Inc., San Diego.

Sellstedt, A. (1986) Nitrogen and carbon utilization in *Alnus incana* fixing N_2 or supplied with NO_3^- at the same rate. *Journal of Experimental Botany* 37, 786-797.

Sellstedt, A., Huss-Danell, K. (1986) Biomass production and nitrogen utilization by *Alnus incana* when grown on N_2 or NH_4^+ made available at the same rate. *Planta* 167, 387-394.

Sellstedt, A., Huss-Danell, K., Ahlqvist, A-S. (1986) Nitrogen fixation and biomass production in symbioses between *Alnus incana* and *Frankia* strains with different hydrogen metabolism. *Physiologia Plantarum* 66, 99-107.

Sheppard, L.J., Hooker, J.E., Wheeler, C.T. *et al.* (1988) Glasshouse evaluation of the growth of *Alnus rubra* and *Alnus glutinosa* on peat and acid brown earth soils when inoculated with four sources of *Frankia*. *Plant and Soil* 110, 187-198.

Simms, E.L., Taylor, D.L. (2002) Partner choice in nitrogen- fixation mutualisms of legumes and rhizobia. *Integrative and Comparative Biology* 42, 369-380.

Simms, E.L., Taylor, D.L., Povich, J. *et al.* (2006) An empirical test of partner choice in a wild legume-rhizobium interaction. *Proceedings of the Royal Society* B 273, 77-81.

Simonet, P., Navarro, E., Rouvier, C. *et al.* (1999) Co-evolution between *Frankia* populations and host plants in the family Casuarinaceae and consequent patterns of global dispersal. *Environmental Microbiology* 1, 525–533.

Stewart, W.D.P., Bond, G. (1961) The effect of ammonium nitrogen on fixation of elemental nitrogen in *Alnus* and *Myrica*. *Plant and Soil* 14, 347-359.

Sturm, M., Racine, C., K. Tape. (2001) Increasing shrub abundance in the Arctic. *Nature* 411, 546.

Tape, K., Sturm, M., Racine, C. (2006) The Evidence for shrub expansion in Northern Alaska and the Pan-Arctic. *Global Change Biology* 12, 686-702.

Tateno, M. (2003) Benefit to N_2-fixing alder of extending growth period at the cost of leaf nitrogen loss without resorption. *Oecologia* 137, 338-343.

Tedersoo, L., Suvi, T., Jairus, T. *et al.* (2009) Revisiting ectomycorrhizal fungi of the genus *Alnus*: differential host specificity, diversity and determinants of the fungal community. *New Phytologist* 182, 727-735.

Thompson, J.N. (1982) *Interaction and Coevolution.* Wiley and Sons, Inc., NY.

Thompson, J.N. (1994) *The Coevolutionary Process.* University of Chicago Press, Chicago.

Thompson, J.N. (2005) *The Geographic Mosaic Theory of Coevolution.* University of Chicago Press, Chicago.

Tjepkema, J.D., Winship, L.J. (1980) Energy requirement for nitrogen fixation in actinorhizal and legume root nodules. *Science* 209, 279-281.

Tremblay, F.M., Nesme, X., Lalonde, M. (1984) Selection and micropropagation of nodulating and non-nodulating clones of *Alnus crispa* (Ait.) Pursh. *Plant and Soil* 78, 171-179.

Uliassi, D.D., Ruess, R.W. (2002) Limitations to symbiotic nitrogen fixation in primary succession on the Tanana River floodplain. *Ecology* 83, 88-103.

U.S. Environmental Protection Agency (2007) Level III ecoregions of the continental United States (revision of Omernik, 1987). Corvallis, Oregon: U.S. Environmental Protection Agency-National Health and Environmental Effects Research Laboratory Map M-1, various scales.

Van Cleve, K., Viereck, L.A., Schlentner, R.L. (1971) Accumulation of nitrogen in alder (*Alnus*) ecosystems near Fairbanks, Alaska. *Arctic and Alpine Research* 3, 101-114.

Van Cleve, K., Viereck, L.A. (1981) Forest succession in relation to nutrient cycling in the boreal forest of Alaska In: *Forest Succession: Concepts and Application.* (eds. D.C. West, H.H. Shugart, D.B. Botkin) Springer-Verlag, NY.

van Dijk, C. (1984) Ecological aspects of spore formation in the *Frankia-Alnus* symbiosis. PhD Thesis. State University of Leiden, Leiden, The Netherlands.

van Dijk, C., Sluimer, A., Weber, A. (1988) Host range differentiation of spore-positive and spore-negative strain types of *Frankia* in stands of *Alnus glutinosa* and *Alnus incana* in Finland. *Physiologia Plantarum* 72, 349-358.

van Dijk, C., Sluimer-Stolk, A. (1990) An ineffective strain type of *Frankia* in the soil of natural stands of *Alnus glutinosa* (L.) Gaertner. *Plant and Soil* 127, 107-121.

van Dijk, C., Sluimer, A. (1994) Resistance to an ineffective *Frankia* strain type in *Alnus glutinosa* (L.) Gaertn. *New Phytologist* 128, 497-504.

Viereck, L.A., Little Jr., E.L. (2007) *Alaska Trees and Shrubs,* 2nd ed. University of Alaska Press, Fairbanks, Alaska.

Walker, L.R., Chapin III, F.S. (1986) Physiological controls over seedling growth in primary succession on an Alaskan floodplain. *Ecology* 67, 1508-1523.

Wall, L.G., Huss-Danell, K. (1997) Regulation of nodulation in *Alnus incana-Frankia* symbiosis. *Physiologia Plantarum* 99, 594-600.

Wall, L.G. (2000) The actinorhizal symbiosis. *Journal of Plant Growth Regulation* 19, 167-182.

Wall, L.G., Berry, A.M. (2008) Early interactions, infection and nodulation in actinorhizal symbiosis. In: *Nitrogen Fixation: Origins, Applications, and Research Progress,* Vol. 6 *Nitrogen-fixing Actinorhizal Symbioses.* (eds. K. Pawlowski, W.E. Newton) Springer, Dordrecht, The Netherlands.

Weber, A. (1986) Distribution of spore-positive and spore- negative nodules in stands of *Alnus glutinosa* and *Alnus incana* in Finland. *Plant and Soil* 96, 205-213.

Weber, A., Sarsa, M-L, Sundman, V. (1989) *Frankia-Alnus incana* symbiosis: effect of endophyte on nitrogen fixation and biomass production. *Plant and Soil* 120, 291-297.

Weber, A. (1990) Host specificity and efficiency of nitrogenase activity of *Frankia* strains from *Alnus incana* and *Alnus glutinosa. Symbiosis* 8, 47-60.

Welsh, A., Mirza, B.S., Reider, J.P. *et al.* (2009) Diversity of frankiae in root nodules of *Morella pensylvanica* grown in soils from five continents. *Systematic and Applied Microbiology* 32, 201-210.

West, S.A., Kiers, E.T., Simms, E.L. *et al.* (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proceedings of the Royal Society of London* **B** 269, 685–694.

Wheeler, C.T., Akkermans, A.D.L., Berry, A.M. (2008) *Frankia* and actinorhizal plants: a historical perspective. In: *Nitrogen Fixation: Origins, Applications, and Research Progress*, Vol. 6 *Nitrogen-fixing Actinorhizal Symbioses*. (eds. K. Pawlowski and W.E. Newton) Springer, Dordrecht, The Netherlands.

Wilson, B.F., Patterson III, W.A., O'Keefe, J.F. (1985) Longevity and persistence of alder west of the tree line on the Seward Peninsula, Alaska. *Canadian Journal of Botany* 63, 1870-1875.

Wipfli, M.S., Deal, R.L., Hennon, P.E. *et al.* (2003) Compatible management of Red Alder-conifer ecosystems in southeastern Alaska. In: *Compatible Forest Management*. (eds. R.A. Monserud, R.W. Haynes, A.C. Johnson) U.S. Government Publication. Kluwer Academic, The Netherlands.

Wolters, D.J., van Dijk, C., Zoetendal, *E.G. et al.* (1997) Phylogenetic characterization of ineffective *Frankia* in *Alnus glutinosa* (L.) Gaertn. nodules from wetland soil inoculants. *Molecular Ecology* 6, 971-981.

Wurtz, T.L. (1995) Understory alder in three boreal forests of Alaska: local distribution and eVects on soil fertility. *Canadian Journal of Forest Research* 25, 987–996

Yamanaka, T., Li, C-Y, Bormann, B.T. *et al.* (2003) Tripartite associations in an alder: effects of *Frankia* and *Alpova diplophloeus* on the growth, nitrogen fixation and mineral nutrition of *Alnus tenuifolia*. *Plant and Soil* 254, 179-186.

Yamanaka, T., Akama, A., Li, C-Y. *et al.* (2005) Growth, nitrogen fixation and mineral acquisition of *Alnus sieboldiana* after inoculation of *Frankia* together with *Gigaspora margarita* and *Pseudomonas putida*. *Journal of Forest Research* 10, 21- 26.

CHAPTER 2:

Estimating N_2 Fixation in Two Species of Alnus in Interior Alaska Using Acetylene Reduction and $^{15}N_2$ Uptake[1]

ABSTRACT

In interior Alaskan boreal forests two species of alder, Alnus tenuifolia and Alnus crispa, represent keystone species in floodplain and upland landscapes, respectively, due to the ability of these plants to form symbiotic associations with the nitrogen-fixing actinomycete, Frankia. It is believed that as much as 70% of the nitrogen (N) accumulated during the 200 year successional development of these forests is derived through atmospheric fixation by these species. Estimates of gross N inputs in these and many other ecosystems have traditionally utilized the acetylene reduction assay (ARA), which requires a conversion factor of the ratio of acetylene to N_2 reduced by nitrogenase, the primary enzyme. Despite the fact that small variations in the reduction ratio can substantially influence estimates of N inputs, few studies have investigated how it varies spatially and temporally. The present study sought to: 1) determine this conversion factor for both species of alder in situ by calibration of the ARA against a $^{15}N_2$ uptake method we developed for field use, and 2) determine whether the conversion factor varied with the successional stage in which the alders occurred.

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[1] Anderson, M.D., R. W. Ruess, D. D. Uliassi & J. S. Mitchell, 2004. Estimating N_2 fixation in two species of Alnus in interior Alaska using acetylene reduction and $^{15}N_2$ uptake. Ecoscience, 11: 102-112.

Specific N fixation (SNF), as measured by $^{15}N_2$ uptake, averaged 36.9 ± 3.4 μmol N g_{DWT} nodule^{-1} hr^{-1} in A. tenuifolia, and 27.5 ± 3.8 μmol N g_{DWT} nodule^{-1} hr^{-1} in A. crispa. Specific acetylene reduction activity (SARA) averaged 15.6 ± 1.6 μmol C_2H_4 g_{DWT} nodule^{-1} hr^{-1} for A. tenuifolia, and 11.3 ± 1.6 μmol C_2H_4 g_{DWT} nodule^{-1} hr^{-1} for A. crispa. Averaged across all plants, the ratio of acetylene to N_2 reduced was 1.92 ± 0.15, and was significantly greater in A. crispa (2.0 ± 0.2) compared with A. tenuifolia (1.81 ± 0.24) ($F_{1,88} = 5.21$, $P < 0.05$). Significant differences ($P < 0.01$) in the value of the conversion factor were observed between early succession and later (mid and late) successional sites for both species. Such differences were also observed among replicate sites within and among stages; site averages ranged from 0.37 ± 0.09 to 5.59 ± 0.76 (n = 10). However, these stage and site differences may also be due to seasonal effects, which could not be controlled for with our design.

SARA was only correlated with $^{15}N_2$ uptake for early successional sites measured early in the growing season, when N_2-fixation rates were lowest and the conversion factor was closest to the theoretical value of four. A significant negative correlation was found between the conversion factor value and the rate of enzyme activity as determined by the $^{15}N_2$ uptake method. Two hypotheses are proposed to explain this result: 1) that it is due to changes in the kinetic properties of nitrogenase at high levels of enzyme activity, resulting in an increased affinity of the enzyme for N_2 relative to C_2H_2, and 2) that the concentration of C_2H_2 used in our ARA was insufficient to saturate nitrogenase, resulting in a higher electron allocation to N_2 relative to C_2H_2 than would be predicted from theoretical considerations. Because of the variation and apparent rate-dependence of the

conversion factor observed in this study, we conclude that previous estimates of ecosystem N inputs based on ARA may need to be reassessed.

INTRODUCTION

Biologically available nitrogen (N) widely limits primary production in terrestrial ecosystems (Vitousek & Field, 1999). The largest non-anthropogenic input of N to the global N cycle is provided by N_2-fixing microbes in symbiotic associations with plants (Paul, 1988; Tate, 1995). These associations are of particular importance to the successional development of interior Alaska boreal forests, where as much as 70% of the N accumulated over the 200 year primary successional sequence on the Tanana River floodplain enters during the first 20-30 years via N_2-fixation by actinomycete bacteria of the genus Frankia in association with Alnus tenuifolia (Viereck, Dyrness & Foote, 1993; Van Cleve et al., 1991; Uliassi & Ruess 2002). The N fixed during this stage has been found to influence the productivity of subsequent succession on the Tanana River floodplain (Van Cleve et al., 1983, 1991). Similarly, it appears that the invasion of alder into interior Alaska during the early Holocene contributed to both the productivity of aquatic systems and N-cycling rates of the soils in the region (Hu, Finney & Brubaker, 2001).

Rate-based estimates of N inputs due to N_2-fixation by interior Alaskan alder have relied on the use of the acetylene reduction assay (ARA) as an estimator of nitrogenase activity (Klingensmith & Van Cleve, 1993; Uliassi & Ruess, 2002). This

technique is based on the assumption that the N_2-fixing enzyme complex, nitrogenase, preferentially reduces acetylene (C_2H_2) to ethylene (C_2H_4) over N_2 to ammonium under an atmosphere containing more than 10% C_2H_2 (Hardy et al., 1968; Shah, Davis & Brill, 1975; Rivera-Ortiz & Burris, 1975). The incubation atmosphere is analyzed for C_2H_4 at varying intervals, providing a simple and low-cost assay of nitrogenase activity (Hardy et al., 1968). A major limitation of the method is the acetylene-induced decline, or inhibition of nitrogenase activity by C_2H_2 within a few minutes of initial exposure (Minchin et al., 1983). However, this effect can be minimized or eliminated using short (2-6 min) incubation times and taking the maximum value of acetylene reduction as representative of pre-assay nitrogenase activity (Minchin et al., 1983; Schwintzer & Tjepkema, 1994).

A second limitation of the ARA as a quantitative indicator of nitrogenase activity is the lack of stoichiometric equivalence between the two reactions catalyzed by the enzyme; i.e., the molar amounts of C_2H_2 converted to C_2H_4, and N_2 converted to NH_3 are not equal. A ratio of 3 moles of C_2H_2 to 1 mole of N_2 is commonly assumed based on the observation that nitrogenase requires one pair of electrons to reduce C_2H_2 to C_2H_4, but three electron pairs to reduce N_2 to two molecules of NH_3 (Hardy et al., 1968). However, based on the discovery that nitrogenase reduces at least one pair of protons to H_2 gas per molecule of N_2, but none to reduce C_2H_2, some authors have proposed a value of 4:1 for this ratio (Simpson & Burris, 1984; Miller, 1991). By calibration against $^{15}N_2$ uptake methods, the ratio has been experimentally determined for a wide variety of species and found to vary by three orders of magnitude, depending on the nature of the

system and the conditions under which the assay is performed (Seitzinger & Garber, 1987; Liengen, 1999). This has led several authors to recommend independent determination of the ratio for each biological system on which the ARA is to be used for quantifying N_2 fixation rates (Saito, Matsui & Salati, 1980; Nohrstedt, 1983, 1985; Seitzinger & Garber, 1987; Schwintzer & Tjepkema, 1994; Montoya et al., 1996). However, such determinations have only been reported for a handful of actinorhizal species in temperate (Fessenden, Knowles & Brouzes, 1973), boreal (Sellstedt, 1986), and tropical ecosystems (Vitousek & Walker, 1989).

Ecosystem level estimates of N inputs based on the ARA are sensitive to the value chosen for the conversion between acetylene reduction (AR) and N_2 fixation. Using a short-term assay to minimize C_2H_2 inhibition and a conversion factor of 3:1, Uliassi & Ruess (2002) estimated N_2-fixation inputs from A. tenuifolia of 59 ±11 kg N ha^{-1} yr^{-1} and 39 ±11 kg N ha^{-1} yr^{-1} in early-successional (alder canopy) and mid-successional (mature balsam poplar canopy) interior Alaskan floodplain forests, respectively. Comparing these values to previously published whole-stand N accumulation data from the same area, they estimated annual losses of 29% (17 kg N ha^{-1} yr^{-1}) and 74% (29 kg N ha^{-1} yr^{-1}) respectively from the two forest types, but acknowledged the strong dependence of their conclusions on the value of the C_2H_2 to N_2 reduction ratio.

The objectives of this study were to 1) determine the C_2H_2 to N_2 reduction ratio for thinleaf alder (Alnus tenuifolia) occurring in primary successional seres of the Tanana River floodplain, and green alder (Alnus crispa), occurring in secondary seres in upland areas adjacent to the Tanana River floodplain, and 2) evaluate the variability of this ratio

within and between the two Alnus species in their respective habitats. To accomplish this, we simultaneously measured AR and $^{15}N_2$ uptake by excised nodules in situ for both plant species growing in replicate early-, mid-, and late-successional floodplain and upland forests in interior Alaska.

METHODS

Study sites

Sites for this study included early, mid- and late-successional boreal forests located within the Bonanza Creek Experimental Forest, approximately 30 km south-west of Fairbanks, Alaska (64° 48' N, 147° 52' W). The general restriction of green alder [Alnus crispa (Ait.) Pursh] to upland forests, and thinleaf alder [Alnus tenuifolia Nutt.] to floodplain forests, while not absolute, is thought to be related to soil moisture content, which is typically greater in floodplain stands (Hultén, 1968; Viereck & Little, 1986; Van Cleve et al., 1991). However, there may also be species-specific differences in response to soil phosphorus and pH, which differ between the two landscapes (Van Cleve et al., 1993). Both species propagate by seed, but vegetative propagation may be more important in explaining the persistence of alder throughout all stages of succession. Half of our study sites were located in forests along the floodplain of the Tanana River, where A. tenuifolia is common. These sites included early-successional stands dominated by A. tenuifolia and balsam poplar (Populus balsamifera) (15-25 yr), mid-successional stands dominated by balsam poplar with a near-continuous understory of A.

tenuifolia (75-100 yr), and late-successional stands of mature white spruce (Picea glauca) with a dense understory of A. tenuifolia (100-175 yr) (Viereck, Dyrness & Foote, 1993). The other half of our study sites were located in upland areas adjacent to the Tanana floodplain, in which the dominant alder species is A. crispa. These sites included early successional post-burn stands dominated by a mixture of A. crispa, paper birch (Betula papyrifera), and trembling aspen (Populus tremuloides) (15-25 yr), mid-successional stands dominated by paper birch and/or aspen, with a sub-canopy of A. crispa (50-85 yr), and late-successional stands of mature white spruce with a mixed sub-canopy of A. crispa and paper birch (75-200 yr).

For each successional stage within both upland and floodplain forests , three replicate stands, separated by up to 5 km, were selected, for a total of 18 sites. Our sites are included within a larger network of permanent sites under study by the Bonanza Creek Long Term Ecological Research Program (BNZ LTER). Further information concerning these sites can be found on the BNZ LTER webpage (http://www.lter.uaf.edu/).

The climate of the region is characterized by an intensely cold snow period averaging 214 days, and annual temperature extremes ranging from -50 to 35 °C. Average daily temperatures range from -24.9 °C in January to 16.4 °C in July, with an average annual temperature of -3.3 °C. Potential evapotranspiration (466 mm) exceeds annual precipitation (269 mm), 65% of which falls during the growing season, which typically extends from mid-May to early September (Viereck, Dyrness & Foote, 1993).

Striking differences in soil climate accompany successional development in both upland (elevation = 270 - 375 m) and floodplain landscapes (elevation = 135 m), where canopy closure and the development of a continuous moss layer in later successional stages reduce average soil temperatures. Landscape differences in soil climate are also strongly influenced by topography. For example, north-facing slopes are usually underlain by permafrost, and contrast sharply with the warm, well-drained soils of south-facing slopes. Poorly-drained black spruce lowlands are also largely underlain by permafrost. Parent material, slope, aspect, time and climate have been used to describe the mosaic of soils found throughout interior Alaska. Soils are uniformly immature, reflecting the characteristics of three parent material categories: bedrock composed of precambrian schist, thick loess deposits originating from glacial periods, and alluvial deposits in floodplain areas. Soils range from poorly-drained cold soils with shallow permafrost to warm well-drained soils in the uplands that support mature white spruce communities. Upland south-facing forests can experience drought stress throughout the growing season, a phenomenon that has become more prevalent during warming of the Alaskan interior over the past 75 years (Barber, Juday & Finney, 2000).

Experimental design

This study was conducted from late June to mid-August, 2001, during the peak of the season for N_2-fixation activity (Uliassi & Ruess 2002). At each of the three replicate stands within each successional stage in both floodplain and upland forests, ten plants (A. tenuifolia on the floodplain and A. crispa in the uplands) were randomly selected for

measurement of AR and $^{15}N_2$ uptake. Sampling dates for each site are given in Table 2.1. All measurements were taken between 0900 and 1400 Alaska Daylight Time. ARA and $^{15}N_2$ uptake were run simultaneously on sub-samples of nodules harvested from each plant, as described below. Most nodules of both species occur within the fibric layer close to the soil surface, and a nodule sample adequate for both assays could be harvested within 10 minutes for each plant.

Acetylene reduction assay

We used a closed-system field assay with a short incubation time (2.5 minutes) to minimize the potential for acetylene-induced decline in nitrogenase activity (Uliassi & Ruess 2002). Acetylene was generated on site by hydration of CaC_2 in a Bliss generator equipped with a rubber septum for convenient gas withdrawal. Approximately 2.5 g (fresh weight) of nodule clusters with subtending fine roots were harvested and placed in a 60 ml polyethylene syringe fitted with a septum to facilitate gas additions and withdrawals. Nodule harvests generally consisted of removal of the root to which the nodule clusters were attached, in order to minimize nodule disturbance. When large root sizes precluded this method, however, clusters were removed by breaking them off at the base along their natural planes of separation.

Nodules were maintained at ambient soil temperature, which was measured with a hand-held digital thermometer (Taylor Thermometers, Oak Brook, Illinois, USA), by placing the incubation syringe within the forest floor until the assay was performed. Within 5-10 minutes of initial nodule harvest, 6 ml of C_2H_2 were added to the syringe,

producing an incubation atmosphere of approximately 10% v/v C_2H_2. This was designated as time zero (T_0). Gas samples were withdrawn from the incubation syringe at 30 s (T_1) and 150 s (T_2) from the time of initial C_2H_2 injection. These samples were transported to the laboratory in Fairbanks within 8 hours of sampling, and immediately analyzed for C_2H_4 content using a Shimadzu 14A gas chromatograph equipped with a 2 m Poropak N column and a back-flush valve to vent C_2H_2 (Shimadzu Scientific, Houston, Texas, USA). Total C_2H_2 reduction was calculated by dividing the increase in molar C_2H_4 concentration from T_1 to T_2 by the time elapsed between the two samples. The result was expressed as specific acetylene reduction activity (SARA = μmol C_2H_4 g_{DWT} nodule^{-1} hr^{-1}) by dividing the result by nodule dry weight of the sample in grams (Eq. 1). Nodule dry weight was determined by thoroughly rinsing each nodule sample, removing the roots, drying the nodules for 48 hours at 65 °C, and weighing to the nearest 0.01 mg.

$$\text{SARA} = \frac{(\text{moles } C_2H_4 \text{ at } T_2 \; - \; \text{moles } C_2H_4 \text{ at } T_1)}{(\text{incubation time (h)} \;\; X \;\; \text{nodule dry wt (g)})} \qquad \text{[Eq. 1]}$$

<u>$^{15}N_2$ uptake</u>

Approximately 2.5 g of fresh nodule with attached fine root were harvested simultaneously with the sample used for SARA determination, placed in a second 60 ml polyethylene syringe fitted with a septum, and placed within the forest floor to maintain the sample at ambient soil temperature, as described above. Ten ml of 99 atom % $^{15}N_2$ (Isotec Inc., Miamisburg, Ohio, USA) was then added to the syringe to produce an

incubation atmosphere of approximately 15% $^{15}N_2$. Immediately after the addition of the $^{15}N_2$, a 15 ml sample of the incubation atmosphere was removed to provide a quantitative measure of atom percent enrichment (APE) of $^{15}N_2$ at T_0. These samples were stored in 10 ml exetainers (Labco, High Wycombe, Buckinghamshire, UK) and transported to Fairbanks for analysis using a dual-inlet isotope ratio mass spectrometer (PDZ Europa Scientific Instruments, Crewe, Chesire, UK). After the removal of the incubation atmosphere sample, the syringe was immediately returned to the forest floor for 10 minutes. Nodules were then removed from the syringe and immediately frozen in liquid N_2. In the laboratory, nodules were thoroughly rinsed through a fine seive of all adhering soil and organic material, dried for 48 hours at 65 °C, and ground using a Wig-L-Bug ball mill (Reflex Analytical, Ridgewood, New Jersey, USA) in preparation for mass spectrometry analysis. The dried nodule samples from the SARA on each plant were used as controls for the determination of atom percent enrichment (APE) for each nodule sample according to the following equation:

$$APE_{nodule} = {}^{15}N_{enriched\ nodules} - {}^{15}N_{control\ nodules} \qquad [Eq. 2]$$

where both ^{15}N content measures are in atom %. By combining APE with total nodule N content, dividing by incubation time, and correcting for the composition of the initial incubation atmosphere as determined by mass spectrometry, we calculated the specific N_2 fixation activity of the nodule samples (SNF = μmol N assimilated g_{DWT} nodule^{-1} h^{-1}) as follows:

$$SNF = \frac{(APE_{nodule} \; X \; \%N_{nodule})}{(\text{incubation time (h)} \; X \; \%^{15}N_{atmosphere})} \qquad \text{[Eq. 3]}$$

where $\%N_{nodule}$ is the mass percent N content of the enriched nodule sample and $\%^{15}N_{atmosphere}$ is the atom percent ^{15}N content of the incubation atmosphere at the beginning of the assay. SNF was divided by 2 to give a measure of N_2 fixation rather than N fixation in all ratio calculations.

C_2H_2 inhibition experiment

To determine whether N_2 fixation was inhibited in the presence of C_2H_2 under our assay conditions, we exposed nodules from twelve greenhouse-grown A. tenuifolia seedlings to both substrates simultaneously, using the assays and sample preparation methods employed in our field study. Three sets of nodules were excised from each plant; one was exposed to approximately 15% v/v $^{15}N_2$, another was exposed to 15% v/v $^{15}N_2$ plus 10% v/v C_2H_2, and the final sample was used to determine ^{15}N natural abundance for the $^{15}N_2$ uptake assay.

Statistical analysis

Differences in parameters related to N_2 fixation were analyzed by ANOVA (PROC GLM) (SAS 1999) using a cross-nested model (Neter et al. 1996). The full model tested for the effects of species, successional stage, ecosystem replicate within

successional stage, and plant within replicate and stage. Species, successional stage, ecosystem replicate, and plant number were included as class variables. Ecosystem replicate within successional stage, and plant number within stage and replicate were included as random effects.

Successional stage and sampling date were confounded, due to the fact that it was not possible to sample both species within all 3 successional stages at the same time. These stage/seasonality effects often accounted for the largest proportion of explained variance in our data. While both successional stage and seasonality have been demonstrated to have strong effects on N_2-fixation rates as measured by ARA in these sites (Schimel, Cates & Ruess, 1998; Uliassi et al., 2000; Uliassi & Ruess, 2002), we were unable to investigate these effects independently in this study. Therefore, to avoid confusion, early succession sites of both species, which were all sampled early in the season, are referred to as Type I sites, rather than as early succession or early season sites. Similarly, mid and late succession/season sites are referred to as Type II and Type III sites, respectively. These site types are defined in Table 2.1.

Because both species were sampled in each of the three successional stages at approximately the same time, we tested for species differences for each successional stage/sampling period by removing successional stage from the model and running each stage separately. In several cases, apparent differences between species in N_2-fixation seemed to be a function of differences in soil temperature. To account for this, ANCOVA was run using soil temperature as a covariate. All species differences reported

here are adjusted for those effects. Data were square-root or $\log_{10}(X+1)$ transformed where necessary to meet ANOVA assumptions.

Data from the greenhouse experiment were compared between treatments ($^{15}N_2$ only vs. $^{15}N_2 + C_2H_2$) using a paired t-test (SAS 1999). Unless otherwise stated, data presented are means (± 1 S.E.) of untransformed data.

RESULTS

$^{15}N_2$ uptake and SARA

Specific N fixation (SNF), as measured by $^{15}N_2$ uptake, was 34% greater in A. tenuifolia (36.9 ± 3.4 μmol N g_{DWT} nodule^{-1} hr^{-1}) compared with A. crispa (27.5 ± 3.8 μmol N g_{DWT} nodule^{-1} hr^{-1}) when averaged across all measurements ($P = 0.02$) (Table 2.2). Part of this difference could be attributed to slightly warmer soils in floodplain stands dominated by A. tenuifolia (11.9 ± 0.3 °C) compared with upland stands dominated by A. crispa (11.2 ± 0.2 °C) ($P = 0.06$), reducing species differences in SNF ($F_{1,88} = 3.50$, $P = 0.06$). Across all sampling dates, SNF rates were positively correlated with soil temperature for both species, but more closely for A. crispa (r^2=0.28, $P < 0.0001$) than for A. tenuifolia (r^2=0.04, $P < 0.05$). The largest proportion of explained variation in SNF was accounted for by site type ($F_{2,88} = 3.04$, $P < 0.05$), and replicate within site type ($F_{6,88} = 14.05$, $P < 0.0001$). For reasons explained earlier, we are uncertain as to whether the apparent successional pattern in SNF (early = 8.2 ± 1.1 μmol

N g_{DWT} nodule^{-1} hr^{-1}; mid = 47.3 ± 5.6 μmol N g_{DWT} nodule^{-1} hr^{-1}; late = 41.2. ± 3.5 μmol N g_{DWT} nodule^{-1} hr^{-1}) is mainly a function of a seasonal pattern, known to be pronounced in these forests (Uliassi & Ruess 2002), given that these stages were sampled around 1 July, 17 July, and 3 August, respectively. The least squares means test indicated significant differences ($P < 0.0005$) in SNF between Type I sites and both Type II and Type III sites, but not between Type II and Type III sites in both A. tenuifolia and A. crispa (Table 2.3).

Species differences in SARA varied to the same degree as SNF, with values for A. tenuifolia (15.6 ± 1.6 μmol C_2H_4 g_{DWT} nodule^{-1} hr^{-1}) being approximately 37% greater than those measured for A. crispa (11.3 ± 1.6 μmol C_2H_4 g_{DWT} nodule^{-1} hr^{-1}) when averaged across all measurements ($F_{1,89} = 5.28$, $P < 0.05$). Again, these species differences were less pronounced when controlled for differences in soil temperature ($F_{1,88} = 2.37$, $P = 0.13$). The overall model explained approximately 59% of the variation in SARA ($F_{91,179} = 1.37$, $P = 0.07$), which was less than the overall explained variation in SNF (69%, $F_{91,179} = 2.18$, $P < 0.0001$). Although variation among replicates within successional stages accounted for a large percentage of explained variation ($F_{6,88} = 5.09$, $P < 0.001$), no apparent differences in SARA among site types were detected ($F_{2,88} = 1.14$, $P = 0.33$). SARA was positively, but weakly correlated with soil temperature for both A. crispa ($r^2=0.06$, $P < 0.05$) and A. tenuifolia ($r^2=0.04$, $P < 0.05$).

C_2H_2 to N_2 reduction ratio

Averaged across all plants, the ratio of C_2H_2 to N_2 reduced was 1.92 ± 0.15, and was significantly greater in A. crispa (2.0 ± 0.2) compared with A. tenuifolia (1.81 ± 0.24) ($F_{1,88} = 5.21$, $P < 0.05$). This ratio was greatest in Type I stands (3.47 ± 0.33), but significantly less in Type II (1.27 ± 0.14), and Type III stands (1.01 ± 0.13) ($F_{2,6} = 10.11$, $P = 0.01$) (Table 2.3). In Type I A. tenuifolia stands the average ratio was 3.82 and was not significantly different from either 3 ($P = 0.1349$) or 4 ($P = 0.7332$). In Type I A. crispa stands the average ratio was 3.13, which was significantly different from 4 ($P = 0.0354$), but not 3 ($P = 0.7559$). In all other stands for both species, the ratio was significantly different from both theoretical values ($P < 0.0001$). The ratio of C_2H_2 to N_2 reduced also varied significantly among replicates within site type within species ($F_{6,97} = 9.03$, $P < 0.0001$).

Rates of SNF and SARA were positively correlated for A. crispa ($r^2 = 0.11$, $P < 0.01$), but not for A. tenuifolia ($P = 0.23$) across all sites. In both species this correlation was strong and highly significant in Type I sites ($r^2 = 0.73$, $P < 0.0001$ for A. tenuifolia; $r^2 = 0.49$, $P < 0.0001$ for A. crispa), but not in Type II or Type III sites (Fig. 2.1).

Our inhibition experiment indicated no significant difference in ^{15}N enrichment of nodule tissue between samples exposed to both C_2H_2 and $^{15}N_2$ and those exposed only to $^{15}N_2$ ($P = 0.7342$), indicating a failure of C_2H_2 to inhibit N_2 fixation under our assay conditions.

Leaf $\delta^{15}N$

Leaf $\delta^{15}N$ was significantly more enriched in A. tenuifolia (0.02 ± .17 ‰) compared with A. crispa (-0.55 ± 0.19 ‰) ($P < 0.02$). This difference was primarily driven by strong species differences in Type II sites, in which leaf $\delta^{15}N$ was significantly more depleted in A. crispa (-1.38 ± 0.16 ‰) relative to A. tenuifolia (1.09 ± 0.20 ‰) ($P < 0.0001$) (Fig. 2.2). This result is consistent with higher N fixation rates in A. tenuifolia compared to A. crispa. These species differences were not found in Type I sites, where values for both species were most depleted and were nearly identical (A. tenuifolia = -1.68 ± 0.05 ‰, A. crispa = -1.63 ± 0.03 ‰) ($P = 0.19$), but were again significantly different in Type III sites (A. tenuifolia = 1.01 ± 0.22 ‰, A. crispa = 1.38 ± 0.36 ‰) ($P = 0.02$). Across all plants, $\delta^{15}N$ and SNF were positively correlated for both A. tenuifolia ($r^2 = 0.19$, $P < 0.0001$) and A. crispa ($r^2 = 0.08$, $P < 0.01$). However, leaf $\delta^{15}N$ was not correlated with SARA in either species.

DISCUSSION

Reduction ratio

In this experiment, the C_2H_2 to N_2 reduction ratio was determined by dividing the rate of C_2H_2 reduction determined for a random sample of nodules from an individual plant by the rate of N_2 reduction determined using $^{15}N_2$ uptake on a second random sample of nodules from the same plant. However the incubation times for the two assays used were not equal; the ARA was carried out for 2.5 minutes while the $^{15}N_2$ uptake

assay was carried out for 10 minutes. We are aware of the possibility of methodological bias this creates in our calculation of the ratio due to the decline in nitrogenase activity after excision of nodules from the source plant (Mague & Burris, 1972). We tested this possibility in a small preliminary greenhouse experiment with 16 A. tenuifolia seedlings and found no difference between mean ARA of nodules assayed immediately after excision and those assayed 10 minutes after excision (data not shown).

The C_2H_2 to N_2 reduction ratio determined in this study was highly variable; site averages ranged from 0.37 ± 0.09 to 5.59 ± 0.76 ($n = 10$), and individual values ranged from 0.02 to 14.0. Previous studies have reported similar variation in the ratio, with values ranging from as low as 0.11 ± 0.01 (Liengen, 1999) to as high as 94 (Seitzinger & Garber, 1987). These upper and lower extremes may be more characteristic of free-living than symbiotic systems, as the above two studies investigated free-living soil and marine cyanobacterial systems, respectively. Previous studies with symbiotic systems have found somewhat less variability. At the low end, van Kessel & Burris (1983) reported a ratio of 0.65 for Trifolium pratense, while Saito, Matsui & Salati (1980) found a ratio of 8.3 ± 0.07 for Phaseolus vulgaris, both of which are Rhizobium-infected species. However, the sample size for both studies was small, and may have inadequately captured the full range of variation in the reduction ratio across broad environmental conditions for the host species.

The few reduction ratios measured for actinorhizal species demonstrate less variability than those for legumes. But again, these studies have been conducted principally on greenhouse-grown plants with low replication. Using hydroponically-

grown Myrica gale seedlings, Schwintzer & Tjepkema (1994) found the reduction ratio ranged from 2.54 ± 0.33 in light-stressed seedlings to 4.32 ± 0.10 in water-stressed seedlings (n=8). Vitousek & Walker (1989) used a $^{15}N_2$ uptake method on naturally-occurring Myrica faya in Hawaii to calibrate their use of the ARA. They reported a value of 3.5 ± 0.7, but only six plants were included in their determination, so the full range of variation in the reduction ratio of this species at their site may not have been captured. Sellstedt (1986) used Kjeldahl-N accumulation, $^{15}N_2$ uptake, and H_2 evolution to calibrate the ARA, and reported reduction ratios for 21 growth chamber-cultivated Alnus incana seedlings ranging from 2.04 to 3.94. Fessenden, Knowles & Brouzes (1973) reported a ratio of 3.14 ± 0.30 for excised nodule samples from 24 naturally-occurring Myrica asplendifolia individuals from the northeastern United States and southeastern Canada. However, because they used a long-term acetylene incubation, it is possible their results underestimated the ratio due to acetylene-induced decline in nitrogenase activity (Minchin et al., 1983).

Within-species variation in the reduction ratio of C_2H_2 to N_2 has been correlated with several environmental and physiological variables for both free-living and symbiotic systems. Among non-symbiotic systems, these variables include soil parent material and vegetation cover (Zechmeister-Boltenstern & Kinzel, 1990), time of season (Liengen, 1999), soil water content (Nohrstedt, 1983), and uptake hydrogenase activity (Paerl, 1982). Among symbiotic systems these variables include SNF (Gibson & Alston, 1984), efficiency of symbiotic nitrogenase (van Kessel & Burris, 1983), and pN_2 (Peters, Toia, Jr. & Lough, 1977) in non-actinorhizal systems, and host plant water and light stresses

(Schwintzer & Tjepkema, 1994) in Myrica gale, an actinorhizal plant. Mechanisms proposed to explain the correlation of soil physical/chemical characteristics with variation in the reduction ratio in non-symbiotic systems, such as differential adsorption of the two substrates to soil minerals (Rennie, Rennie & Fried, 1978; Nohrstedt, 1985) and differential diffusion rates of the two substrates in aqueous solution (Zechmeister-Boltenstern & Kinzel, 1990; Nohrstedt, 1983; Rice & Paul, 1971), may not be relevant to our study which utilized excised root nodules, since these explanations rely on direct effects of soil properties exerted during the assay.

In the present study, significant intra-specific differences in the value of the C_2H_2:N_2 reduction ratio were observed among replicate sites both within a site type and among site types. The site type effect probably contains components of both successional stage and seasonality. Due to the inherent confounding of these two factors in our experimental design, successional stage effects cannot be separated from seasonality effects in our analysis. Schwintzer & Tjepkema (1994) reported significant effects of both light and water availability on C_2H_2:N_2 reduction ratio in Myrica gale seedlings. Because these factors vary considerably both spatially and temporally in interior Alaskan boreal forests, it is possible that both site and site-type variations in the value of the C_2H_2:N_2 reduction ratio observed in our study are related to variation in one or both of these factors. In particular, Schwintzer & Tjepkema (1994) found that the C_2H_2:N_2 reduction ratio was significantly lower in light stressed plants, which is consistent with our finding that the value of the ratio decreased significantly between Type I sites where alder dominated the canopy, and both of the other site types, where alder was limited to

the understory. However, the degree to which variation in light and/or water availability was associated with variation in the C_2H_2:N_2 reduction ratio in our study cannot be directly assessed because no data were collected for either of these variables.

The pattern of variation in the C_2H_2:N_2 reduction ratio observed in our study suggests this variation might be largely accounted for by differences in N_2-fixation rate among sites. Both SNF and the C_2H_2:N_2 reduction ratio varied significantly between Type I sites and both other site types, but not between Type II and Type III sites (Table 2.3). Similarly, the relationship between ARA and SNF was most significant in Type I stands of both A. tenuifolia ($r^2 = 0.73$, $P < 0.0001$) and A. crispa ($r^2 = 0.49$, $P < 0.0001$), but was not significant in either species in Type II or Type III sites (Fig. 2.1). Apparent rate-dependence of the C_2H_2:N_2 reduction ratio has been previously reported in Australian Lupinus angustifolius crops (Gibson & Alston, 1984). These authors suggested this observation could be accounted for by a change in kinetics of nitrogenase at high specific N_2 reduction rates, resulting in the preferential reduction of N_2 over C_2H_2. This hypothesis is supported by a number of studies of nitrogenase in vitro, in which the enzyme appeared to favor N_2 as a substrate over C_2H_2 at high or non-limiting levels of electron flux (Davis, Shah & Brill, 1975; Shah, Davis & Brill, 1975; Sadkov & Likhtenshtein, 1990) or simply to decrease affinity for C_2H_2 at relatively high levels of electron flux (Eady & Postgate, 1974; Lowe, Fisher & Thorneley, 1990).

The apparent rate-dependence of the C_2H_2:N_2 ratio can be explained by the operating mechanisms of the nitrogenase complex. The complex is composed of two enzymes; nitrogenase, which binds either N_2 or C_2H_2, and nitrogenase reductase, which

passes electrons one at a time to the nitrogenase enzyme/substrate complex. Each one-electron transfer from nitrogenase reductase to nitrogenase/substrate is followed by a dissociation of the two enzymes, after which the now-oxidized reductase is again reduced while the nitrogenase associates with another reduced molecule of reductase. Thus, the reductase exists in two oxidation states, while the nitrogenase must pass through several oxidation states before the bound substrate is completely reduced (Burgess & Lowe, 1996).

Some oxidation states of nitrogenase have been found to have differing affinities for the enzyme's various substrates. For example, N_2 cannot bind to nitrogenase until the enzyme has been reduced at least three electrons above its ground state, while C_2H_2 can bind to nitrogenase in a less reduced state (Lowe, Fisher & Thorneley, 1990; Burgess & Lowe, 1996). Thus, at high N fixation rates, and presumably high levels of electron flux, a relatively high amount of nitrogenase may exist at a sufficiently reduced level to bind N_2, leading to a preferential reduction of this substrate relative to C_2H_2. At low fixation rates, the opposite substrate preference would be expected. This hypothesis relies on the assumption that high rates of fixation are largely due to high rates of electron flux through the nitrogenase complex.

A second hypothesis that may account for the negative correlation between the C_2H_2:N_2 reduction ratio and N_2-fixation observed in the present study is that a saturating level of C_2H_2 may not have been achieved during the assay. Hardy et al. (1968) proposed that 3–10% C_2H_2 would be sufficient to saturate the enzyme. This concentration is consistent with the apparent Michaelis constants of nitrogenase for the two substrates,

which suggest an approximately 10 to 20-fold greater affinity of the enzyme for C_2H_2 relative to N_2 (Hardy et al., 1968; Davis, Shah & Brill, 1975; Christiansen, Seefeldt & Dean, 2000).

Several authors report a near total transfer of electron flux to the reduction of C_2H_2 under a 10-20% C_2H_2 atmosphere, as indicated by the inhibition of H_2 evolution normally accompanying N_2 fixation (Rivera-Ortiz & Burris, 1975; Schubert & Evans, 1976; Paerl, 1982). This is why an incubation atmosphere of 10-20% C_2H_2 is generally recommended for the ARA, and was used in the present study. Zuckermann et al. (1997), however, using a highly sensitive real-time method for monitoring C_2H_4 production, failed to achieve complete saturation of in vivo nitrogenase in cultures of the cyanobacterium Nodularia spumigena under an atmosphere of 25% C_2H_2. Considering the greater diffusion barrier presented by excised nodules compared to heterocystous cyanobacteria, it is possible that even higher C_2H_2 concentrations might be necessary to achieve enzyme saturation in these systems. At sufficiently high levels of nitrogenase activity the reduction of C_2H_2 might become diffusion rather than enzyme-limited, causing the concentration of C_2H_2 in the immediate vicinity of nitrogenase to drop to levels insufficient to inhibit electron allocation to N_2. Thus, electron reallocation to C_2H_2 might be minimized at high levels of enzyme activity, allowing nitrogenase to reduce more N_2 relative to C_2H_2 than would be predicted from electron allocation stoichiometry. Such a diffusion limitation would be expected to be less pronounced for N_2, given the much higher concentration of this substrate compared to C_2H_2.

The failure of C_2H_2 to inhibit N_2 fixation under our assay conditions, as indicated by our greenhouse experiment, could be explained by the rate-dependence of the ratio; i.e., that a saturating level of C_2H_2 was not achieved during the assay or, given the high fixation rates observed in these greenhouse-grown plants (57.9 ± 9.5 µmol N g_{DWT} nodule^{-1} hr^{-1}), that nitrogenase favors nitrogen as a substrate at high levels of enzyme activity. Our greenhouse study demonstrated that $^{15}N_2$ uptake was not inhibited by C_2H_2 in the assay, but it is not clear which of our hypotheses, if either, explains this result.

It is interesting that the strongest correlation between the two methods for measuring N_2 fixation in the field occurred at a time when fixation rates were lowest and the C_2H_2:N_2 reduction ratio was closest to the theoretical value (Fig 2.1, Table 2.2). To our knowledge similar results have not been reported. This decoupling of the two assays can be accounted for by either of the two mechanisms proposed to explain the rate-dependence of the C_2H_2:N_2 ratio, and is probably a condition necessary for the observed rate-dependence. If the two assay methods were not decoupled at high enzyme activities, that is, if all electron flux through the nitrogenase complex were allocated to one substrate or the other at all rates of enzyme activity, it would not be possible for the ratio to be rate-dependent.

Implications for ecosystem N cycling

Because of the simplicity and cost-effectiveness of the method, ARA has been widely used for estimating N_2-fixation rates across a broad spectrum of terrestrial systems, from boreal (Klingensmith & Van Cleve, 1993; Uliassi & Ruess, 2002),

temperate (Tripp, Bezdicek & Heilman, 1979; Huss-Danell, Lundquist & Ohlsson, 1991; Hurd, Raynal & Schwintzer, 2001), and tropical forests (Vitousek & Walker, 1989; Pearson & Vitousek, 2001), to arctic and alpine tundra (Holzmann & Haselwandter, 1988; Kay & Virginia, 1989; Liengen & Olsen, 1997). The primary uncertainties in scaling ARA-based data to the stand level include 1) adequate characterization of the seasonal variation in nitrogenase activity, 2) accurate values for the C_2H_2:N_2 reduction ratio, and 3) reliable estimates of live nodule biomass.

Our finding that there is likely variation in the C_2H_2:N_2 reduction ratio at both temporal and spatial scales within and among conspecific hosts suggests that assessments of ecosystem N cycling based on ARA may require reexamination. For example, Pearson & Vitousek (2001) found that rates of N accumulation in vegetation biomass exceeded ARA-based N_2-fixation inputs in Hawaiian Acacia koa plantations, and concluded that the largest proportion of plant N uptake was derived from soil organic matter turnover. A much different conclusion was presented by Uliassi & Ruess (2002), who found that ARA-based N_2-fixation inputs to interior Alaskan floodplain forests by A. tenuifolia significantly exceeded rates of biomass N accumulation, and suggested that these forests were not retaining a large proportion of fixed N.

Even though the reduction ratio for A. tenuifolia derived from the current study (1.81 ± 0.24) is significantly less than the 3.0 used by Uliassi & Ruess (2002), we hesitate to conclude that these previous estimates of N_2 fixation and ecosystem N retention were conservative. The apparent rate-dependence of the C_2H_2:N_2 reduction ratio suggests that it may be impossible to rely on any one C_2H_2:N_2 reduction ratio value for A. tenuifolia,

and perhaps any species. Moreover, the apparent uncoupling of SNF and SARA observed at high rates of SNF in the present study suggests the ARA may not be well-suited for even relative comparisons of N_2-fixation rates between organisms. Further studies duplicating the scale of the present one with other N_2-fixing species and/or in other ecosystems will be necessary to determine whether the pattern we observed is typical. Despite the greater cost of the $^{15}N_2$ uptake method compared to the ARA, our results call for a reassessment of N_2 fixation inputs using $^{15}N_2$ uptake for all ecosystems where N_2-fixing organisms contribute a significant amount to net primary production.

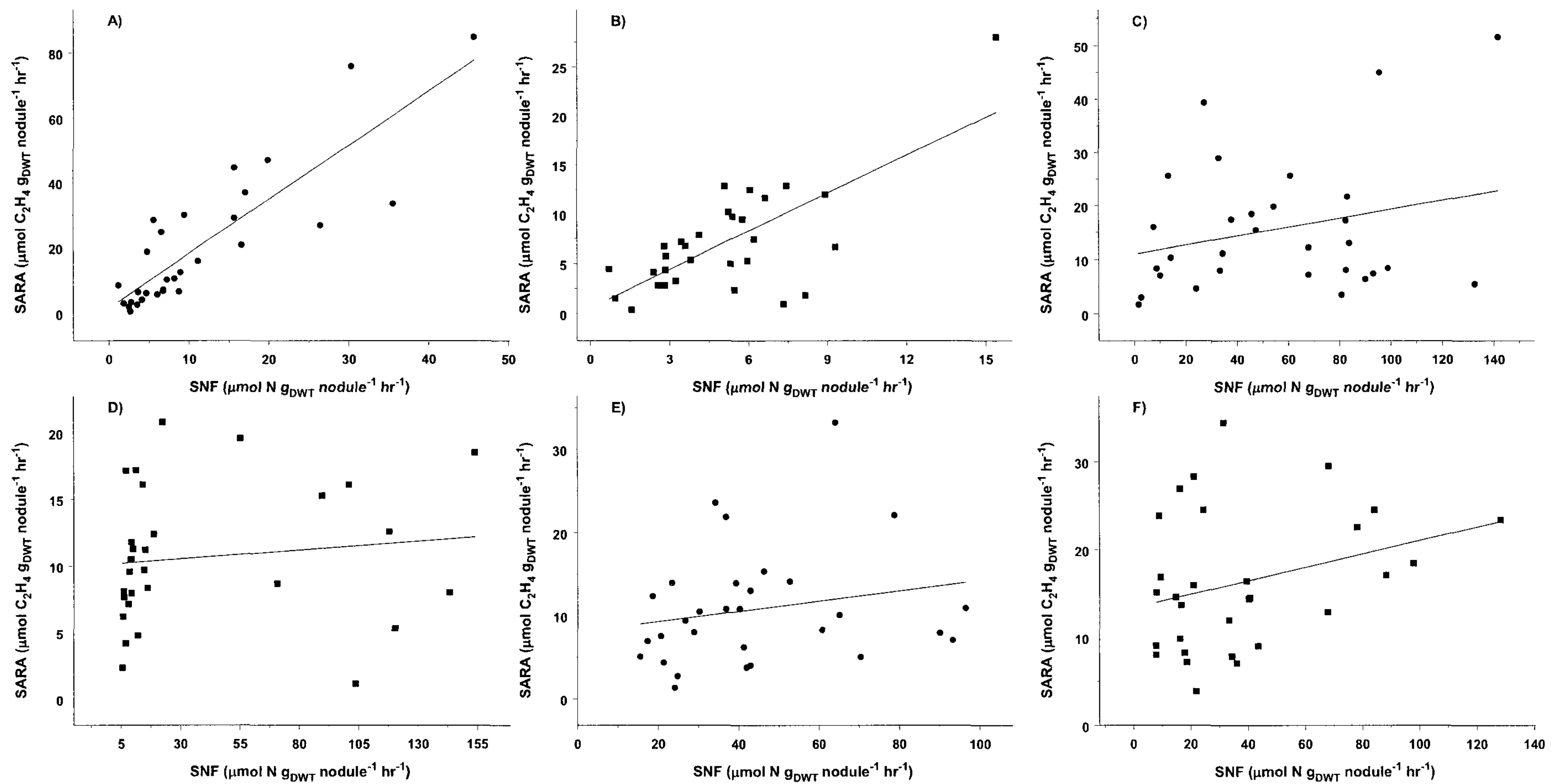

Figure 2.1. Linear regressions between SARA and SNF: for A. tenuifolia (A, C, E) and A. crispa (B, D, F) measured in Type I (A, B), Type II (C,D) and Type III (E, F) site types. Each point represents an individual plant. Relationships are significant for Type I site types only (A. tenuifolia, $r^2 = 0.73$, $P < 0.0001$; A. crispa, $r^2 = 0.49$, $P < 0.0001$).

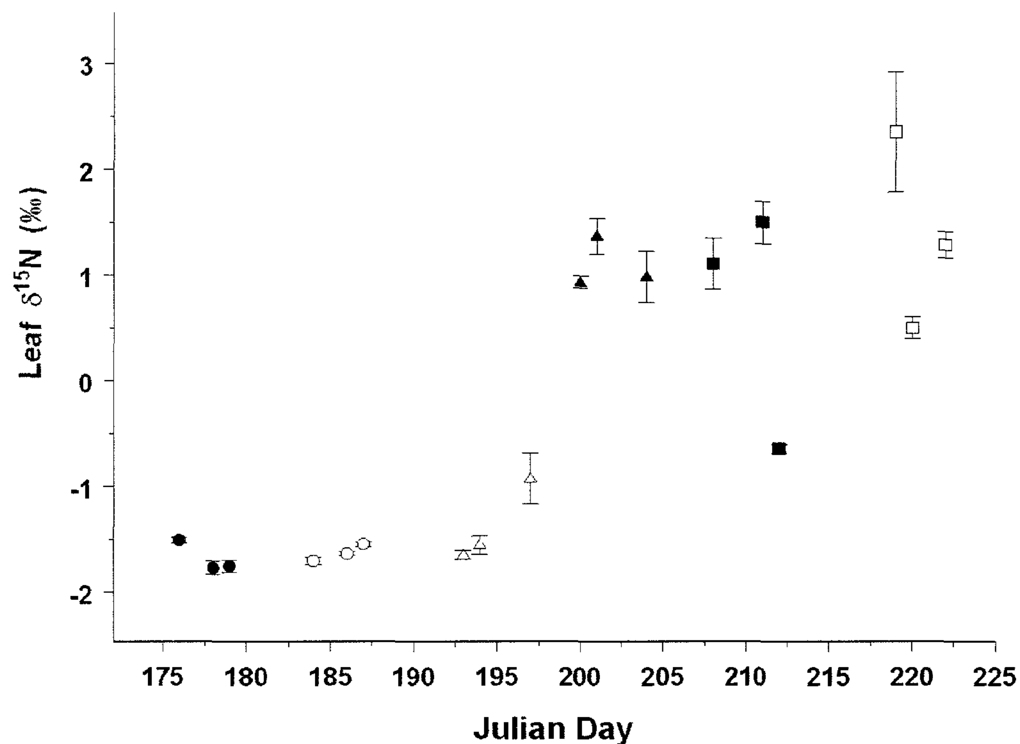

Figure 2.2. Leaf $\delta^{15}N$ values. Means for 3 replicate stands of A. tenuifolia (closed symbols) and A. crispa (open symbols) measured in Type I (circles), Type II (triangles) and Type III (squares) site types. Each point represents the mean (± 1 standard error) of 10 plants. Significant differences between species exist only for Type II sites ($P < 0.0001$).

Table 2.1. Sampling schedule and definition of site types.

Site Type	Successional Stage	Plant Species	Replicate Site	Sampling Date
I	Early	A. tenuifolia	1	25 June
			2	27 June
			3	28 June
		A. crispa	1	3 July
			2	5 July
			3	6 July
II	Mid	A. crispa	1	12 July
			2	13 July
			3	16 July
		A. tenuifolia	1	19 July
			2	20 July
			3	23 July
III	Late	A. tenuifolia	1	27 July
			2	30 July
			3	31 July
		A. crispa	1	7 August
			2	8 August
			3	10 August

Table 2.2. Sampling dates and mean measured values by site type. Site type averages (mean ± 1 SE, n=30) for data collected during measurements of nitrogen fixation, listing soil temperature (°C), specific leaf area (SLA = $cm^2\ g^{-1}$), leaf natural ^{15}N abundance ($\delta^{15}N$, ‰), specific N assimilation rate determined from $^{15}N_2$ uptake (SNF = μmol N g_{DWT} nodule^{-1} hr^{-1}), specific acetylene reduction activity (SARA = μmol C_2H_4 g_{DWT} nodule^{-1} hr^{-1}), and the molar reduction ratio of C_2H_2 to N_2.

Species	Site Type	Sampling Dates	Soil Temperature	SLA	Leaf $\delta^{15}N$	SNF	SARA	Ratio
A. tenuifolia	I	25-28 June	11.7 ± 0.6	151.1 ± 8.4	-1.68 ± 0.05	11.3 ± 2.0	20.4 ± 3.8	3.8 ± 0.5
	II	19-23 July	13.4 ± 0.5	237.0 ± 7.3	1.09 ± 0.20	55.2 ± 7.0	15.6 ± 2.3	1.1 ± 0.2
	III	27-31 July	10.5 ± 0.4	171.2 ± 5.7	0.65 ± 0.25	44.2 ± 4.3	10.8 ± 1.3	0.6 ± 0.1
A. crispa	I	3-6 July	9.3 ± 0.3	123.0 ± 4.4	-1.63 ± 0.03	5.1 ± 0.6	7.0 ± 1.0	3.1 ± 0.4
	II	12-16 July	12.8 ± 0.4	195.0 ± 7.6	-1.38 ± 0.16	39.4 ± 8.7	10.7 ± 0.9	1.5 ± 0.2
	III	7-10 August	11.5 ± 0.2	173.8 ± 3.8	1.38 ± 0.03	33.8 ± 5.7	16.3 ± 1.4	1.4 ± 0.2

Table 2.3. SARA and SNF post-hoc results. Pair-wise least-square means comparisons of SARA, SNF, and ratio values between all species and site type combinations. Values given are P-values of pair-wise t-tests (n = 30). NS indicates no significant difference between the mean values compared.

Species			A. tenuifolia			A. crispa		
	Site Type		I	II	III	I	II	III
A. tenuifolia	I	SARA	--	NS	0.0013	<0.0001	0.0012	NS
		SNF	--	<0.0001	<0.0001	NS	0.0002	0.0004
		Ratio	--	<0.0001	<0.0001	NS	<0.0001	<0.0001
	II	SARA		--	NS	0.0040	NS	NS
		SNF		--	NS	<0.0001	0.0304	0.0197
		Ratio		--	NS	<0.0001	NS	NS
	III	SARA			--	NS	NS	0.0580
		SNF			--	<0.0001	NS	NS
		Ratio			--	<0.0001	0.0446	0.0561
A. crispa	I	SARA				--	NS	0.0018
		SNF				--	<0.0001	<0.0001
		Ratio				--	0.0005	0.0003
	II	SARA					--	0.0530
		SNF					--	NS
		Ratio					--	NS
	III	SARA						--
		SNF						--
		Ratio						--

LITERATURE CITED

Barber, V.A., G.P. Juday & B.P. Finney. 2000. Reduced growth of Alaskan white spruce in the twentieth century from temperature-induced drought stress. Nature, 405: 668-673.

Burgess, B. K. & D. J. Lowe, 1996. Mechanism of molybdenum nitrogenase. Chemical Reviews, 96: 2983-3011.

Christiansen, J., L. C. Seefeldt & D. R. Dean, 2000. Competitive substrate and inhibitor interactions at the physiologically relevant active site of nitrogenase. The Journal of Biological Chemistry, 275: 36104-36107.

Davis, L. C., V. K. Shah & W. J. Brill, 1975. Nitrogenase VII: effect of component ratio, ATP and H_2 on the distribution of electrons to alternative substrates. Biochimica et Biophysica Acta, 403: 67-78.

Eady, R. R. & J. R. Postgate, 1974. Nitrogenase. Nature, 249: 85-810.

Fessenden, R. J., R. Knowles & R. Brouzes, 1973. Acetylene-ethylene assay studies on excised root nodules of Myrica asplenifolia L. Soil Science Society of America Proceedings, 37: 893-898.

Gibson, P. R. & A. M. Alston, 1984. The acetylene to nitrogen ratio in Lupinus angustifolius L. Page 33 in C. Veeger and W. E. Newton (eds.). Advances in Nitrogen Fixation Research. Martinus Nijhoff and Dr. W. Junk, The Hague.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson & R. C. Burns, 1968. The acetylene ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiology, 43: 1185-1207.

Holzmann, H. P. & K. Haselwandter, 1988. Contribution of nitrogen fixation to nitrogen nutrition in an alpine sedge community (Caricetum curvulae). Oecologia, 76: 298-302.

Hu, F., B. P. Finney & L. B. Brubaker, 2001. Effects of Holocene Alnus expansion on aquatic productivity, nitrogen cycling, and soil development in southwestern Alaska. Ecosystems, 4: 358-368.

Hultén, E. 1968. Flora of Alaska and neighboring territories. Stanford University Press, Stanford, California, USA.

Hurd, T. M., D. J. Raynal & C. R. Schwintzer, 2001. Symbiotic N_2 fixation of Alnus incana ssp. rugosa in shrub wetlands of the Adirondack Mountains, New York, USA. Oecologia, 126: 94-103.

Huss-Danell, K., P. Lundquist & H. Ohlsson, 1991. N_2 fixation in a young Alnus incana stand, based on seasonal and diurnal variation in whole plant nitrogenase activity. Canadian Journal of Botany, 70: 1537-1544.

Kay, I. S. & R. A. Virginia, 1989. Asymbiotic dinitrogen fixation by tundra in the Imnavait Creek drainage, Alaska, USA. Biology and Fertility of Soils, 7: 207-212.

Klingensmith, K. M. & K. Van Cleve, 1993. Denitrification and nitrogen fixation in floodplain successional soils along the Tanana River, interior Alaska. Canadian Journal of Forest Research, 23: 956-963.

Liengen, T., 1999. Conversion factor between acetylene reduction and nitrogen fixation in free-living cyanobacteria from high arctic habitats. Canadian Journal of Microbiology, 45: 223-229.

Liengen, T. & R. A. Olsen, 1997. Nitrogen fixation by free-living cyanobacteria from different coastal sites in a high arctic tundra, Spitsbergen. Arctic and Alpine Research, 29: 470-477.

Lowe, D. J., K. Fisher & R. N. F. Thorneley, 1990. Klebsiella pneumoniae nitrogenase: mechanism of acetylene reduction and its inhibition by carbon monoxide. Biochemistry Journal, 272: 621-625.

Mague, T. H. & R. H. Burris, 1972. Reduction of acetylene and nitrogen by field grown soybeans. New Phytologist, 71: 275-286.

Miller, R. W., 1991. Molybdenum nitrogenase. Pages 9-36 in M. J. Dilworth and A. R. Glenn (eds.). Biology and biochemistry of nitrogen fixation. Elsevier, Amsterdam.

Minchin, F. R., J. F. Witty, J. E. Sheehy & M. Miller, 1983. A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. Journal of Experimental Botany, 34: 641-649.

Montoya, J. P., M. Voss, P. Kahler & D. C. Capone, 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Applied and Environmental Microbiology, 62: 986-993.

Neter, J., M.H. Kutner, W. Wasserman & C.J. Nachtsheim (eds.), 1996. Applied linear regression models, Third Edition. McGraw-Hill, New York.

Nohrstedt, H. O., 1983. Conversion factor between acetylene reduction and nitrogen fixation in soil: effect of water content and nitrogenase activity. Soil Biology and Biochemistry, 15: 275-279.

Nohrstedt, H. O., 1985. Nonsymbiotic nitrogen fixation in the topsoil of some forest stands in central Sweden. Canadian Journal of Forest Research, 15: 715-722.

Paerl, H. W., 1982. In situ H_2 production and utilization by natural populations of N_2 fixing blue-green algae. Canadian Journal of Botany, 60: 2542-2546.

Paul, E. A., 1988. Towards the year 2000: directions for future nitrogen research. Pages 417-425 in J. R. Wilson (ed.). Advances in nitrogen cycling in agricultural ecosystems. C A B international, Wallingford, UK.

Pearson, H. L. & P. M. Vitousek, 2001. Stand dynamics, nitrogen accumulation, and symbiotic nitrogen fixation in regenerating stands of Acacia koa. Ecological Applications, 11: 1381-1394.

Peters, G. A., R. E. Toia, Jr. & S. M. Lough, 1977. Azolla-Anabaena azollae relationship. Plant Physiology, 59: 1021-1025.

Rennie, R. J., D. A. Rennie & M. Fried, 1978. Concepts of ^{15}N usage in dinitrogen fixation studies. Pages 107-133 in Isotopes in biological dinitrogen fixation. Proceedings of an Advisory Group Meeting Organized by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, Vienna, 1977. International Agency of Atomic Energy, Vienna.

Rice, W. A. & E. A. Paul, 1971. The acetylene reduction assay for measuring nitrogen fixation in waterlogged soil. Canadian Journal of Microbiology, 17: 1049-1056.

Rivera-Ortiz, J. M. & R. H. Burris, 1975. Interactions among substrates and inhibitors of nitrogenase. Journal of Bacteriology, 123: 537-545.

Sadkov, A. P. & G. I. Likhtenshtein, 1990. Ratio of the nitrogen-fixing and acetylene reducing activities in a nitrogenase complex. Biochemistry (USSR), 55: 446-449.

Saito, S. M. T., E. Matsui & E. Salati, 1980. $^{15}N_2$ fixation, H_2 evolution and C_2H_2 reduction relationships in Phaseolus vulgaris. Physiologia Plantarum, 49: 37-42.

SAS Institute, 1999. The SAS System for Windows. Version 8. SAS Institute, Inc., Cary, NC, US.

Schimel, J. P., R. G. Cates & R. Ruess, 1998. The role of balsam poplar secondary chemicals in controlling soil nutrient dynamics through succession in the Alaskan taiga. Biogeochemistry, 42: 221-234.

Schubert, K. R. & H. J. Evans, 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proceedings of the National Academy of the Sciences of the United States, 73: 1207-1211.

Schwintzer, C. R. & J. D. Tjepkema, 1994. Factors affecting the acetylene to $^{15}N_2$ conversion ratio in root nodules of Myrica gale L. Plant Physiology, 106: 1041-1047.

Seitzinger, S. P. & J. H. Garber, 1987. Nitrogen fixation and $^{15}N_2$ calibration of the acetylene reduction assay in coastal marine sediments. Marine Ecology Progress Series, 37: 65-73.

Sellstedt, A., 1986. Acetylene reduction, H_2 evolution and $^{15}N_2$ fixation in the Alnus incana-Frankia symbiosis. Planta, 167: 382-386.

Shah, V. K., L. C. Davis & W. J. Brill, 1975. Nitrogenase VI: acetylene reduction assay: dependence of nitrogen fixation estimates on component ratio and acetylene concentration. Biochimica et Biophysica Acta, 384: 353-359.

Simpson, F. B. & R. H. Burris, 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science, 224: 1094-1097.

Tate, R. L.,1995. Soil Microbiology. Wiley & Sons, New York.

Tripp, L. N., D. F. Bezdicek & P. E. Heilman, 1979. Seasonal and diurnal patterns and rates of nitrogen fixation by young red alder. Forest Science, 25: 371-380.

Uliassi, D. D. & R. W. Ruess, 2002. Limitations to symbiotic nitrogen fixation in primary succession on the Tanana river floodplain. Ecology, 83: 88-103.

Uliassi, D. D., K. Huss-Danell, R. W. Ruess & K. Doran, 2000. Biomass allocation and nitrogenase activity in Alnus tenuifolia: responses to successional soil type and phosphorus availability. Ecoscience 7: 73-79.

Van Cleve, K., C. T. Dyrness, L. A. Viereck, J. Fox, F. S. Chapin III & W. Oechel, 1983. Taiga ecosystems in interior Alaska. Bioscience, 33: 39-44.

Van Cleve, K., F. S. Chapin III, C. T. Dyrness & L. A. Viereck, 1991. Element cycling in taiga forests: state-factor control. Bioscience, 41: 78-88.

Van Cleve, K., C. T. Dyrness, G. M. Marion & R. Erickson, 1993. Control of soil development on the Tanana River floodplain, interior Alaska. Canadian Journal of Forest Research, 23: 941-955.

van Kessel, C. & R. H. Burris, 1983. Effect of H_2 evolution on $^{15}N_2$ fixation, C_2H_2 reduction and relative efficiency of leguminous symbionts. Physiologia Plantarum, 59: 329-334.

Viereck, L. A., C. T. Dyrness & M. J. Foote, 1993. An overview of the vegetation and soils of the floodplain ecosystems of the Tanana River, interior Alaska. Canadian Journal of Forest Research, 23: 889-898.

Viereck, L.A. & E.L. Little, 1986. Alaska trees and shrubs. University of Alaska Press, Fairbanks, Alaska.

Viereck, L. A., K. Van Cleve, P. C. Adams, and R. E. Schlentner. 1993. Climate of the Tanana River floodplain near Fairbanks, Alaska. Canadian Journal of Forest Research. 23: 899-913.

Vitousek, P. M. & L. R. Walker, 1989. Biological invasion by Myrica faya in Hawaii: plant demography, nitrogen fixation, ecosystem effects. Ecological Monographs, 59: 265-274.

Vitousek, P. M. & C. B. Field, 1999. Ecosystem constraints to symbiotic nitrogen fixers: a simple model and its implications. Biogeochemistry, 46: 179-202.

Zechmeister-Boltenstern, S. & H. Kinzel, 1990. Non-symbiotic nitrogen fixation associated with temperate soils in relation to soil properties and vegetation. Soil Biology and Biochemistry, 22: 1075-1084.

Zuckermann. H., M. Stahl, L. J. Stal, J. Reuss, S. TE Lintel Hekkert, F. Harren & D. Parker, 1997. On-line monitoring of nitrogenase activity in cyanobacteria by sensitive laser photoacoustic detection of ethylene. Applied and Environmental Microbiology, 63: 4243-4251.

CHAPTER 3:

Host Species and Habitat Affect Nodulation by Specific *Frankia* Genotypes in Two Species of *Alnus* in Interior Alaska[1]

ABSTRACT

Alders (*Alnus spp.*) are important components of northern ecosystems due to their ability to fix nitrogen (N) in symbiosis with *Frankia* bacteria. Availability of optimal *Frankia* may be a contributing factor in limiting the performance and ecological effects of *Alnus*, but the factors underlying distribution of *Alnus*-infective *Frankia* are not well understood. This study examined the genetic structure (*nif*D-K spacer RFLP haplotypes) of *Frankia* assemblages symbiotic with two species of *Alnus* (*A. tenuifolia* and *A. viridis*) in four successional habitats in interior Alaska. We used one habitat in which both hosts occurred to observe differences between host species independent of habitat, and we used replicate sites for each habitat and host to assess the consistency of symbiont structure related to both factors. We also measured leaf N content and specific N-fixation rate (SNF) of nodules (^{15}N uptake) to determine whether either covaried with *Frankia* structure, and whether *Frankia* genotypes differed in SNF *in situ*. *Frankia* structure differed between sympatric hosts and among habitats, particularly for *A. tenuifolia*, and was largely consistent among replicate sites representing both factors. Leaf N differed between host species and among habitats for both hosts. SNF did not differ among

[1] Anderson MD, Ruess RW, Myrold DD, Taylor DL (2009) Host species and habitat affect nodulation by specific *Frankia* genotypes in two species of *Alnus* in interior Alaska. Oecologia 160:619-630.

habitats or host species, and little evidence for differences in SNF among *Frankia* genotypes was found, due largely to high variation in SNF. Consistency of *Frankia* structure among replicate sites suggests a consistent relationship between both host species and habitat among these sites. Correlations with specific environmental variables and possible underlying mechanisms are discussed.

INTRODUCTION

In terrestrial ecosystems of the Northern hemisphere, alders (*Alnus spp.,* Betulaceae) are important early colonizing plants in both primary and secondary seral systems (*e.g.*, Chapin *et al.*, 1994; Van Cleve and Viereck, 1981), due largely to their ability to form root nodule-based, nitrogen (N) fixing symbioses with actinomycete bacteria of the genus *Frankia*. This symbiosis allows alder to maintain high N content and growth rates in low nutrient and/or disturbed habitats, which can result in both strong competitive effects of alder growth and facilitative effects of alder-derived N on associated plant species throughout succession (*e.g.*, Vogel and Gower, 1998; Wurtz, 1995; Chapin *et al.*, 1994; Walker and Chapin, 1986; Van Cleve and Viereck, 1981). Controlled inoculation studies between *Alnus* species and *Frankia* strains indicate that specificity of associations is variable for both organisms, and that infection by different *Frankia* inocula can have large effects on host growth, N content and N-fixation (Martin *et al.*, 2003; Prat, 1989; Hooker and Wheeler, 1987; Sellstedt *et al.*, 1986; Dillon and Baker, 1982; Dawson and Sun, 1981). Moreover, isolates from a given host species are

not necessarily the highest-performing on that host in the lab (Sellstedt *et al.*, 1986; Dillon and Baker, 1982; Dawson and Sun, 1981), and the relative performance of specific *Alnus* spp.-*Frankia* combinations can vary widely based on soil conditions (Kurdali *et al.*, 1990; Sheppard *et al.*, 1988). Thus, availability of optimal symbiont genotypes due to host specificity and/or spatial heterogeneity of *Frankia* may contribute to factors limiting the establishment, performance and ecological effects of alder in natural habitats. However, current knowledge of the distribution of alder-nodulating *Frankia* among habitats and host species, and associated patterns in host physiology, is limited.

Genetic variation in symbiotic *Frankia* is subject to a wide range of selective effects exerted by both environmental factors and host plants. *Frankia* appear to disperse more readily than their host plants and to maintain a free-living existence in soil (*reviewed in* Benson and Dawson, 2007; Benson and Silvester, 1993). Plant-trapping studies indicate that population size of soil-dwelling *Frankia* can covary with a number of environmental factors related to soil conditions (*e.g.*, Batzli *et al.*, 2004; Huguet *et al.*, 2004a,b; Myrold and Huss-Danell, 1994; Zitzer and Dawson, 1992; Dawson *et al.*, 1989) and non-host vegetative cover (*e.g.*, Maunuksela *et al.*, 1999; Paschke *et al.*, 1994; Zitzer and Dawson, 1992; Smolander and Sundman, 1987), and some of these factors appear to differentially affect *Frankia* genotypes (Huguet *et al.*, 2004a,b; Zitzer and Dawson, 1992). Given the evolutionary trade-off expected to occur between genetically-determined symbiotic and free-living bacterial lifestyles (Denison and Kiers, 2004; West *et al.*, 2002), such environmental selection is unlikely to favor optimal symbionts. However, host plants may also exert considerable influence over symbiont assemblages.

Evolutionary models of root-nodule symbioses predict the existence of physiological mechanisms in host plants for selective allocation of resources to optimal symbionts and/or withholding of resources from poor mutualists (West *et al.*, 2002), and both processes have been observed in legume hosts under greenhouse conditions (Simms *et al.*, 2006; Kiers *et al.*, 2003). Since bacteria inhabiting nodules can outnumber their soil-dwelling conspecifics in the same area by several orders of magnitude (West *et al.*, 2002), such host selection has strong potential to feed back to soil-dwelling populations. Some evidence for such positive feedback has been reported for *Frankia* hosts; positive correlations between nodulation potential of soils on host seedlings in the greenhouse and presence of the same host in the field have been observed in *Alnus* (Myrold and Huss-Danell, 1994), *Casuarina* (Zimpfer *et al.*, 1999) and *Ceanothus* (Jeong and Myrold, 2001), and in the latter two genera the positive effect of host presence differed among *Frankia* genotypes.

Field surveys of *Frankia* variation in alder nodules point to the importance of both environmental factors and host species in the distribution of particular *Frankia* types (*e.g.*, Dai *et al.*, 2005; Weber, 1990; van Dijk *et al.*, 1988). The patterns revealed by the studies to date, however, are ambiguous in a number of respects. First, the majority of such studies have not been concerned with genetic variation of symbionts *per se*, but with geographic patterns in the presence of *Frankia* spores in host nodules. However, in addition to the low resolution of this distinction, which sorts symbionts only into 'spore-positive' (Sp+) and 'spore-negative' (Sp-) categories, the genetic basis of this dichotomy is considered unresolved (Benson and Silvester, 1993), so it is unclear whether the

observed patterns represent distribution of symbiont genotypes or phenotypic plasticity of symbionts or hosts. Second, in the handful of studies that include more than one *Alnus* species, the contributions of host and habitat to the observed patterns are confounded due to non-overlapping distributions of host species at regional scales (Dai *et al.*, 2005) or among different microhabitats within a region (*e.g.*, Weber, 1990; Weber *et al.*, 1987; Weber, 1986). Third, the consistency of *Frankia* structure on single host species in specific habitats is unclear, since most studies aim to describe variation across relatively large areas, and do not include *pre hoc* replicate sites for each habitat examined (*e.g.*, Igual *et al.*, 2006; Dai *et al.*, 2004; Huguet *et al.*, 2004b; Markham and Chanway, 1998). Exceptions are provided by Holman and Schwintzer (1987), who report significant differences between replicated 'streamside' and 'disturbed' habitats in the frequency of Sp+ *Frankia* in *A. incana* ssp. *rugosa* nodules in the state of Maine, and Khan *et al.* (2007), who found that *Frankia* structure in Himalayan *A. nepalensis* nodules was correlated with elevation in replicate sampling locales. Fourth, it does not appear that any survey of alder symbiont variation has examined correlations between observed *Frankia* variation and host physiology in the field.

The Tanana River floodplain in the boreal forest of interior Alaska provides a 'natural laboratory' in which some of these ambiguities can be addressed. Low regional plant diversity and repetition of ecosystem-controlling factors such as soil parent material, topography and cycles of flood and fire across the landscape (Van Cleve *et al.*, 1996) create a mosaic of intermixed patches of successional habitats, making for relatively straightforward habitat replication. The sympatric occurrence of two alder

species – *Alnus viridis* ssp. *fruticosa* (Ruprecht) Regel (formerly *Alnus crispa*; hereafter, *Alnus viridis*) and *Alnus incana* ssp. *tenuifolia* (Nuttall) Breitung (hereafter, *Alnus tenuifolia*) – in some habitats also allows for examination of host-based differences without the confounding effects of habitat or location. Additionally, the presence of sites belonging to the Long Term Ecological Research (LTER) program allows access to environmental data continuously collected for each of the sites that are included in the program. The present study sought to characterize genetic variation in symbiotic *Frankia* and host physiology among habitats and host species within a restricted area in the Bonanza Creek LTER. Specifically, we sought to determine whether: 1) genetic structure of *Frankia* is consistently associated with habitat for either host species, 2) genetic structure of *Frankia* is consistently associated with host species between sympatric hosts in one of these habitats, 3) variation among *Frankia* assemblages parallels variation in host physiology among habitats and host species and, 4) different *Frankia* genotypes differ in specific N_2 fixation rate (SNF) *in situ* in either host species. For objective (3) we chose to measure SNF and leaf N content because both are ecologically important, readily measured in the field, and known to vary among host species and *Frankia* genotypes.

MATERIALS AND METHODS

Study sites

The Bonanza Creek Long Term Ecological Research area (BNZ LTER) is located approximately 30 km south-west of Fairbanks, Alaska (64° 48' N, 147° 52' W). The

dominant feature of the region is the active floodplain of the Tanana River, but upland forests occur adjacent to the north bank. Soil development and successional dynamics differ between floodplain (FP) and upland (UP) forests. On the floodplain, primary succession begins with the formation of alluvial silt bars, which are rapidly colonized by *Salix* spp., balsam poplar (*Populus balsamifera* L.), and *A. tenuifolia*. *A. tenuifolia* grows rapidly and forms a dense closed canopy approximately 5-10 years after initial colonization which persists for 25-30 years. Nitrogen fixed during this alder-dominated stage may account for 60-70% of the N accumulated during 200 years of progressive succession (Van Cleve *et al.*, 1993, 1971). Approximately 50 years after bar formation, balsam poplar overtops the alder canopy. Alder abundance declines as the poplar canopy matures, and as poplar is eventually replaced by white spruce (*Picea glauca* (Moench) Voss) ~125 years after substrate formation. *A. tenuifolia* persists in the understory of these spruce forests, and in some floodplain spruce stands *A. viridis* also occurs.

In secondary successional ecosystems on south-facing slopes adjacent to the Tanana floodplain *A. viridis* appears shortly after fire, along with Alaska paper birch (*Betula neoalaskana* (Sarg.)), trembling aspen (*Populus tremuloides* Michx) and a few species of *Salix*. Approximately 25-50 years post-fire, an overstory of paper birch and/or aspen develops, which yields to white spruce dominance 100-200 years post-fire. *A. viridis* persists throughout this sequence, and continues to provide significant amounts of fixed N (Mitchell, 2006; Van Cleve and Viereck, 1981).

Experimental design and sample collection

In June 2002, 12 study sites were selected; three replicate sites representing each of four habitat types: 1) early succession floodplain with dense (~1200 stems/ha) *A. tenuifolia* canopy (FPE sites 1, 2 and 3), 2) late succession floodplain with white spruce canopy and moderately dense understory (~400 stems/ha) of *A. tenuifolia* and *A. viridis* (FPL sites 1, 2 and 3), 3) early succession upland with scattered (~200 stems/ha) *A. viridis* individuals (UPE sites 1, 2 and 3), and 4) late succession upland with white spruce canopy and moderately dense (~400 stems/ha) *A. viridis* understory (UPL sites 1, 2 and 3). Where possible, these sites were selected to avoid geographic clustering with respect to habitat (Table 3.1). This was not possible for UPE sites, which were all located in the same burn area (Table 3.1). In the sympatric sites (FPL1-3) individuals of the two species were intermixed and generally within 5m of each other. All but four of these sites – the three UPE sites and FPL3 – are established long-term monitoring sites defined and maintained by the BNZ LTER project (http://www.lter.uaf.edu/).

Each time a site was sampled ten mature plants (*A. tenuifolia* in FPE, and *A. viridis* in UPE and UPL sites) were haphazardly chosen for measurement of SNF, and collection of nodule and leaf tissue. At the sympatric (FPL) sites ten plants of each species were sampled. In general, one site was sampled per field day (between 0900 and 1400 Alaska Daylight Time). In order to account for the known effect of seasonality on SNF (Anderson *et al.*, 2004; Uliassi and Ruess, 2002) in our statistical model, we used a Latin Square sampling design (Neter *et al.*, 1996) in which each site was sampled at three separate time periods over the growing season – period 1 extended from 19 June to 2

July, period 2 from 22 July to 10 August and period 3 from 19 August to 1 September – for a total of 30 plants of each species at each site. At each sampling period we selected plants not chosen during the previous sampling period(s) in order to avoid any effect of previous sampling disturbance on SNF. At each site the combined area sampled for all three periods was approximately 2000 m^2.

From each plant, approximately 5-10 g (fresh weight) of nodule tissue was harvested for field measurement of SNF. In the sympatric (FPL) sites, roots were traced to a host stem prior to nodule harvest in order to identify host species. Leaf tissue samples, consisting of five 13 mm diameter leaf punches, were taken from fully-emerged leaves located at the periphery of the canopy of each plant. Leaf punches were dried at 40 °C for at least 48 hours, weighed for measurement of specific leaf weight (SLW = g/cm^2 dry leaf tissue), then ball-milled and analyzed via mass spectrometry for N content on a dual-inlet isotope ratio mass spectrometer (PDZ Europa Scientific Instruments, Crewe, Chesire, UK). Soil temperature and moisture data were also collected for each plant in order to include these variables in the statistical model for SNF. One soil core (5 cm diameter X 10 cm depth) per plant was taken within 1 m of each sampled nodule for determination of soil water content. Cores were dried to constant weight at 65 °C, and moisture content determined as the difference between fresh and dry weight, expressed as a percentage of fresh weight. Soil temperatures at 1 cm and 5 cm depths were recorded at each plant using a hand-held digital thermometer (Taylor Thermometers, Oak Brook, Illinois, USA).

From each nodule sample (1 per plant) two subsamples consisting of 2-3 nodule lobes were collected in the lab. Mass spectrometry was performed on one subsample for measurement of SNF, and the other subsample was used for PCR-RFLP analysis. When possible, both subsamples were taken from the same nodule cluster to minimize the chance that they contained different strains of *Frankia*. For 51 of the 177 plants included in the final SNF-RFLP analysis, small nodule cluster size prevented the use of this strategy. For these a subsample was chosen from a short section of root, thoroughly mixed, and split in half. SNF was then examined on one half, and RFLP analysis was performed on the other. All samples which appeared to contain more than one RF pattern were excluded from the final analysis, providing an additional barrier against the possibility that SNF and RFLP analyses included different *Frankia* strains.

Difficulty locating plants at some field sites, and unsuccessful sample processing for some samples, particularly during PCR-RFLP analysis, resulted in unequal amounts of data among the assays performed and the sites sampled (Table 3.2).

SNF measurement

To avoid uncertainties related to the commonly-used acetylene reduction assay (Anderson *et al.*, 2004), we measured SNF using a $^{15}N_2$ uptake assay we developed previously. This method is described in detail in Anderson *et al.* (2004). Briefly, half of each field nodule sample was exposed to an atmosphere of ~15% (atom %) $^{15}N_2$ for 10 minutes immediately after harvest. $^{15}N_2$ fixation rate was determined via mass spectrometry by comparing ^{15}N enrichment in assayed nodules to natural abundance of

^{15}N in non-assayed nodules, and dividing by elapsed time for the assay. $^{15}N_2$ fixation rate was converted to N_2 fixation rate by adjusting for the initial ^{15}N concentration (15%) in the assay atmosphere, which was determined via mass spectrometry.

PCR-RFLP analysis

Prior to DNA extraction, nodule subsamples were surface sterilized in 10% H_2O_2 for 1 min, rinsed continuously for 5 min with RO water, and stored at -80 °C. Nodules were ground on a shaker mill (Retsch, Inc., Newtown, PA) in 200 μL of AP1 extraction buffer from the Plant DNeasy 96 kit (Qiagen, Carlsbad, CA) in the plates provided. The remainder of the extraction was performed according to the manufacturer's instructions, with the addition of 20 mg/mL lysozyme followed by a 30 min incubation at 37° C, as recommended for Gram-positive bacteria.

The *nif*D-K intergenic spacer (IGS) locus was selectively amplified via polymerase chain reaction (PCR) (Mullis *et al.*, 1986) using newly designed primers: forward, *nif*D1310fr-CAGATGCACTCCTGGGACTACTC; reverse, *nif*KR331fr – CGGGCGAAGTGGCTGC. Maximum PCR success rate was achieved for each species using different reaction compositions: *A. tenuifolia* samples: 25 μL final volume (18 μL ultrapure H_2O, 1 μL each primer (10 μM) and 5 μL undiluted DNA extract added to dehydrated PCR "beads" (Amersham Biosciences, Piscataway, N.J.)); *A. viridis* samples: 25 μL final volume (2.5 μL of AmpliTaq 10X Buffer II (Applied Biosystems, Foster City, CA), 0.25 μL AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 0.4 μM of each primer, and 4.0 μL of 0.4% bovine

serum albumin. The possibility of bias introduced by this PCR difference was examined on 19 samples (eight *A. tenuifolia* and 11 *A. viridis*) that yielded successful PCR reactions with both compositions. All of these samples yielded the same restriction fragment patterns for both PCR reaction compositions. For all samples, initial denaturation was carried out at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s. A final 5 min extension at 72° C was also included. Product yield and length were checked by electrophoresis on 1.5% SeaKem agarose (Cambrex, Rockland, ME) and visualized by ethidium bromide/UV fluorescence.

Restriction fragments were generated via separate digests of PCR product with *Cfo* I and *Hae* III (5 U/rxn) (Promega, Madison, WI). All digests included 10-12 µL of PCR product, and were carried out at 37° C for at least 5 h. Digestion products were electrophoresed at 10 V/cm on 3% (1% SeaKem/2% NuSieve; Cambrex, Rockland, ME) agarose gels and visualized by ethidium bromide/UV fluorescence. Restriction fragment patterns obtained for each enzyme were compared visually within a gel and scored as unique based on the presence or absence of bands of particular length. Fragments shorter than 80 bp were excluded from the analysis, since this is the shortest length measurable with the standard used (MassRuler™ DNA Ladder, Low Range; Fermentas Life Sciences, Burlington, Ontario). Restriction patterns occurring on more than one gel were verified on a second gel, and all RFLP patterns were checked against PCR product length to ensure additivity of fragments. Each unique combination of restriction patterns across

the two enzymes was given a single numerical restriction fragment pattern (RF) designation.

In order to check that our PCR was selective for *Frankia*, and to examine whether sequence variation existed within our RF groups, we performed DNA sequencing on a subset of nodules which included multiple representatives of the most frequent RF groups. Cycle sequencing was performed on cleaned (QiaQuick PCR purification kit, Qiagen, Carlsbad, CA) PCR product using 2.0 µl BigDye and 3.0 µl 10X buffer (Applied Biosystems, Foster City, CA) together with 3.2 µl of 1.0 µM PCR primer and 40-60 ng of DNA per 20 µl reaction, which were subjected to 98 °C for 1 min followed by 25 cycles of 98 °C for 10 s, 58 °C for 5 s, and 60 °C for 4 min. Cycle sequencing products were cleaned using Centri-Sep sephadex columns (Princeton Separations, Inc., Adelphia, NJ), and capillary sequencing was performed on an ABI3100 analyzer (Applied Biosystems, Foster City, CA). Raw sequences were edited using CodonCode Aligner (CodonCode Corp., Dedham, MA), aligned in BioEdit (Hall, 1999) and compared with the NCBI nucleotide database using the BLASTn utility (Altschul *et al.*, 1997).

Data analysis

Because the two host species were unequally distributed among our landscapes and stages, and our desired comparisons were between sampling blocks in which a given host species occurred in a given habitat (floodplain/upland landscapes and early/late succession) we consolidated landscape, stage and host species into a single variable, "HABSPEC" (habitat||species), with five levels: *A. tenuifolia* in early and late succession

on the floodplain (FPE AT and FPL AT, respectively), *A. viridis* in late succession on the floodplain (FPL AV) and in early and late succession in the uplands (UPE AV and UPL AV, respectively). Composition of symbiotic *Frankia* assemblages was examined using correspondence analysis (PROC CORRESP; SAS Institute, 2001) to estimate the relative contributions of habitat and host species, and to visually assess whether *Frankia* composition was more similar among replicate sites within or among HABSPEC levels.

Continuous variables (SNF, leaf N, soil moisture and soil temperature) were analyzed using general linear models (PROC GLM; SAS Institute, 2001) with *post-hoc* comparisons performed using Tukey's HSD on unadjusted or least-square adjusted means, as appropriate. When necessary, raw data were square-root or log transformed to meet the assumptions of the GLM. For soil variables, sampling period and HABSPEC were included as class variables, with replicate site nested within HABSPEC and the interaction between HABSPEC and sampling period also included. For SNF and leaf N models were built according to a backward elimination protocol (Neter *et al*, 1996) which initially included HABSPEC, sampling period, and replicate site nested within HABSPEC as class variables, soil moisture and soil temperature (quadratic) as covariates, and interaction terms for HABSPEC and sampling period, and among all class and continuous variables. At each step of the elimination the independent variable with the smallest F value was dropped from the model, until $P < 0.05$ for all remaining variables. All F and P values used were based on type III sums of squares due to the presence of covariates, and empty cells for SNF. For SNF, sample losses resulted in low sample sizes for sites within each sampling period and unbalanced distribution of RF

groups with respect to sampling period. RF effects on SNF were therefore only examined using data from the peak sampling period of SNF for each site.

We examined environmental variation among the habitats included in this study via principal-components-analysis (PCA) (PROC PRINCOMP; SAS Institute, 2001) using unpublished data publicly available on the BNZ-LTER website (http://www.lter.uaf.edu/). These data included measurements of 13 physical and chemical soil characteristics in sites representing the same (FPE, FPL and UPL), or very similar (UPE), stand types as our sites within the BNZ-LTER. The soil characteristics included in this analysis were total carbon, organic matter content, cation exchange capacity, pH, Kjeldahl N, total P, Mg, K, Ca, bulk density, percent sand, percent silt and percent clay. Information on the specific methods used for collecting these data is available on the BNZ-LTER website. Site scores for the first two principal components were regressed against site scores from the correspondence analysis of RF pattern distribution using simple linear regression in JMP 7.0.

RESULTS

Frankia distribution, host specificity and diversity

RFLP analysis was successful for 216 of the 444 nodule samples collected, and yielded 10 RF patterns (Table 3.2). DNA sequence data was obtained from 18 nodules which included all RF groups except RF2 and RF5, and >1 nodule for RF1, RF4, RF7, RF8 and RF9. In BLASTn searches, the closest match for all sequences was to *Frankia*

alni strain ACN14A (accession #CT573213.2); E values for this match ranged from 10^{-48} for RF7 to 0.0 for RF8 and RF9. All RF groups for which >1 nodule was sequenced yielded identical sequences. All sequences are available on the NCBI website under accession numbers FJ655072-FJ655090.

Seven RF patterns were found in *A. tenuifolia* nodules on the floodplain, three of which were unique to a particular successional stage: RF5 to early succession; RF4 and RF6 to late succession. RF5 and RF6 were rare, each occurring in only one *A. tenuifolia* nodule in a given site, but RF4 occurred in all three FPL sites, at moderate frequency in two sites and co-dominant in the third. RF7 was dominant in *A. tenuifolia* nodules in all FPE sites, whereas in all three FPL sites RF1 was the most frequent pattern, though it occurred at much lower frequency than RF7 in FPE sites. Within a successional stage, the most common RF pattern was the same in all sites, but the most common in FPE sites, RF7, was present in only two FPL sites, while the most common RF group in FPL sites, RF1, was only found in one FPE site. In all FPE sites the most common RF pattern occurred with at least three times the frequency of the second most frequent pattern, but this level of dominance only occurred in one FPL site.

The majority of *A. viridis* nodules yielded either RF8 or RF9, and these patterns were mostly evenly distributed. Over 90% of all nodules collected from this host yielded one of these two RF patterns, and in six of the nine sites in which this host occurred, the two patterns were co-dominant. The exceptions were two FPL sites, in which RF9 was dominant, and one UPE site, in which RF4 was dominant. However, the latter site was one of two sites – both UPE3 and UPL2 – yielding very low sample success, < 10

samples. Thus, while the occurrence of RF4 in this site appears unique among upland sites, the proportion of this genotype may be overrepresented in such a small sample, and the occurrence of RF4 in other upland sites may have been missed due to the generally low sample size in upland sites (Table 3.2).

In the sympatric (FPL) sites, three of the nine RF patterns found in the two host species occurred on both hosts (RF2, RF4, RF6). The remaining six patterns were unique to a particular host species: three to *A. tenuifolia* (RF1, RF3, RF7) and three to *A. viridis* (RF8, RF9, RF10). The shared patterns together accounted for 26% (27 of 103) of the nodules collected in the sympatric sites. RF patterns from these three groups occurred in 22 of 59 (37%) of the *A. tenuifolia* nodules sampled in these sites, but only five of 44 (11%) of the sympatric *A. viridis* nodules. In all sites, the most frequent pattern occurring on each host species was unique to the host.

Correspondence analysis yielded three distinct clusters along the first two dimensions (Figure 3.1), which account for nearly 75% of the total χ^2 in the analysis. Dimension 1 accounts for 44% of the overall χ^2, and is clearly associated with host species; all points associated with *A. tenuifolia* lie to the right of the origin, while all but one *A. viridis* point lie to the left. The exception is site UPE3, which was dominated by RF4, a pattern more common in *A. tenuifolia* nodules. Dimension 2 accounts for another 30% of the overall χ^2, and is primarily associated with floodplain successional stage: all points associated with *A. tenuifolia* in FPE sites fall below the origin and all those in FPL sites fall above, with UP sites in the center. With the exception of UPE3, very little variation is evident among *A. viridis* points, which form a single small cluster.

Continuous variables

Analysis of variance indicated significant effects of sampling period and replicate site nested within HABSPEC on SNF, leaf N, soil moisture and soil temperature. Additionally there was a small but significant interaction between HABSPEC and sampling period for all dependent variables. A significant main effect of HABSPEC was observed for all variables except SNF (Tables 3.3, 3.4), which was highly variable (Coefficient of Variation = 122.8) and mostly correlated with sampling period (Table 3.4).

Evidence for differences in SNF among RF groups was equivocal, and was hampered by large variation in SNF and small sample sizes for RF groups within peak sampling periods. At peak sampling period only replicate site within HABSPEC was significant in the GLM, both with all data included ($n = 143$, $r^2 = 0.38$, $F = 5.5$, $P < 0.0001$) and with only samples for which ≥ 5 RF data points were available ($n = 53$, $df = 13$ $r^2 = 0.45$, $F = 2.5$, $P = 0.015$). When RF was included in the latter model it was the only other class variable retained ($df = 16$, $F = 3.1$, $P = 0.037$) by backward elimination and explained a further 12% of the variance in SNF ($df = 3$, $r^2 = 0.57$, $F = 2.9$, $P = 0.004$). T-tests on unadjusted means indicated significant differences between RF7 and RFs 4, 8 and 9, and between RF1 and RF8, but when Tukey's HSD was used only RF7 and RF8 were significantly different.

LTER soil data

The clustering pattern yielded by the PCA of the LTER soil data (Figure 3.2) was very similar to the pattern yielded by correspondence analysis of RF data (Figure 3.1), with three clusters separated by the first two principal components (PC). FPE and FPL sites occupy opposite ends of PC1and, with the exception of site FPL3, upland sites form a smaller cluster separate from floodplain sites. PC1 accounts for over half the variance in the PCA, and correlates strongly with exchangeable cations (loading value (LV) = 0.37), N (LV = 0.36), Mg (LV = 0.35), C (LV = 0.34) and organic matter content (LV = 0.35), and negatively with pH (LV = -0.34) and bulk density (LV = -0.35). PC2, which separates floodplain from upland sites, accounts for nearly 22% of the variance in the data and is correlated with sand (LV = 0.51) and P content (LV = 0.35), and negatively with silt (LV = -0.47) and clay content (LV = -0.39). Site scores for PC1 were significantly correlated with dimension 1 (P = 0.021) in the correspondence analysis, which separates *Frankia* assemblages by both host species and landscape (FP vs. UP), and with dimension 2 (P = 0.011) which separates assemblages by floodplain habitat. Both PC1 and dimension 2 yield similar site ordinations, with FPE and FPL sites at opposite ends and UP sites between them. PC2 scores did not correlate with either dimension in the correspondence analysis.

DISCUSSION

Frankia genetic structure

In the present study, we used a surface-sterilization procedure in order to minimize the chance of contamination in our molecular analysis. Such sterilization has been suggested to be ineffective in removing *Frankia* and non-*Frankia* organisms (Valdés *et al.*, 2005) from the nodule surface, and peeling of the nodule periderm is favored by a number of workers (*e.g.*, Rouvier *et al.*, 1996). While we cannot definitively discount the possibility of surface contamination in our study, the low occurrence of multiple RF patterns in single nodules (2 out of 218) suggests that most nodules were occupied by a single genotype, and our BLAST results closely match alder-infective *Frankia*. We therefore think that the possibility of contamination in our study is minimal.

This study confirms the results of previous field studies reporting differences in genetic structure of symbiotic *Frankia* assemblages associated with differences in alder host species and habitat conditions. Our study complements previous studies by examining sympatric host species and replicated examples of habitat conditions. Our most significant findings in this respect are: 1) intermixed sympatric hosts differed in *Frankia* structure to essentially the same degree as different host species in different habitats (Figure 3.1, Table 3.2), demonstrating significant influence of host species over symbiotic *Frankia* structure independent of habitat, 2) the two host species differed in the degree to which habitat appeared to influence *Frankia* structure, with relatively large

differences evident in *A. tenuifolia* compared to *A. viridis* nodules from different habitats, and 3) *Frankia* structure was largely consistent among replicate sites representing host species and habitat sampling blocks, suggesting consistent relationships between both factors and *Frankia* structure among our study sites.

Differences in *Frankia* structure between sympatric hosts were the largest of any of the comparisons we examined, and were consistent among replicate sites. These differences were largely due to apparent reciprocal specificity between hosts and symbionts; *i.e.*, the most common RF pattern found on each sympatric host species was unique to the host. Since the two species were largely intermixed within these sites, this difference cannot be wholly attributed to differences in microhabitat. While we cannot discount the possible interactive effect of habitat – that one or both hosts may associate with the dominant RF group from the other host under different environmental conditions (Simonet *et al.*, 1999) – the fact that this was not observed in any of the other habitats in this study, nor in a wider survey of *A. viridis* in Alaskan tundra habitats of the Brooks Range and Seward Peninsula (Taylor, MacFarland and Ruess, unpublished data), casts doubt on this possibility. It is nevertheless possible that the specificity we observed in the field does not reflect potential associations that may occur under cross-inoculation conditions, which can result in broader associations between host species and *Frankia* than those observed in the field (Huguet *et al.*, 2005; Simonet *et al.*, 1999), and which typically indicate relative promiscuity of both *Alnus* and *Frankia* (Prat, 1989; Du and Baker, 1992; Sheppard *et al.*, 1988; Dillon and Baker, 1982; Dawson and Sun, 1981). Alternatively, this apparent reciprocal specificity may represent genetic differences

between alder species and/or among *Frankia* genotypes in the range of symbiotic partners with which they are compatible. Large such differences between the host species in this study would be congruent with the large phylogenetic distance between them (Chen and Li, 2004; Navarro *et al.*, 2003). Interspecific host-symbiont specificity in *Alnus* is apparent in some cross-inoculation studies (Du and Baker, 1992; Weber, 1990; van Dijk *et al.*, 1988; Weber *et al.*, 1987), and genetic variation even within a host species may affect the level of infection by specific symbiont genotypes (van Dijk and Sluimer, 1994). Comparison of the potential and field specificities between our host species and *Frankia* groups will require a controlled inoculation experiment.

Frankia structure and soil conditions exhibited similar patterns of variation among habitats in this study. Large differences in both occurred between floodplain successional stages for *A. tenuifolia* and, with the exception of site UPE3, the largest differences in both for *A. viridis* occurred between upland and floodplain habitats (Figure 3.1, 3.2, Table 3.2, 3.3). The consistency of *Frankia* structure among replicate sites for most habitats suggests a close relationship between symbiont structure *in planta* and habitat conditions. Soil moisture and temperature patterns among habitats are similar to patterns in *Frankia* structure, and the correlation between dimension 2 in the correspondence analysis and PC1 in the PCA suggests that soil organic matter, N, exchangeable cations and pH are also important correlates. Many of these factors have been suggested to affect the size of infective *Frankia* populations in soil (*e.g.*, Huguet *et al.*, 2004a; Martin *et al.*, 2003; Zitzer and Dawson, 1992; Smolander, 1990), and some appear to differentially affect *Frankia* genotypes (Huguet *et al.*, 2004a,b; Zitzer and

Dawson, 1992). However, the mechanisms underlying such correlations are likely to be complex, involving covariation (*e.g.*, soil N and organic matter), or synergistic interactions among soil variables (*e.g.*, pH and nutrient content), as well as direct effects on soil bacteria and/or indirect effects acting through either host growth and nodulation (Uliassi and Ruess, 2002; Wall, 2000; Crannell *et al.*, 1994), or non-host plant cover (Maunuksela *et al.*, 1999; Paschke *et al.*, 1994; Zitzer and Dawson, 1992; Smolander and Sundman, 1987). Nevertheless, such mechanisms are probably limited in their action to one or more points along the sequence of events which must occur for *Frankia* to colonize a host in a new site, a process which requires essentially three steps: 1) dispersal to the site and, within the site, to the rhizosphere of a potential host plant and/or, 2) maintenance of viability in the soil until the opportunity for a host interaction occurs, and 3) securing and maintaining a host interaction. In the following discussion, we envision these steps as a series of 'filters' acting on an initially random *Frankia* assemblage, and present hypotheses for our results based on this scenario.

The largest difference in *Frankia* structure within a single host species in this study occurred between *A. tenuifolia* habitats, which also differed in environmental conditions to the largest degree of all habitats in the study. Given that these sites are not geographically clustered (Table 3.1), and the fact that the Tanana River is known to transport *Alnus*-infective *Frankia* propagules (Huss-Danell *et al.*, 1997), the contribution of dispersal to this difference does not seem likely to be important, although the greater age of FPL (≥150 years) vs. FPE (~25 years) soils may contribute to the larger number of RF patterns found in the former habitat (Burleigh and Dawson, 1994b; Huston, 1994).

The large differences in soil characteristics between *A. tenuifolia* habitats in this study, and the reported effects of such factors on soil-dwelling *Frankia*, suggest a significant contribution of differential soil viability among bacterial genotypes to differences in symbiotic *Frankia* structure between these habitats. Higher richness in FPL sites may result from these sites supporting larger numbers of infective soil *Frankia* due to their lower pH (Zitzer and Dawson, 1992) and salt content (Van Cleve *et al.*, 1993; Young *et al.*, 1992), higher organic matter (Burleigh and Dawson, 1994b) and exchangeable cation content (Smolander, 1990), and greater moisture and aeration (Dawson *et al.*, 1989) than FPE sites. Soil pH has been suggested as a particularly important factor affecting soil-dwelling *Frankia*. While opposite correlations to the one suggested here between pH and number of *Alnus*-infective *Frankia* units in soil have been reported (Martin *et al.*, 2003; Smolander, 1990; Smolander and Sundman, 1987), soils in these studies were more acidic than in the present study, in which site means ranged from 5.0 to 7.4. In this range, Zitzer and Dawson (1992) observed negative correlation with number of *Alnus*-infective *Frankia* units in soil. Interestingly, these authors also report a positive correlation with *Frankia* infective on *Elaeagnus angustifolia* across the same pH range, indicating the effects of pH may be strain-specific. Soil temperature and moisture have also been observed to differentially affect host-infection groups of *Frankia* (Sayed *et al.*, 1997), and structure of *Frankia* within a host infection group has been observed in relation to soil depth (Nalin *et al.*, 1997). Considering the difference in soil temperature and moisture between our *A. tenuifolia* habitats, and the greater vertical soil development

and more complex vertical structure of FPL than FPE soils, these factors may also have contributed to the differences we observed in symbiotic *Frankia* structure.

In addition to direct effects on soil *Frankia*, it is also possible that differences in environmental conditions affect host genetic structure and/or physiology, and that compatibility with/selection of specific symbionts by the host in response to genetic constraints or physiological demands contributes to genetic structure of symbionts in the nodules. While we cannot discount the possibility of genetic structure in alder hosts among our habitats, such structure seems unlikely given the restricted geographic range of this study and fact that alder is pollinated and dispersed by wind. Host physiology may differ greatly between habitats, however. This is suggested by the parallel differences observed in leaf N and canopy position in *A. tenuifolia* between FPE and FPL sites, which probably allows greater peak photosynthetic rates in canopy versus understory leaves (Dawson and Gordon, 1979). If alder is able to select genotypes based on optimal physiological benefit in a given environment, such host choice would be a strong contributing 'filter' on the genetic structure of the symbionts. Both positive and negative selection of symbionts have been observed in legume-rhizobia interactions (Simms *et al.*, 2006; Kiers *et al.*, 2003) and *A. glutinosa* appears to exert negative selection on ineffective (non-N-fixing) *Frankia* genotypes (Wolters *et al.*, 1997; van Dijk and Sluimer, 1994). Positive symbiont selection by *Alnus* does not appear to have been investigated. The basis of such choice – *e.g.*, differences in rate or cost of N-fixation among symbiont genotypes – may be difficult to detect in the field. The present study was hampered in this regard by high variability of SNF, low sample size within a site,

and lack of prior knowledge of *Frankia* distribution patterns, but we hope that the information we collected will help to design more powerful field inquiries into host-relevant differences among *Frankia* genotypes in the region.

Both *Frankia* structure and soil conditions exhibited less variation among habitats for *A.viridis* than for *A. tenuifolia* (Table 3.2, Figure 3.2). Among-habitat comparisons for *A. viridis* are hampered by low sample size in upland sites. However, reasonable, comparably-sized samples were collected for each landscape (UP and FP) and appear to indicate landscape-level differences in *Frankia* richness. This difference may be related to host plant distribution; RF groups not detected in UP sites were generally more frequently associated with *A. tenuifolia* than *A. viridis*, and may depend on the former host to maintain appreciable soil populations. Alternatively, this difference may be due to restricted survival of genotypes in uplands related to topography or lower soil moisture (Dawson *et al.*, 1989), or to restricted dispersal of genotypes between landscapes. Both wind and birds have been suggested as *Frankia* vectors and both appear able to differentially affect *Frankia* strains (Burleigh and Dawson, 1994a,b; Paschke and Dawson, 1993; Burleigh and Torrey, 1990), so it is possible that the lower diversity of upland sites is due in part to such dispersal limitations on some genotypes. Given the generally low variation in *Frankia* structure observed in *A. viridis* nodules and the low frequency with which RF2, RF4 and RF6 occur in FPL sites, host selection seems unlikely to be an important contributor to symbiont structure for this host.

This study was motivated by the possibility that limitations in availability of optimal *Frankia* genotypes due to host specificity and/or heterogeneous distribution of

bacteria among habitats, together with differences among bacterial genotypes in host performance, may represent a limitation on performance and ecological effects of alder in boreal habitats of interior Alaska. We found evidence for strong and consistent field specificity between alder species and *Frankia* genotypes and, in *A. tenuifolia*, for differences in distribution of symbiont genotypes among habitats. In the latter host leaf N covaried with *Frankia* structure, but we found little evidence for variation in SNF among symbiont genotypes for either host. Further studies are needed to determine whether the distribution patterns we observed in symbiotic *Frankia* are primarily due to differences in corresponding soil populations or to non-random associations with hosts, and whether the *Frankia* genotypes observed in this study vary in physiological benefit to their hosts.

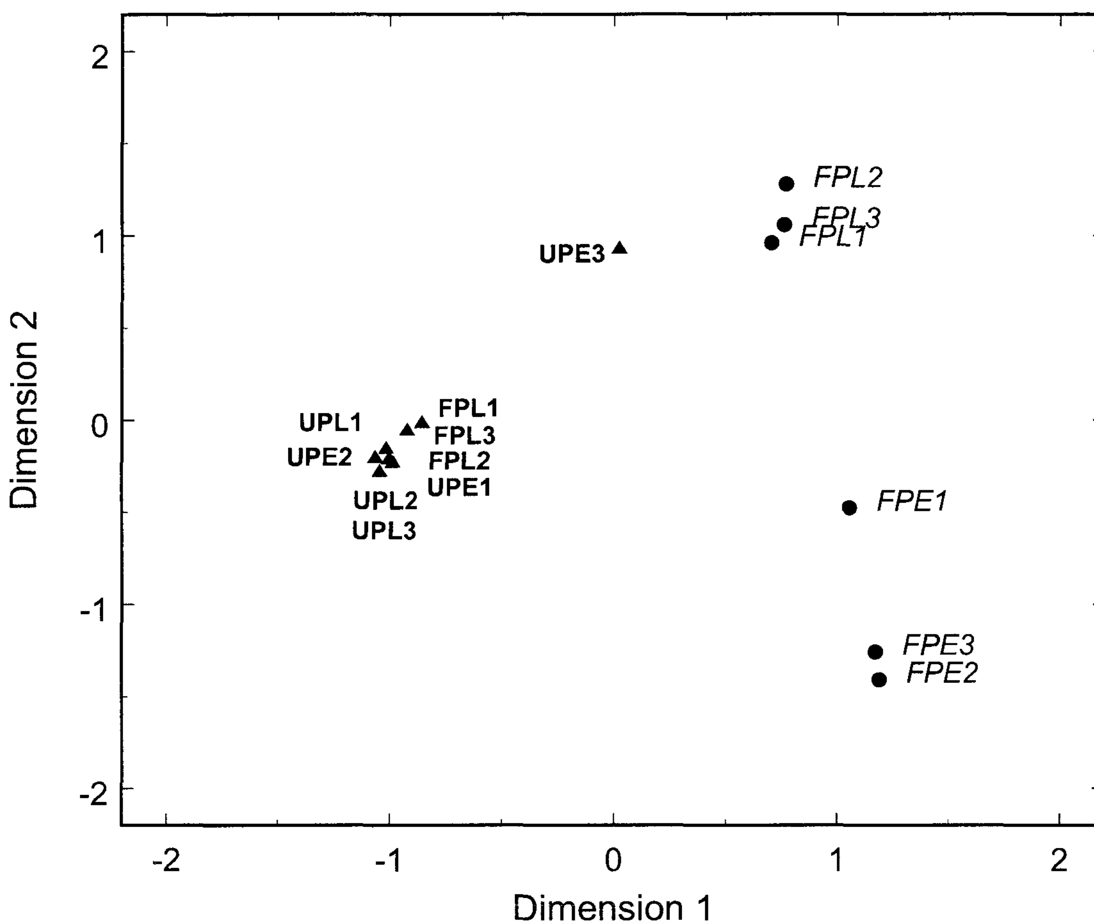

Figure 3.1. Correspondence analysis of *Frankia* genetic structure. Graph of the first 2 dimensions of correspondence analysis performed on RFLP pattern (*nif*D-K spacer) abundance data from *Alnus tenuifolia* (circle symbols) and *A. viridis* (triangle symbols) nodules collected from three replicate sites (1-3) representing early (E) and late (L) successional habitats in floodplain (FP) and upland (UP) landscapes in the BNZ-LTER. Dimension 1 accounts for 44.3% and dimension 2 for 30.4% of the total χ^2 for the analysis.

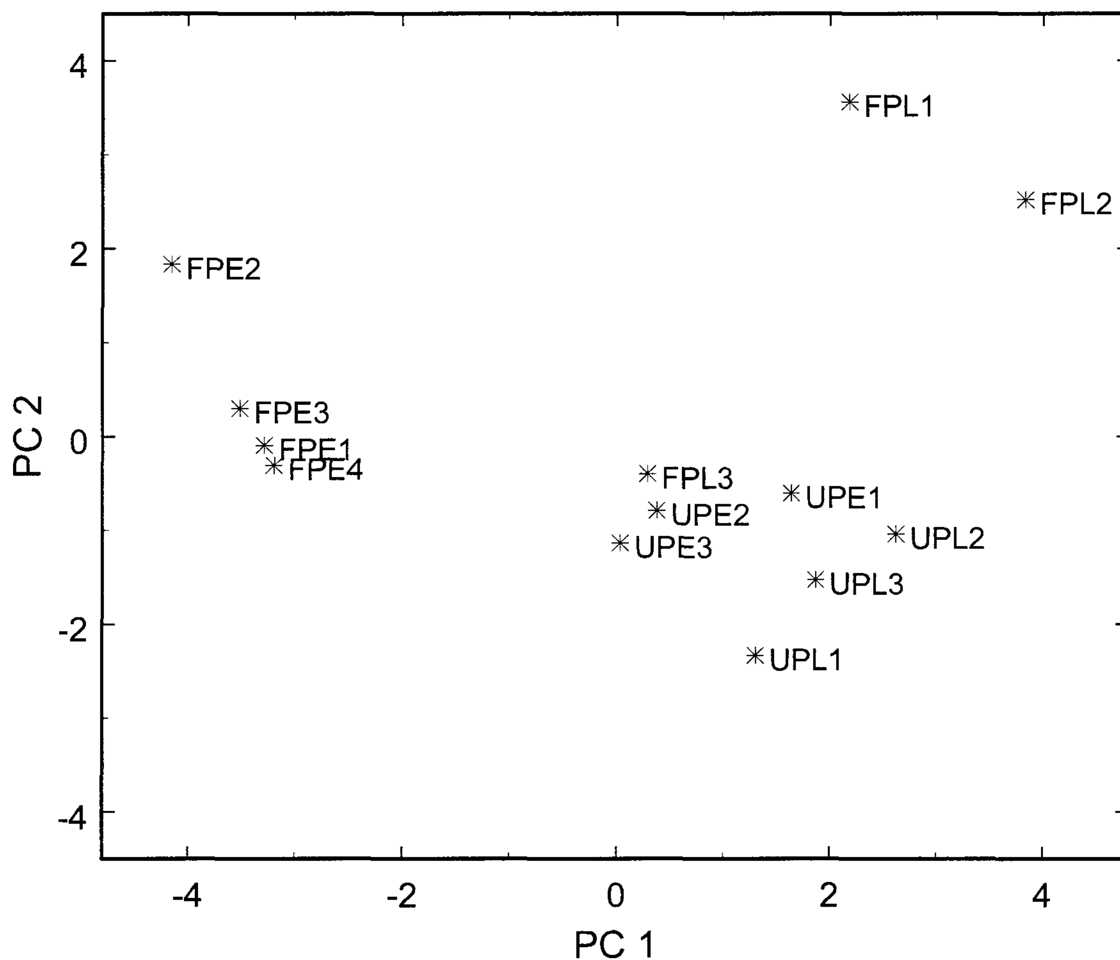

Figure 3.2. Principal components analysis of soil data. Graph of the first 2 principal components of a principal components analysis performed on public domain data (http://www.lter.uaf.edu/) collected for 13 edaphic characteristics in the same habitat types sampled in the present study in the Bonanza Creek Experimental Forest. FP and UP indicate floodplain and upland sites, respectively and E and L indicate early and late succession habitats. Numbers refer to replicate sites for each habitat. Principal component 1 accounts for 54.5%, and principal component 2 for 21.7 % of the total variance in the analysis.

Table 3.1. Mean geographic distances among study sites (km ± 1 standard deviation).

LANDSCAPE			FLOODPLAIN		UPLAND	
	STAGE		EARLY	LATE	EARLY	LATE
		HABITAT	FPE	FPL	UPE	UPL
FLOODPLAIN	EARLY	FPE	8.7 ± 6.1	5.7 ± 4.4	7.5 ± 3.7	8.5 ± 2.9
	LATE	FPL		3.9 ± 2.1	7.0 ± 0.7	8.5 ± 1.7
UPLAND	EARLY	UPE			1.2 ± 0.6	3.2 ± 1.2
	LATE	UPL				4.1 ± 2.3

Table 3.2. *Frankia* genotypes and physiological data recovery by HABSPEC. a) Number of nodules from each site and host species yielding each PCR-RFLP pattern (*nif*D-K spacer) observed in the Bonanza Creek Experimental Forest. Site designations include landscape (FP = floodplain, UP = upland), successional stage (E = early, L = late), and replicate site number 1-3. b) Number of nodule samples, out of 30 included in the experimental design, yielding data for PCR-RFLP (RF) (*nif*D-K spacer), specific N fixation (SNF) and leaf N content for each experimental site in the Bonanza Creek Experimental Forest.

SITE	HOST SPECIES	a. RESTRICTION FRAGMENT (RF) PATTERN										b. TOTALS		
		RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9	RF10	RF	SNF	Leaf N
FPE1	*A. tenuifolia*	4	2					12				18	30	30
FPE2						1		17				18	17	30
FPE3				1		1		13				15	27	28
FPL1	*A. viridis*		1		1				6	7	1	16	29	29
	A. tenuifolia	8	4		8			3				23	30	30
FPL2	*A. viridis*				2				2	16		20	30	30
	A. tenuifolia	7	4	1	1							13	20	30
FPL3	*A. viridis*						1		3	14		18	30	30
	A. tenuifolia	13		3	4		1	2				23	19	30
UPE1	*A. viridis*								6	6		12	30	30
UPE2									4	6		10	26	27
UPE3					3				1			4	30	30
UPL1									7	4		11	30	30
UPL2									2	3		5	20	25
UPL3									5	5		10	30	30

Table 3.3. Physiological and soil variable means by HABSPEC. Mean values of continuous variables (Mean ± Standard Error of the Mean) measured in the present study for plant physiological and environmental factors in the Bonanza Creek Experimental Forest. Common superscripts indicate homogeneous subsets (Tukey HSD, P > 0.05) (PROC GLM; SAS Institute, 2001). C = alder canopy; U = alder in understory.

HABITAT/HOST SPECIES			PHYSIOLOGICAL VARIABLES			ENVIRONMENTAL VARIABLES		
Landscape	Stage	Species	SNF (μmol N_2 g noddwt^{-1}×h^{-1})	Leaf N (% by mass)	SLW (gdwt leaf tissue/cm^2)	Soil Moisture (% by mass)	Soil Temperature (°C)	Canopy Position
Floodplain	Early	*A. tenuifolia*	28.3 ± 5.5^{a}	2.64 ± 0.05^{a}	6.21 ± 0.19^{a}	28.7 ± 0.5^{bc}	9.5 ± 0.3^{a}	C
	Late		22.4 ± 3.2^{a}	2.46 ± 0.03^{b}	5.06 ± 0.12^{b}	33.4 ± 1.2^{a}	7.5 ± 0.2^{b}	U
		A. viridis	30.3 ± 3.3^{a}	2.26 ± 0.03^{c}	4.79 ± 0.11^{b}	32.0 ± 1.2^{ab}	8.2 ± 0.2^{bd}	U
Upland	Early		33.9 ± 4.5^{a}	2.50 ± 0.04^{b}	7.17 ± 0.80^{c}	25.8 ± 0.7^{de}	9.3 ± 0.2^{ac}	C
	Late		34.0 ± 3.9^{a}	2.49 ± 0.03^{b}	4.24 ± 0.09^{d}	26.5 ± 0.6^{ce}	8.8 ± 0.2^{cd}	U

Table 3.4. Regression statistics for continuous variables. Analysis of variance (PROC GLM; SAS Institute, 2001) table for selected continuous variables measured in the experiment. Independent variables were retained by backward elimination starting with a model which contained all class and continuous variables and interactions. NS = not significant, *** P < 0.0001, ** P < 0.001, * P < 0.01.

Independent Variables	SNF (μmol N_2 g noddwt^{-1}*h^{-1})				Leaf N by mass (%)				Soil Moisture (% by mass)				Soil Temperature (°C, 5 cm depth)			
	R^2	P	F	df	R^2	P	F	df	R^2	P	F	df	R^2	P	F	df
Whole Model	0.65	***	25.6	27	0.47	***	15.0	24	0.41	***	12.3	24	0.60	***	26.5	24
Sampling Period		***	63.1	2		***	48.9	2		***	46.1	2		***	70.3	2
HABSPEC		NS	-	-		***	21.6	4		***	16.4	4		***	20.8	4
Replicate Site (HABSPEC)		***	4.9	10		***	14.8	10		***	11.4	10		***	37.7	10
HABSPEC × Sampling Period		***	7.6	8		**	3.7	8		*	2.6	8		***	4.3	8
Soil Temperature (quadratic)		*	9.5	1			---				---				---	

REFERENCES

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402.

Anderson MD, Ruess RW, Uliassi DD, Mitchell JS (2004) Estimating N_2 fixation in two species of *Alnus* in interior Alaska using acetylene reduction and $^{15}N_2$ uptake. Ecoscience 11: 102-112.

Batzli JM, Zimpfer JF, Huguet V, Smyth CA, Fernandez M, Dawson JO (2004) Distribution and abundance of infective, soilborne *Frankia* and host symbionts *Shepherdia*, *Alnus*, and *Myrica* in a sand dune ecosystem. Can J Bot 82: 700-709.

Benson DR, Dawson JO (2007) Recent advances in the biogeography and genecology of symbiotic *Frankia* and its host plants. Physiol Plant 130: 318-330.

Benson DR, Silvester WB (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 57: 293-319.

Burleigh SH, Dawson JO (1994a) Desiccation tolerance and trehalose production in *Frankia* hyphae. Soil Biol Biochem 26: 593-598.

Burleigh SH, Dawson JO (1994b) Occurrence of *Myrica* nodulating *Frankia* in Hawaiian volcanic soils. Plant Soil 164: 283-289.

Burleigh S, Torrey JG (1990) Effectiveness of different *Frankia* cell types as inocula for the actinorhizal plant *Casuarina*. Appl and Environ Microbiol 56: 2565-2567.

Chapin III FS, Walker LR Fastie CL, Sharman LC (1994) Mechanisms of primary succession following deglaciation at Glacier Bay, Alaska. Ecol Monographs 64: 149-175.

Chen Z, Li J (2004) Phylogenetics and biogeography of *Alnus* (Betulaceae) inferred from sequences of nuclear ribosomal DNA ITS region. Int J Plant Sci 165: 325-335.

Crannell WK, Tanaka Y, Myrold DD (1994) Calcium and pH interaction on root nodulation of nursery-grown red alder (*Alnus rubra* Bong.) seedlings by *Frankia*. Soil Biol Biochem 26: 607-614.

Dai Y, Zhang C, Xiong Z, Zhang Z (2005) Correlations between the ages of *Alnus* host species and the genetic diversity of associated endosymbiotic *Frankia* strains from nodules. Science in China C life sci 48S: 76-81.

Dai YM, He XY, Zhang CG, Zhang ZZ (2004) Characterization of genetic diversity of *Frankia* strains in nodules of *Alnus nepalensis* (D. Don) from the Hengduan Mountains on the basis of PCR-RFLP analysis of the *nifD-nifK* IGS. Plant Soil 267: 207-212.

Dawson JO, Gordon JC (1979) Nitrogen fixation in relation to photosynthesis in *Alnus glutinosa*. Bot Gaz 140S: S70-S75.

Dawson JO, Kowalski DG, Dart PJ (1989) Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant Soil 118: 1-11.

Dawson JO, Sun S-H (1981) The effect of *Frankia* isolates from *Comptonia peregrina* and *Alnus crispa* on the growth of *Alnus glutinosa*, *A. cordata*, and *A. incana* clones. Can J For Res 11: 758-762.

Denison RF, Kiers ET (2004) Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. FEMS Microbiol Let 237: 187-193.

Dillon JT, Baker D (1982) Variations in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. New Phyt 92: 215-219.

Du D, Baker DD (1992) Actinorhizal host-specificity of Chinese *Frankia* strains. Plant Soil 144: 113-116.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Holman RM , Schwintzer CR (1987) Distribution of spore-positive and spore-negative nodules of *Alnus incana* ssp. *rugosa* in Maine, USA. Plant Soil 104: 103-111.

Hooker JE, Wheeler CT (1987) The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. Physiol Plant 70: 333-341.

Huguet V, Gouy M, Normand P, Zimpfer JF, Fernandez MP (2005) Molecular phylogeny of Myricaceae: a reexamination of host-symbiont specificity. Mol Phyl Evol 34: 557-568.

Huguet V, Batzli JM, Zimpfer JF, Gourbière F, Dawson JO, Fernandez MP (2004a) Nodular symbionts of *Shepherdia*, *Alnus*, and *Myrica* from a sand dune ecosystem: trends in occurrence of soilborne *Frankia* genotypes. Can J Bot 82: 691-699.

Huguet V, Mergeay M, Cervantes E, Fernandez MP (2004b) Diversity of *Frankia* strains associated to *Myrica gale* in Western Europe: impact of host plant (*Myrica* vs. *Alnus*) and of edaphic factors. Env Microbiol 6: 1032-1041.

Huss-Danell K, Uliassi D, Renberg I (1997) River and lake sediments as sources of infective *Frankia* (*Alnus*). Plant Soil 197: 35-39.

Huston MA (1994) Biological diversity: the coexistence of species on changing landscapes. Cambridge University Press: Cambridge, UK.

Igual JM, Valverde A, Valázquez E, Santa Regina I, Rodríguez-Barrueco C (2006) Natural diversity of nodular microsymbionts of *Alnus glutinosa* in the Tormes River basin. Plant Soil 280: 373-383.

Jeong S-C, Myrold DD (2001) Population size and diversity of *Frankia* in soils of *Ceanothus velutinus* and Douglas-fir stands. Soil Biol and Biochem 33: 931-941.

JMP version 7.0 (1989-2007) SAS Institute, Inc. Cary, NC.

Kahn A, Myrold DD, Misra AK (2007) Distribution of *Frankia* genotypes occupying *Alnus nepalensis* nodules with respect to altitude and soil characteristics in the Sikkim Himalayas. Physiol Plant 130: 364-371.

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425: 78-81.

Kurdali F, Rinaudo G, Moiroud A, Domenach AM (1990) Competition for nodulation and $^{15}N_2$-fixation between a Sp^+ and a Sp^- *Frankia* strain in *Alnus incana*. Soil Biol Biochem 22: 57-64.

Markham J, Chanway CP (1998) *Alnus rubra* (Bong.) nodule spore type distribution in southwestern British Columbia. Plant Ecol 135: 197-205.

Martin KJ, Posavatz NJ, Myrold DD (2003) Nodulation potential of soils from red alder stands covering a wide age range. Plant Soil 254: 187-192.

Maunuksela L, Zepp K, Koivula T, Zeyer J, Haahtela K, Hahn D (1999) Analysis of *Frankia* populations in three soils devoid of actinorhizal plants. FEMS Microbiol Ecol 28: 11-21.

Mitchell JS (2006) Patterns and controls over N inputs by green alder (*Alnus viridis*) to a secondary successional chronosequence in interior Alaska. M. S. Thesis University of Alaska Fairbanks.

Mullis KB, Faloona FA, Scharf S, Saiki RK, Horn G, Erlich HA (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor symposia on quantitative biology.

Myrold DD, Huss-Danell K (1994) Population dynamics of *Alnus*-infective *Frankia* in a forest soil with and without host trees. Soil Biol Biochem 26: 533-540.

Nalin R, Normand P, Domenach A-M (1997) Distribution and N_2-fixing activity of *Frankia* strains in relation to soil depth. Physiol Plant 99: 732-738.

Navarro E, Bousquet J, Moirod A, Munive A, Piou D, Normand P (2003) Molecular phylogeny of *Alnus* (Betulaceae), inferred from nuclear ribosomal DNA ITS sequences. Plant Soil 254: 207-217.

Neter J, Kutner MH, Nachtsheim CJ, Wasserman W (1996) Applied linear statistical models 4th edn. WCB McGraw-Hill: Boston, MA.

Paschke MW, Dawson JO, Condon BM (1994) *Frankia* in prairie, forest, and cultivated soils of central Illinois, USA. Pedobiol 38: 546-551.

Paschke MW, Dawson JO (1993) Avian dispersal of *Frankia*. Can. J. Bot. 71: 1128-1131.

Prat D (1989) Effects of some pure and mixed *Frankia* strains on seedling growth in different *Alnus* species. Plant Soil 113: 31-38.

Rouvier C, Prin Y, Reddell P, Normand P, Simonet P (1996) Genetic diversity among *Frankia* strains nodulating members of the family Casuarinaceae in Australia revealed by PCR and restriction fragment length polymorphism analysis with crushed root nodules. Appl Env Microbiol 62: 979-985.

SAS Institute (2001) The SAS system for Windows. Version 8.2. SAS Institute, Inc: Cary, NC.

Sayed WF, Wheeler CT, Zahran HH, Shoreit AAM (1997) Effect of temperature and soil moisture on the survival and symbiotic effectiveness of *Frankia* spp. Biol Fert Soils 25: 349-353.

Sellstedt A, Huss-Danell K, Ahlqvist A-S (1986) Nitrogen fixation and biomass production in symbioses between *Alnus incana* and *Frankia* strains with different hydrogen metabolism. Physiol Plant 66: 99-107.

Sheppard LJ, Hooker JE, Wheeler CT, Smith RI (1988) Glasshouse evaluation of the growth of *Alnus rubra* and *Alnus glutinosa* on peat and acid brown earth soils when inoculated with four sources of *Frankia*. Plant Soil 110: 187-198.

Simms EL, Taylor DL, Povich J, Shefferson RP, Sachs JL, Urbina M, Tausczik Y (2006) An empirical test of partner choice mechanisms in a wild legume-rhizobium interaction. Proc Roy Soc B 273: 77-81.

Simonet P, Navarro E, Rouvier C, Reddell P, Zimpfer J, Dommergues Y, Bardin R, Combarro P, Hamelin J, Domenach A-M, Gourbière, Prin Y, Dawson JO, Normand P (1999) Co-evolution between *Frankia* populations and host plants in the family Casuarinaceae and consequent patterns of global dispersal. Env Microbiol 1: 525-533.

Smolander A (1990) *Frankia* populations in soils under different tree species – with special emphasis on soils under *Betula pendula*. Plant Soil 121: 1-10.

Smolander A, Sundman V (1987) *Frankia* in acid soils of forests devoid of actinorhizal plants. Physiol Plant 70: 297-303.

Uliassi DD, Ruess RW (2002) Limitations to symbiotic nitrogen fixation in primary succession on the Tanana river floodplain. Ecology 83: 88-103.

Valdés M, Pérez N-O, Estrada-de los Santos P, Caballero-Mellado J, Peña-Cabriales JJ, Normand P, Hirsch AM (2005) Non-*Frankia* actinomycetes isolated from surface-sterilized roots of *Casuarina equisetifolia* fix nitrogen. Appl and Env Microbiol 71: 460-466.

Van Cleve K, Viereck LA, Dyrness CT (1996) State factor control of soils and forest succession along the Tanana River in interior Alaska, USA. Arc Alp Res 28: 388-400.

Van Cleve K, Dyrness CT, Marion GM, Erickson R (1993) Control of soil development on the Tanana River floodplain, interior Alaska. Can J For Res 23: 941-955.

Van Cleve K, Viereck LA (1981) Forest succession in relation to nutrient cycling in the boreal forest of Alaska. *In* Forest succession: concepts and application. *Edited by* West DC, Shugart HH, Botkin DB. Springer-Verlag, New York, pp. 179-211.

Van Cleve K, Viereck LA, Schlentner RL (1971) Accumulation of nitrogen in alder (*Alnus*) ecosystems near Fairbanks, Alaska. Arc Alp Res 3: 101-114.

van Dijk C, Sluimer A (1994) Resistance to an ineffective *Frankia* strain type in *Alnus glutinosa* (L.) Gaertn. New Phyt 128: 497-504.

van Dijk C, Sluimer A, Weber A (1988) Host range differentiation of spore-positive and spore-negative strain types of *Frankia* in stands of *Alnus glutinosa* and *Alnus incana* in Finland. Physiol Plant 72: 349-358.

Vogel JG, Gower ST (1998) Carbon and nitrogen dynamics of boreal Jack pine stands with and without a green alder understory. Ecosystems 1: 386-400.

Walker LR, Chapin III FS (1986) Physiological controls over seedling growth in primary succession on an Alaskan floodplain. Ecology 67: 1508-1523.

Wall LG (2000) The actinorhizal symbiosis. J Plant Growth Reg 19: 167-182.

Weber A (1986) Distribution of spore-positive and spore-negative nodules in stands of *Alnus glutinosa* and *Alnus incana* in Finland. Plant Soil 96: 205-213.

Weber A (1990) Host specificity and efficiency of nitrogenase activity of *Frankia* strains from *Alnus incana* and *Alnus glutinosa*. Symbiosis 8: 47-60.

Weber A, Nurmiaho-Lassila E-L, Sundman V (1987) Features of the intrageneric *Alnus-Frankia* specificity. Physiol Plant 70: 289-296.

West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc Roy Soc Lon B 269: 685-694.

Wolters DJ, van Dijk C, Zoetendal EG, Akkermans DL (1997) Phylogenetic characterization of ineffective *Frankia* in *Alnus glutinosa* (L.) Gaertn. nodules from wetland soil inoculants. Mol Ecol 6: 971-981.

Wurtz TL (1995) Understory alder in three boreal forests of Alaska: local distribution and effects on soil fertility. Can J For Res 25: 987-996.

Young DR, Sande E, Peters GA (1992) Spatial relationships of *Frankia* and *Myrica cerifera* on a Virginia, USA barrier island. Symbiosis 12: 209-220.

Zimpfer JF, Kennedy GJ, Smyth CA, Hamelin J, Navarro E, Dawson JO (1999) Localization of *Casuarina*-infective *Frankia* near *Casuarina cunninghamiana* trees in Jamaica. Can J Bot 77: 1248-1256.

Zitzer SF, Dawson JO (1992) Soil properties and actinorhizal vegetation influence nodulation of *Alnus glutinosa* and *Elaeagnus angustifolia* by *Frankia*. Plant Soil 140: 197-204.

CHAPTER 4:

Phylogeny and Assemblage Composition of *Frankia* in *Alnus tenuifolia* Nodules across a Primary Successional Sere in Interior Alaska[1]

ABSTRACT

In nitrogen (N) fixing symbioses between plants and bacteria, genetic variation in bacterial symbionts, host-symbiont specificity, and environmental variation represent fundamental constraints on the ecology and evolution of plant and bacterial partners. Detailed information on specificity and environmental variation is lacking in many naturally-occurring N-fixing systems. This study examined patterns of host specificity of *Frankia* in naturally-occurring root nodules of two species of *Alnus* in interior Alaska, and genetic variation in endosymbiotic *Frankia* associated with environment in one host. Our objectives were: 1) to determine phylogenetic relationships among *Frankia* occurring in nodules of *Alnus tenuifolia* and *A. viridis* and, for *A. tenuifolia*, to examine: 2) genetic differences in symbiotic *Frankia* between early and late primary successional habitats, 3) distribution of *Frankia* diversity within vs. among host plants in individual sites, and whether this differs between habitats, 4) spatial patterns in *Frankia* distribution within sites, and 5) whether variation in *Frankia* is correlated with specific soil factors, and whether such correlations differ between habitats. Symbiont genotypes most commonly associated with each host belonged to different clades within the *Alnus*-

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[1] Anderson MD, Ruess RW, Taylor DL (in preparation) Phylogeny and assemblage composition of *Frankia* in *Alnus tenuifolia* nodules across a primary successional sere in interior Alaska. *Molecular Ecology*.

infective group of *Frankia*, and for *A. tenuifolia*, appeared to be divergent from previously described *Frankia* in this group. *A. tenuifolia* nodules from early and late succession habitats harbored distinct assemblages of *Frankia* genotypes which were largely similar among replicate sites representing each habitat. Nodules from early succession sites generally contained a single dominant symbiont genotype with no discernable clumping of symbiont genotypes at any spatial scale. Assemblages on late succession plants were more diverse, differed widely among plants within a site, and displayed significant autocorrelation of symbiont genotypes both within and among host plants. In early succession, occurrence of the dominant genotype was strongly correlated with carbon : nitrogen ratio in the mineral soil fraction, while in late succession occurrence of the most common genotypes was generally associated with carbon and nitrogen content of the organic soil fraction. The results of this study are consistent with either direct structuring of symbiont assemblages in soil, or host-mediated effects such as host choice. Evidence for both scenarios is discussed.

INTRODUCTION

Root nodule-based nitrogen (N) fixing symbioses are ecologically and economically important interactions, providing important natural inputs to the global N cycle (Reed *et al.* 2011) and including many plant species important in agriculture, forestry and bioremediation (Wheeler & Miller 1990; Graham & Vance 2003). Thirteen bacterial genera and plants from ten families participate in these symbioses (Sawada *et al.*

2003; Dawson 2008). Across this broad group, specificity, in terms of phylogenetic breadth of the partner associated with a given plant or bacterial taxon, varies widely (Young & Johnston 1989; Swensen & Benson 2008), but even within relatively specific associations, genetic variation in both plant and bacterial partners exists (*e.g.*, Simonet *et al.* 1999; Béna *et al.* 2005). Such variation among bacterial symbionts can affect growth, N-fixation and reproduction of host plants (*e.g.*, Parker 1995; Markham 2008; Heath 2010), and conversely, variation among plant genotypes within a host species can affect bacterial reproduction (Heath 2010). Further, the benefits of the symbiosis for both partners can be affected by environmental variables such as nutrient concentration (Heath & Tiffin 2007; Heath *et al.* 2010) and herbivory (Heath & Lau 2011). Host specificity, genetic variation within suites of compatible bacteria, and environmental variation thus represent important potential constraints on the coevolution of N-fixing symbioses (Thompson 2005; Heath 2010), on the colonization of new habitats (Parker *et al.* 2006), on growth and ecosystem functioning of N-fixing plants (Anderson 2011), and on the practical applications of specific N-fixing systems (*e.g.*, Thrall *et al.* 2000). Detailed field descriptions of bacterial diversity with respect to host taxa and environmental variation are fundamental to understanding the role of such constraints in natural habitats. The main objective of this study was to provide such a description for the *Alnus-Frankia* symbiosis in interior Alaska.

In root-nodule symbioses, plant and bacterial partners disperse independently and most bacterial taxa are capable of survival in the soil independent of the host plant. Distribution of bacterial genotypes in naturally-occurring host nodules therefore reflects

the cumulative effects of differences in dispersal between plant and bacterial propagules, specificity of plant and microbial partners, and environmental factors acting independently on each partner and on the interaction between them. At regional scales, decoupled dispersal may limit the ability of host plants to colonize new habitats (Larson & Siemann 1998; Parker *et al.* 2006), while at more local scales colonization and competitive exclusion can create patchy structure among genets in symbiotic microbes such as mycorrhizal fungi (Bruns 1995). Specificity may reflect phylogenetic barriers to nodulation between plant and bacterial taxa (*e.g.*, Mirza *et al.* 2009) and, on the plant side, may additionally reflect an ability to preferentially associate with specific bacterial genotypes (Kiers *et al.* 2003; Simms *et al.* 2006; Heath & Tiffin 2009). Environmental variation in distribution of N-fixing plant species (Dawson 2008) and soil-dwelling fractions of symbiotic bacteria (McInnes *et al.* 2004; Chaia *et al.* 2010) are well-described. Environmental variation may also act on the interaction between specific plant and bacterial genotypes, as when the optimally-performing pairing of host and symbiont genotypes differs under different environmental conditions (Heath & Tiffin 2007).

Field studies of N-fixing systems often focus on variation in mutualistic benefits received by plants from anonymous bacteria inhabiting soils from different locations (*e.g.*, Burdon *et al.* 1999; Thrall *et al.* 2000), or variation in genetically characterized bacteria in host nodules collected in single locations (*e.g.*, Wang *et al.* 2009), across large geographic areas (*e.g.*, Simonet *et al.* 1999), or across assortments of unreplicated habitats (*e.g.*, Navarro *et al.* 1999). While each approach can provide useful information

about some aspects of an interaction, each also has limitations, and a multi-faceted approach will, of course, provide the most complete picture. Such an approach, involving both field and laboratory studies, indicated that intra-specific specificity was the most important factor determining distribution of genotypes of *Bradyrhizobium* spp. symbiotic with the annual, self-fertilizing legume *Amphicarpaea bracteata* at local (< 1 km) and regional (~1000 km) scales in North America (Spoerke *et al.* 1996; Wilkinson *et al.* 1996; Wilkinson & Parker 1996; Parker & Spoerke 1998). This result, while compelling, may not apply to many other N-fixing plants such as *Alnus* spp., which are quite different from *Amphicarpaea* in terms of both population structure – *Amphicarpaea bracteata* is a largely self-fertilizing annual with populations that can be strongly differentiated at sub-kilometer scales (Parker & Spoerke 1998), while *Alnus* spp. are highly outcrossing perennials with generally panmictic populations (Bousquet & Lalonde 1990) – and lifespan: *Alnus* may live well over 100 years, over which time considerable environmental variation may occur which can alter selective pressures on both partners (Thompson 1994). Further, considering the relatively large ecophysiological differences among *Alnus* species, the factors structuring symbiont assemblages may even differ between species of *Alnus* (Anderson 2011).

In interior Alaska, symbiotic *Frankia* assemblages can differ widely between sympatric *Alnus* spp., and between successional habitats occupied by a given host species (Anderson *et al.* 2009). The present study aimed to characterize this variation in detail, focusing on five types of pattern: 1) phylogenetic relationships among *Frankia* occurring in nodules of *A.incana* ssp. *tenuifolia* (hereafter *A. tenuifolia*) and *A. viridis* ssp.

fruticosa (syn. *A. crispa*, hereafter *A. viridis*) and, in *A. tenuifolia*: 2) differences between early and late primary successional habitats, utilizing replicated and intermixed sites representing each habitat, 3) distribution of *Frankia* diversity within vs. among host plants in individual sites, and whether this differs between habitats, 4) spatial patterns in *Frankia* distribution within sites, and 5) whether variation in *Frankia* is correlated with specific soil factors, and whether such correlations differ between habitats. This study was conducted at a relatively restricted spatial scale (<15 km) in order to minimize the confounding effects of location, and is part of a larger project examining the possible role of host-choice in the ecology of the *Alnus-Frankia* system in interior Alaska.

MATERIALS AND METHODS

Study system and field sites

Study sites were located within the Bonanza Creek Experimental Forest (BNZ), part of the Long-Term Ecological Research (LTER) network and located approximately 30 km south-west of Fairbanks, Alaska, USA (64°48' N, 147°52' W). Detailed descriptions of the sites are available in Anderson *et al.* (2009), and further details are on the BNZ-LTER website (http://www.lter.uaf.edu/). Briefly, sites represent early and late-successional endpoints of a primary sere that initiates on alluvial deposits of the Tanana River in interior Alaska. *A. tenuifolia* colonizes these deposits and forms a closed canopy ~5-10 years after initial substrate formation (Hollingsworth *et al.* 2010; Nossov *et al.* 2011). Further succession proceeds through a stage dominated by balsam poplar

(*Populus balsamifera* L.) beginning ~50 years post-substrate, then white spruce (*Picea glauca* (Moench) Voss) after ~120 years (Chapin *et al.* 2006). *A. tenuifolia* persists in the understory in these later stages, and *A. viridis*, which is generally absent in early stages, is relatively common in white spruce-dominated stands, where both species of *Alnus* often co-occur.

Design and field methods

This study included six sites: three early (*A. tenuifolia*-dominated) and three late (white spruce-dominated) succession sites located on the floodplain of the Tanana River; these sites are FPE1-3 and FPL1-3, respectively, in Anderson *et al.* (2009). All sites were sampled between 21 and 28 July, 2005. At each site 20 *A. tenuifolia* plants were randomly selected by choosing the closest plant to a random point generated using three variables: 1) distance along (3-7 m), 2) direction (right or left) and 3) distance from (0-20 m) a 100 m transect line. In four of the six sites this line was contiguous and oriented perpendicular to the trend of the river in order to capture the widest available diversity of habitat. At site FPE1 the narrowness of the alder stand made it necessary to orient the transect parallel to the river, and at site FPL1 the absence of *A. tenuifolia* from a large portion of the site made it necessary to construct the plot using two shorter (~50 m) transects perpendicular to the river but paralleling each other 250 m apart. Distance and compass bearing from each random point to the nearest plant were recorded, and bearing and GPS coordinates were recorded for the origin of each transect. To examine the diversity of *Frankia* occurring within individual plants, five of the twenty plants at each

site were randomly chosen for 'intensive' sampling; ten nodule clusters were sampled from each of these plants and used for genetic characterization of symbionts. From each of the remaining 15 'extensive' plants, one nodule cluster was collected. For extensive plants, the first nodule cluster encountered for a plant was collected; for intensive plants nodule clusters were collected haphazardly from all four quadrants in a 1 m radius circular plot surrounding the base of the plant. When possible, nodule clusters from these plants were collected from different lateral root branches, but in late succession sites low nodule numbers on plants made it necessary to collect multiple nodule clusters from individual lateral branches on most plants. For all collected nodules, lateral roots were traced to the plant stem prior to collection to ensure collection from the correct plant. At ten plants (every other plant along the transect), a soil core (5 cm diameter X 10 cm depth) was collected within 0.5 m of the base of the plant, separated into organic and mineral fractions by hand, and transported to the lab in plastic zipper bags. Soil samples were dried at 65°C for 72 hours and stored at -20°C until chemical analysis could be performed. Soil pH was measured with a pH meter on a 1:1 slurry of soil and deionized water. Total N and C were determined with a CN autoanalyzer (LECO Corporation, St. Joseph MI). Total phosphorus was determined colorimetrically using the molydenum blue method with an amino-napthtol-sulfonic acid reducing agent (Jackson 1958). All soil variables were determined separately for organic and mineral horizons.

Frankia characterization

Nodule clusters were sterilized in the field immediately after collection by shaking in 10% bleach with Alconox for 3 min, followed by three 1-min rinses in DI water. After sterilization nodules were stored on dry ice until transport to the lab, then stored at -80° C until DNA extraction could be performed. Prior to extraction, nodules were freeze-dried to constant weight and ground on a shaker mill (Retsch, Inc., Newtown, PA). DNA was extracted from a subsample of each nodule cluster (≤ 3 lobes) with the Plant DNeasy 96 kit (Qiagen, Carlsbad, CA), with the addition of 20 mg/mL lysozyme followed by a 30 minute incubation at 37° C, as recommended for Gram-positive bacteria. Polymerase chain reaction (PCR) (Mullis *et al.* 1986) was performed using dehydrated PCR "beads" (Amersham Biosciences, Piscataway, N.J.), newly designed primers for the *nif*D-K intergenic spacer (IGS) region – forward, *nif*D1310frGC – CGCCAGATGCACTCCTGGGACTACT, reverse, *nif*KR331frGC – CGGGCGAAGTGGCTGCGGAA – and 35 cycles of 95°C for 1 min, and 65°C for 5 min. All successful PCR reactions were examined for sequence variation via restriction fragment length polymorphism (RFLP) based on separate digests (37° C for 8 hr) with *Cfo* I and *Hae* III (Promega, Madison, WI), as described in Anderson *et al.* (2009). PCR and restriction digest products were measured via electrophoresis on 1.5% (SeaKem; Cambrex, Rockland, ME) and 3% (1% SeaKem/2% NuSieve; Cambrex, Rockland, ME) agarose, respectively, and visualized via ethidium bromide/UV fluorescence. Digest fragments shorter than 50 bp were excluded from the analysis. Each unique combination of fragment patterns across both enzymes was given a numerical 'RF' designation.

To verify DNA sequence variation of *nif*D-K PCR product indicated by RFLP, and to examine each RF group for undetected variation, a subset of nodules from each RF group was sequenced which included multiple nodules from the most frequent RF groups. DNA extracts from nodules representing a subset of the *nif*D-K-based RF groups were also examined for variation in the 16S-23S intergenic spacer locus (rIGS), to verify diversity patterns observed for the *nif* locus, and to aid in phylogenetic placement of the RF types. Primers used for rIGS were 16Sfr1 – GTCACGAAAGTCGGTAACA, and 23SRfr1 – CCAGTGAGCTAATACGCAC, with a temperature program consisting of 30 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 4 min, with a 4 min 94°C initial denaturing and 20 min 72°C final extension step. For both loci, PCR product was cleaned using the QiaQuick PCR cleanup kit (Qiagen, Carlsbad, CA) and quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington DE). Cycle sequencing reactions were performed with ABI BigDye (2.0 μL/rxn) (Applied Biosystems Inc., Carlsbad CA), using PCR primers and an annealing temperature of 58°C. Capillary sequencing was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems Inc., Carlsbad CA).

Data analysis

To determine whether most of the *Frankia* diversity in each sampling site had been detected, we generated sample-based rarefaction curves based on both interpolation- (Mau Tau (Colwell *et al.* 2004)) and extrapolation-based (Chao1 and Chao2 (Chao 1984)) richness estimates. All estimates were calculated using Estimate S version 8.2

(Colwell 2009), with plant treated as sample. We concluded that saturation had been reached if the 95% confidence intervals for the interpolation- and extrapolation-based estimators overlapped in the final sample.

The contributions of among-plant (within-site) and among-site (within-stage) differences to variation in symbiotic *Frankia* assemblages were compared using both nominal logistic regression with the program JMP 8.0 (SAS Institute) and analysis of molecular variance (AMOVA)(Excoffier *et al.* 1992) with Arlequin 3.1 (Excoffier *et al.* 2005). For both methods the two factors were directly compared in a single analysis for the intensively sampled plants, with site and plant (nested within site) included as independent variables and *Frankia* RF type as the dependent. The results of these analyses were compared to separate analyses run on extensively sampled plants which only included site as an independent variable. Separate analyses were run for each successional stage for both methods. In the AMOVA for the intensively sampled dataset, plants were included as populations and sites as groups; for the extensive dataset sites were included as populations and only within- and among-population variation was examined. Both sequence and RFLP data were examined in the AMOVA, and both gave qualitatively similar results.

Prior to spatial analysis all field distances (along-transect, from-transect, and from-random-point) and compass bearings for the location of all plants and nodules in each site were converted to x-y coordinates. Global spatial autocorrelation for each site was assessed using Mantel tests (Mantel 1967) of correlation between a Euclidean distance matrix calculated for all nodules collected in a site (n=51-61 nodules) and a

binary connectivity matrix which coded nodules with shared RF haplotype as '1', and '0' otherwise. Significance of the correlation was determined using a permutation test which included 999 permutations. Local autocorrelation within each site was assessed using Mantel correlograms, with ten distance classes defined so that each class contained the same number of pairwise comparisons, but with the lowest distance class adjusted to ≤ 1 m to encompass all nodules collected within intensively sampled plants. Correlogram analyses were performed on both multivariate data (all RF groups included in the connectivity matrix) and separately for each of the most common individual RF types in each site. For all correlogram analyses significance was assessed examining Bonferroni-adjusted P-values from a permutation test that included 999 permutations. Geographic distance and distance class matrices were generated, and global and local Mantel tests performed, using the program PASSaGE2 (Rosenberg & Anderson 2011).

Correlations between abundance of RF types and measured soil variables at the level of individual plants were investigated using multiple nominal logistic regression with RF type as the dependent variable. Separate analyses were performed for each successional stage. Variables included in the analysis were pH, N, C, and P content, as well as C:N, C:P, and N:P ratios for both organic and mineral horizons. For each model, initial construction utilized a backward stepwise procedure which began with all variables in the model, then removed the variable with the smallest χ^2 at each step until $P \leq 0.05$ for all independent variables. Previously removed variables were retested in the resulting model until the whole model χ^2 and log likelihood difference between the full and reduced models were maximized, and $P \leq 0.05$ for all included variables. Selection

of variables to add during the forward stepwise phase was informed by bivariate logistic regressions performed between RF type and each soil variable prior to model construction, which aided in selection of promising variables during the forward procedure. Bivariate linear regressions were also performed between all independent variables prior to model construction, and the results used to determine multicollinearity among dependent variables in the regression model. Independent variables found to be significant in the regression model for all RF types were then tested in separate models constructed for the most common individual RF types in a successional stage.

Phylogenetic analysis

Separate phylogenetic analyses were performed for *nif*D-K and rIGS loci. Comparison sequences included several derived from nodules of *Alnus viridis* ssp. *fruticosa* that co-occurred with *A. tenuifolia* in our FPL sites (sampling methods described in Anderson *et al.* 2009), as well as all sequences of comparable length that were available on the NCBI database and found via BLASTn searches (accession numbers provided in tree figures). For all analyses, multiple sequence alignments were generated using ClustalX (Thompson *et al.* 1997) and corrected by eye using SeaView (Galtier *et al.* 1996) and BioEdit (Hall 1999). Corrected alignments were trimmed to a common length for all sequences. Prior to phylogenetic analysis, substitution model selection was performed for each locus using the program jModeltest (Posada 2008), with all 88 available models included in the assessment, and the base tree ML optimized. For the rIGS data, both likelihood and corrected Akaike information criteria (AIC_C) indicated

a best fit for the General Time-Reversible model (Tavaré 1986) with gamma-distributed rate heterogeneity (GTR+G), while Bayesian information criterion (BIC) selected the transitional model (Posada 2003) with gamma-distributed rate heterogeneity and proportion of invariant sites (TIM+G+I). For the *nif*D-K data, likelihood measures indicated GTR+G as the best fit, and both AIC_C and BIC indicated Kimura's three-parameter (Kimura 1981) with unequal base frequencies and gamma-distributed rate heterogeneity (TPM3uf+G).

Three phylogenetic analysis programs were utilized in order to assess the robustness of our results to different tree selection criteria and tree search algorithms. All programs were run on the CIPRES network (Miller *et al.* 2010). Maximum-likelihood analyses were performed using both RAxML (Stamatakis 2006) and GARLI (Zwickl 2006), which utilize different search algorithms: RAxML uses a fast hill-climbing search strategy while GARLI implements a genetic algorithm. RAxML was run using the GTR+G substitution model for both loci, as the program does not offer a GTR+G+I option on the CIPRES interface. GTR+G was used for both final tree selection and bootstrap analysis, which was run using the rapid option and 100 replicates. GARLI was run with default population and termination settings, and the general model recommended by jModeltest for each locus. Bayesian analysis was performed using the program MrBayes on the CIPRES network, with the most general model settings offered (GTR+G+I) and flat Dirichlet priors. Markov chain Monte Carlo simulations were set to 5 million generations, sampled every 1000 generations with the first 25% of samples discarded as burn-in.

Because the spacer portion of the *nif*D-K locus was highly divergent and difficult to align, we also assessed the robustness of our phylogenetic analysis to inclusion of different portions of the alignment. We used the program Gblocks (Castresana 2000) to remove portions of the alignment deemed unreliable according to the most stringent, the least stringent, and an intermediate set of parameters. The resulting alignments were then analyzed using RAxML and MrBayes, and the best tree compared for qualitative results with the tree based on the entire alignment.

RESULTS

Frankia characterization and phylogenetic host specificity patterns

PCR/RFLP characterization of nodule *Frankia* yielded interpretable data for 342 of 390 nodules (88%) collected from *A. tenuifolia*. The majority of RF types observed were identical to those previously reported from *Alnus* species in these sites (Anderson *et al.* 2009), with the exception of RF11-RF16, which were new to this study. *Nif*D-K sequence data were obtained from nodules representing all RF types except RF12 and RF14, and for multiple representatives of RF1-RF4 and RF7. In all cases in which multiple sequences were obtained for the same RF group, all sequences were identical. Ribosomal IGS sequences were obtained from a subset of the *nif*D-K-based RF types which included RF1-4, RF7, RF9, and RF16.

Phylogenetic analysis of the rIGS locus using both ML and Bayesian approaches placed all sequences from both host species we examined in the ‘Clade 2’ group of

Frankia, which includes strains infective on *Alnus*, *Myrica* and *Casuarina* (Clawson *et al.* 2004)(Fig. 4.1). RF9, from *A. viridis*, and RF4, which infects both *A. viridis* and *A. tenuifolia* in our sites, are relatively similar to sequences derived from other alder host species. By contrast, the majority of sequences derived from *A. tenuifolia*, including RFs 1, 2, 3, 6, 7 and 16, form a well-supported clade in all analyses that is comparatively distant from other Clade 2 *Frankia*.

For the *nif*D-K locus, the two ML programs and the Bayesian analysis placed RF8-10 from *A. viridis*, and RF4, which infects both hosts, in distinct clades within the Clade 2 *Frankia*. Similar to the results from the rIGS locus, all three programs also yielded a well-supported third clade consisting of sequences representing RF groups 1-3, 5-7 and 11-16 from *A. tenuifolia* that is relatively distant from other *Alnus*-infective strains (Fig. 4.2). Placement of this clade relative to other groups differed among analytical methods, however; both ML-based methods placed this clade on a relatively long branch within the Clade 2 *Frankia*, while in the Bayesian analysis it formed a sister group to Clade 2 (Fig. 4.2). Placement of this clade was also sensitive to the characters included in the analysis for the ML programs: with the least stringent (348 of 757characters) and intermediate (291 characters) settings both RAxML and GARLI still placed the group within the Clade 2, but with the most stringent settings (156 characters) both programs placed it as a sister to known Clade 2 sequences. Placement of this clade by Bayesian analysis was not affected by the portion of the alignment included.

Variation in *Frankia* assemblage structure

At the broadest scale, the genetic composition of symbiotic *Frankia* assemblages observed in the present study is very similar to that previously reported for *A. tenuifolia* in these sites (Anderson *et al.* 2009), which suggests assemblage composition may be temporally stable, at least at the scale of time between the two sampling efforts (3 years). As in the earlier study, early succession sites were dominated by a single RF haplotype (RF7), and late succession sites supported a much higher overall diversity (Table 4.1), with differences in *Frankia* structure paralleled by differences in soil chemistry between successional habitats (Table 4.2). The largest difference with the earlier study is the appearance in this study of RF16 as a dominant component of late succession assemblages, which may be related either to the larger sampling area and/or sample size per site, or the much higher success rate of the PCR-RFLP procedure in the current study. Given the scale at which this haplotype is clumped in most sites (see 'spatial structure of *Frankia* assemblages' subsection), the latter explanation seems more likely.

Accumulation curves for RF richness indicated that saturation had been reached for all six sites, based on overlapping 95% confidence intervals between Mau Tau (interpolation-based) and Chao1 and Chao2 (extrapolation-based) indices. Mau Tau richness estimates were also always within one 'species' of all other estimators calculated by Estimate S, which included ACE, ICE, bootstrap, and jackknife-based estimates. Thus, it appears that this study captured the available RF diversity in each site.

Comparison of among-plant with among-site contributions to *Frankia* assemblage structure via both logistic regression and AMOVA gave largely similar results, and

indicate differences in the partitioning of symbiont genetic variation between the two habitats. The results of the AMOVA for the intensive dataset indicate that a nearly equal proportion of molecular variation among *Frankia* genotypes occurs within populations (plants) for both early (79%) and late (77%) succession *A. tenuifolia*, but total variation was much higher in late succession (sum of squares (SS) = 47.6, n=128) than in early (SS = 22.05, n=129). The partitioning of the remaining ~20% of the proportional variation also differs between the two habitats. In early succession it is split nearly equally between among-site and among-plant within-site components, both of which contribute significantly ($P < 0.02$) to the overall variation in the AMOVA. Both components were also significant in bivariate logistic regressions, but neither was significant in the multiple regression model. In late succession nearly all of the remaining 20% is accounted for by the among-plant within-site component, which is the only one of the two to contribute significantly to the overall variation. The results of the logistic regression are similar, with the only significant variation discernable being that among plants nested within sites in the late succession habitat. These results contrast somewhat with the analyses of the extensive dataset, which indicates no significant contribution of among-site variation in early succession for either analytical method. Among late succession sites, both AMOVA and logistic regression indicate small but significant differences in *Frankia* structure for the extensive dataset.

Spatial structure of *Frankia* assemblages

Among early succession sites, global Mantel tests were only significant for site FPE2 ($P < 0.02$), but were not significant for the other two early succession sites. Correlograms detected no significant local autocorrelation for any of the early succession sites, including FPE2. These results were not surprising, given the level of dominance of RF7 that occurred in these sites (Table 4.1).

All late succession sites yielded significant global autocorrelation when all RF types were included in the analysis ($P_{perm} = 0.002$ for FPL1, 0.006 for FPL2, and 0.03 for FPL3). For all three sites, multivariate correlograms produced the largest peaks in autocorrelation values in the first distance class (<1 m), which corresponds to within-plant distances (Table 4.3), although for site FPL2 this peak was not significant. For the other two sites, autocorrelation values dropped to insignificance in the second distance class, which corresponded closely with first nearest-neighbor distances among plants. Together with the significant autocorrelation from 0-1 m, this suggests a patch size for all RF types that is roughly equivalent with individual plants (Legendre & Fortin 1989) in these sites. By contrast, in FPL2 significant autocorrelation was only observed in the third distance class (13 to 24 m), suggesting patchiness of symbiotic *Frankia* at a scale which included more than one plant. In correlograms for FPL1 and FPL3 additional significant peaks were observed after the drop to insignificance beyond the smallest distance classes, suggesting the occurrence of multiple patches among all RF types (Legendre & Fortin 1989). In FPL1, the second peak occurred in distance class 5 (30 to 50 m), while in FPL3 the second peak occurred in the class 6 (56 to 63 m).

Autocorrelation patterns for the most frequent RF types in each site largely paralleled the multivariate patterns (Table 4.3), with a few differences. In site FPL1 the within-plant peak (distance class 1) in the multivariate correlogram was reflected in correlograms for three of the four most common RF types (RF1, RF4 and RF7), with the fourth (RF16) producing a peak that corresponded with the second peak in the multivariate correlogram for this site. By contrast, in FPL2 the significant multivariate peak corresponded with a peak in RF16, but a significant within-plant (< 1 m) peak also occurred for this RF. A significant peak occurred for RF6 which was not apparent in the multivariate correlogram for this site. In site FPL3 both the first and second peaks from the multivariate correlogram also occur for RF16, and RF2 also displayed significant autocorrelation in the first distance class. Taken together, these results suggest that clumping of RFs occurs both within and among plants in late-succession sites, and that clump size is variable both among RFs within a site and among sites.

Correlation with soil variables

In early succession soils, the best logistic regression model indicated RF occurrence was significantly correlated with N and C content, pH and C:N ratio in the mineral horizon (Table 4.4A). The strongest correlation was with mineral C:N ratio, which was the only significant independent variable in bivariate regressions, and which also correlated significantly with the occurrence of each of the most common individual RF types. This relationship was particularly strong in the dominant RF type, RF7, which

was positively related to C:N ratio, while the other two RF types tested only occurred at low C:N ratios.

Regression analysis with all RF types in late succession yielded two nearly identical best models, each with three explanatory variables (Table 4.4B). Both models included N and C content in the organic horizon, but one also included organic C:N ratio, while in the other pH in the mineral horizon was the third significant variable. Analysis of individual RF types indicated correlations with specific soil variables that differed among RF types (Table 4.4). In addition to RF1, the two other RF types that occurred in early succession soils were correlated with a different suite of variables in late succession. RF3 occurrence in late succession correlated with C content and C:N ratio of the organic horizon, while RF7 was more strongly correlated with C and N content than C:N ratio, all in the organic horizon. Similar to its occurrence in early succession, RF7 retained a positive relationship with C:N ratio, although this relationship was much weaker in late succession sites. RF6 was also positively correlated with C:N ratio in the organic horizon.

DISCUSSION

The present study had two major objectives: 1) to characterize the phylogenetic affinities of *Frankia* associated with *A. tenuifolia* and *A. viridis* in our interior Alaskan field sites, and 2) to provide a detailed description of habitat-based differences in symbiotic *Frankia* assemblages for the former host which includes consistency of

structure among replicate sites, scale and spatial pattern of structure within sites, and correlations with edaphic factors. Our most significant findings are: 1) *Frankia* symbionts from *A. tenuifolia* appear to belong to a single clade that is relatively divergent from other described *Frankia*, while *A. viridis* symbionts appear to be closely related to the majority of *Alnus*-infective *Frankia*, and 2) large habitat-based differences in *A. tenuifolia* symbiont assemblage structure were observed which included differences in the scale and spatial pattern of *Frankia* assemblages, as well as correlations with specific soil variables. These differences were largely consistent among replicate sites, and overall structure was largely independent of sampling year and sampling intensity.

Genetic structure in natural assemblages of nodule-dwelling bacteria can be envisioned as resulting from a hierarchy of factors which act directly and separately on plant and bacterial partners, as well as effects acting on the interaction between the two partners. At the broadest level, independent dispersal of plant and bacteria is likely to result in non-overlapping distributions of plant and bacterial genotypes across a range of spatial scales, limiting the range of possible associations to genotypes of each partner that share distributions. A further constraint is provided by evolved patterns of host-symbiont specificity, which includes both 'hard' barriers preventing nodule initiation in particular host-symbiont pairings, as well as softer barriers which result in differences among bacterial genotypes in the proportion of nodules occupied on a given host (Anderson 2011). Among compatible bacterial strains, habitat suitability can also directly structure soil-dwelling assemblages of N-fixing bacteria (McInnes *et al.* 2004; Chaia *et al.* 2010), limiting the pool of bacterial genotypes available to plants in specific macro-habitats, and

potentially contributing to micro-habitat (within and among individual plant rhizospheres) structure, if environmental variation occurs at this scale. Finally, within the range of genetically compatible symbiotic partners in a given habitat, interactive effects such as context-dependence of symbiotic outcomes (Heath & Tiffin 2007; Heath *et al.* 2010), and any ability plants might have to select optimal symbionts (Simms & Taylor 2002), can further limit the realized range of associations in natural settings. The limited geographic scale of the present study (all sites were within 14 km of each other) and the highly outcrossing habit of *Alnus* spp. make it unlikely that geographic range or genetic structure in plant populations explain much of the observed variation. Rather, our observations are more consistent with mechanisms related to host specificity, direct environmental effects on *Frankia*, and interactions between *Alnus* and *Frankia*.

The results of our phylogenetic analysis, together with data on the occurrence of the different RF types on both host species examined in this and a prior study (Anderson *et al.* 2009), suggest a relatively high degree of specificity between *Alnus* species and *Frankia* genotypes in our sites. *A. tenuifolia* and *A. viridis* harbor distinct symbiont assemblages, with the dominant RF types for each host occurring only rarely on the other host even in sympatry (Anderson *et al.* 2009). In the present study the same pattern was observed in a much larger sample from *A. tenuifolia*, and also found to contain a significant phylogenetic component; RF types dominant on *A. tenuifolia* appear to be fairly divergent from other described strains for the loci we examined. Phylogenetic host specificity thus appears to be a significant constraint on the structure of *Frankia* assemblages in *Alnus* nodules in our study sites.

Previous studies of host specificity patterns between *Alnus* and *Frankia* have yielded few general patterns for comparison with our results. A long history of cross-inoculation studies collectively suggests little absolute specificity between host species and bacterial strains in terms of the ability to form nodules (*reviewed in* Anderson 2011). More recent phylogenetic studies of *Alnus*-infective *Frankia* have yielded both clades that appear specific to one host species and clades made up of samples from many host species, although host-specific clades appear to be the exception rather than the rule (*e.g.*, Hahn 2008; Welsh *et al.* 2009a; Kennedy *et al.* 2010; Pokharel *et al.* 2010). Few field studies that include multiple *Alnus* species have been conducted, and those that exist have yielded somewhat conflicting results. Lipus & Kennedy (2011) found large differences in the frequency of occurrence of two dominant *Frankia* genotypes in nodules of *A. viridis* (ssp. not specified) and *A. rubra* in different sites, and this result was largely supported in cross-inoculation tests using both host species grown in soil from beneath each host in the field. By contrast, Pokharel *et al.* (2010), who characterized nodule symbionts from 12 sympatrically-occurring *Alnus* taxa in an Illinois arboretum using rep-PCR and *nif*H sequencing, found a single *Frankia* genotype in all nodules of nine host taxa (75% of all collected nodules) from all three subgenera of *Alnus*, suggesting an extremely low degree of host-symbiont specificity. Interestingly, this dominant genotype was found in all 14 nodules collected from *A. tenuifolia* in their study. While the major results of these two studies differ, both studies also report that the apparent specificity observed was modified to some degree by environmental conditions. It is worth noting in this context that in the present study RF8 and RF9, which are extremely common in

Alaskan *A. viridis* nodules, were observed at low frequency in nodules of *A. tenuifolia*. And RF2 and RF6, which belong to the *A. tenuifolia*-specific clade, have been observed in *A. viridis* nodules (Anderson *et al.* 2009), suggesting that the specificity we observed is not due to an inability to form nodules, but may be due to selection by plants. Further, both of these exceptions were only observed in sites containing both host species, suggesting that formation of nodules between uncommonly nodulating host-symbiont pairs may be enhanced by maintenance of *Frankia* genotypes in soils under the preferred hosts. Thus, while host species clearly plays a large role in structuring symbiont assemblages in our sites, a lack of hard barriers to nodulation also leaves open the possibility of modulation by physiological selection and/or environmental factors.

Within a species of *Alnus*, variation in structure of symbiotic *Frankia* assemblages across different environments is widely described (*e.g.*, Khan *et al.* 2007; Welsh *et al.* 2009b; Kennedy *et al.* 2010), but generally not characterized in terms of within- vs. among-plant variance components, spatial structure, consistency across multiple sites with similar environmental conditions, or with respect to specific edaphic factors. The large, consistent differences we have observed between early and late successional habitats in this and an earlier study (Anderson *et al.* 2009) suggest a strong relationship between habitat-related factors and symbiont structure in *A. tenuifolia* nodules. These habitat-related differences, and their consistency among replicate sites, are paralleled in the present study by patterns in within- vs. among-plant structure, spatial structure, and correlations with edaphic factors. Again, considering the geographic scale of the study, direct structuring of soil *Frankia* assemblages by environmental factors

and/or non-random selection of symbiont genotypes by hosts are more likely explanations for the observed patterns than host genetic structure and specificity. Assessing the degree to which direct effects on soil-dwelling *Frankia* vs. plant-mediated effects contribute to *Frankia* structure is impossible in the current study, since the two are inextricably confounded, particularly in early succession sites which display very few patterns in *Frankia* distribution within sampling sites. However, in late succession, comparison of the scale and spatial patterns of variation with respect to individual plants, sites, and edaphic factors is suggestive.

Early succession sites varied much less than late succession sites in terms of *Frankia* structure – within plants, among plants within sites, and among sites. However, both AMOVA and simple logistic regressions indicated significant contributions of all three components to variation in *Frankia* structure, despite the overwhelming dominance of RF7 in these sites. The similar contributions made by these components suggest that the factor(s) determining *Frankia* structure act similarly within and among plants, and among replicate early succession sites. Such factors can include both direct effects on soil bacteria and effects mediated by host plants. Our early succession sites are much more homogeneous than late succession sites in terms of plant community structure, vertical soil development, and soil chemistry, all of which appear to directly affect *Frankia* assemblages in soils (*reviewed in* Anderson *et al.* 2009; Chaia *et al.* 2010). However, this environmental homogeneity also occurs at the plant level, so the response of *Frankia* in nodules could be largely mediated by host plants. Many theoretical studies of the evolutionary stability of mutualisms predict host plants should be able to select

among symbionts based on mutualistic quality (*e.g.*, Simms & Taylor 2002; Kiers & Denison 2008, but see Marco *et al.* 2009), and this prediction has experimental support in some rhizobial systems (Kiers *et al.* 2003, 2006; Simms *et al.* 2006). If *A. tenuifolia* plants are able to select optimal symbionts under a given set of environmental conditions, and optimal behavior is a genetically-determined trait for the bacterium, this ability may account for much of the between-habitat structure we have observed. Given the high N demand of plants and low N supply of soils in early compared to late succession habitats, any host choice should be most stringent in the former habitat (Anderson *et al.* 2009).

The clearest specific factor correlated with *Frankia* structure in this study was C:N ratio of the mineral horizon, high values of which appear to favor RF7 at the expense of RF1 and RF3. Again, this pattern may reflect either direct effects on soil *Frankia,* or plant-mediated effects via non-random nodulation. C:N ratio is a well-known indicator of substrate quality for saprotrophic microbes such as *Frankia*, and also affects N-availability for plants by controlling N-release by N-mineralizing bacteria (*e.g.*, Yamakura & Sahunalu 1990). Thus, the correlation between C:N and RF7 may indicate that RF7 is a specialist on low-quality substrate and therefore makes up a larger fraction of *Frankia* available in soil for nodulation, or that plants in high C:N areas have a greater reliance on N-fixation which, under the plant-choice hypothesis, will result in greater selection of RF7 by host plants. It is also possible that the correlation stems from an underlying factor that simultaneously affects both C:N and RF7; e.g., differences in flood-related moisture regimes due to terrace height differences among sites, which could simultaneously affect both microbial community structure (*e.g.*, proportion of RF7) and

metabolic activity (*e.g.*, C:N ratio) (Schimel & Chapin 2006). Discriminating among such hypotheses will require a carefully controlled inoculation study.

Compared to early succession, *Frankia* structure in late succession sites was much more heterogeneous: RF diversity was higher in both richness and evenness, and strong patterns were observed in the scale and spatial distribution of variation. A greater proportion of the variation in symbiotic *Frankia* occurred among plants within a site than among sites in the AMOVA for this habitat type, and variation among plants nested within sites was the only significant component in multiple logistic regressions. Distribution of *Frankia* RF types had a strong spatial component within plants in two sites, and among plants in all three sites. Collectively, these results suggest that the factors underlying symbiont structure in these sites act both within plants (strong spatial structure) and among plants within a site (spatial structure and AMOVA/logistic regression results), and that these factors are largely consistent among replicate late succession sites (very little variation at this level in AMOVA/logistic regression). The among-plant vs. among-site pattern is consistent with the pattern of environmental variation in these sites, in which individual plants often occupy distinct microhabitats within a site – e.g., riverbanks at the forest edge, canopy gaps, patches of deciduous overstory, wet depressions from old sloughs, and even patches underlain with permafrost – but similar mixtures of microhabitats occur in different sites. Similar to the situation in early succession sites, among-plant variation in late succession sites does not favor hypothetical mechanisms acting on soil *Frankia* directly over hypotheses invoking plant-mediated mechanisms.

However, several additional observations suggest a more important role for direct effects on soil *Frankia* in these sites. The significant correlations with edaphic factors for several RF types suggest that habitat suitability in soil may be the primary factor determining both within- and among-plant spatial structure of *Frankia* in these sites, rather than resource-neutral or host-related factors such as colonization history and competitive exclusion, which can also create patchy distributions of microbial genets on host root systems (Bruns 1995). It is conceivable that this effect is mediated by plant selection, *i.e.*, that plants in specific microhabitats select symbionts optimal for their physiological requirements from among spatially structured soil *Frankia*. However, in addition to the non-parsimonious nature of this explanation, which posits both spatial structure in soil *Frankia* and a selective mechanism in plants, this seems unlikely for several reasons: 1) the high diversity of RF types within plants runs counter to the idea of one or a few optimal genotypes for a given set of conditions, 2) the wide variability in identity and relative abundance of RF types among even closely occurring plants also suggests a lack of optimal symbiont(s) in a given microhabitat, and 3) the high soil N and low leaf N compared to early succession sites (Anderson *et al.* 2009) suggest that late succession plants may rely less on fixed N than their early succession counterparts, which is also supported by the lower frequency and smaller size of nodules on plant roots in late succession sites (Anderson & Ruess, personal observations). The latter point would undermine both the need for, and the effect of, any plant selection in these sites. Nevertheless, in the absence of experimental data we cannot definitively discount the influence of host plant on structure of nodulating *Frankia* in these sites.

We have previously hypothesized that differences in symbiotic *Frankia* assemblages between our early and late succession habitats may be primarily due to active choice of optimal symbiotic genotypes by *A. tenuifolia* in early succession sites (Anderson *et al.* 2009). In these sites, low soil N content and high plant N demand should place a high premium on symbiotically fixed N, and host choice for optimal symbionts (*e.g.*, those with highest N-fixation rates and/or lowest costs) would be expected to result in low diversity in nodules if only one or a few genotypes meet host criteria. In late succession sites, by contrast, lower N demand due to lower light in the understory, and higher N:P ratio in soils should be expected to lower allocation to nodules overall (Wall 2000), and may result in less stringent host choice (*i.e.*, random sampling of soil *Frankia* by alder). While the results of this study could not differentiate between host-related indirect and soil-related direct effects in early succession sites, our results in late succession sites suggest direct effects on soil assemblages are most important in this habitat, which is consistent with the second part of our hypothesis. Further work is needed to investigate: 1) whether *A. tenuifolia* is capable of selecting optimal symbionts under controlled laboratory conditions, and whether selection varies with environmental conditions, particularly those suggested to be important in field studies, and 2) whether nodule assemblages in early and late succession sites are random or non-random samples of soil assemblages with respect to *Frankia* genetic structure.

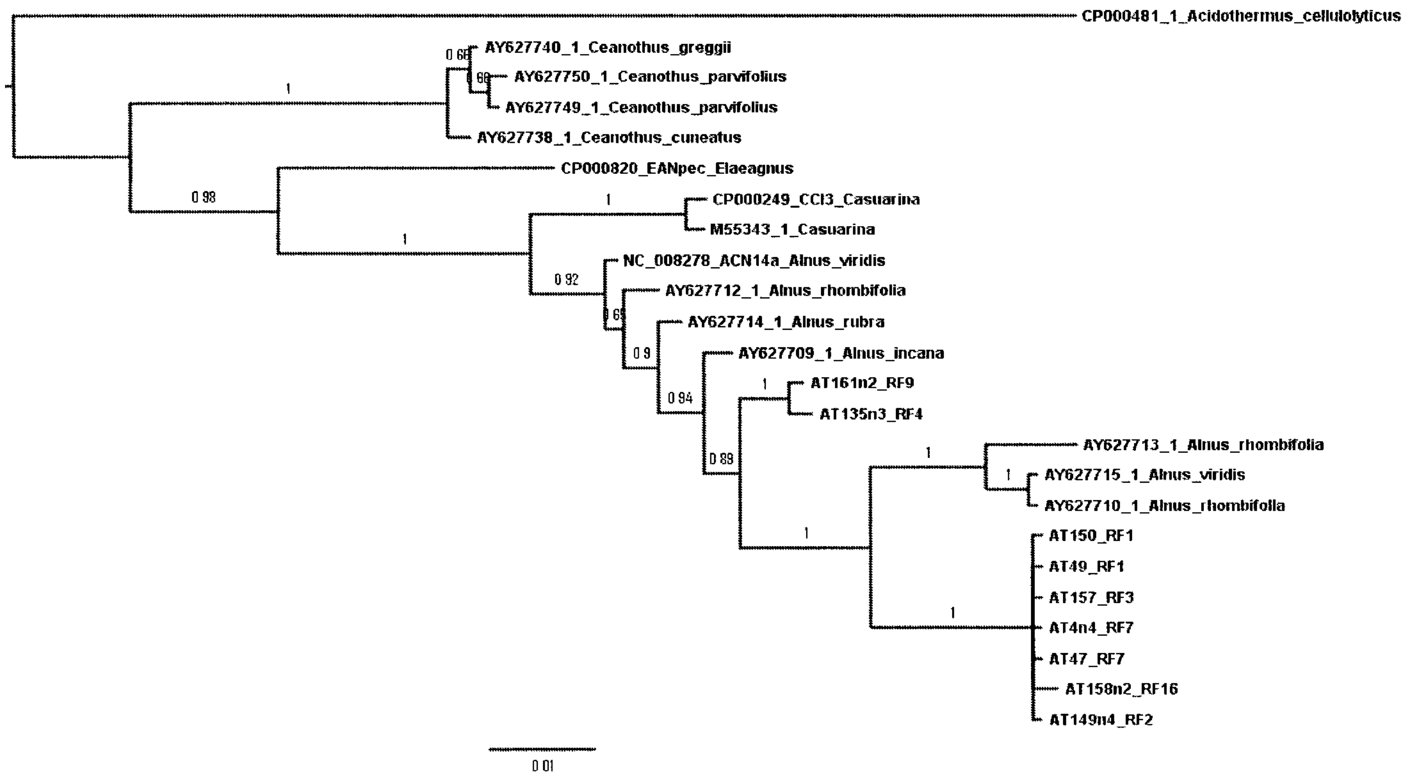

Figure 4.1. *Frankia* phylogeny based on rIGS locus. Bayesian consensus tree based on DNA sequences (1143 characters) of the ribosomal intergenic spacer (rIGS) locus derived from *Alnus tenuifolia* ('AT__') and *A. viridis* ('AC__') nodules collected in early and late succession habitats in the Bonanza Creek Experimental Forest, interior Alaska. 'RF' designations refer to restriction fragment patterns for each sample based on PCR-RFLP of the *nif*D-K locus. Host species and accession numbers for are given in the tree for comparison sequences downloaded from Genbank. Branch labels are Bayesian posterior probabilities.

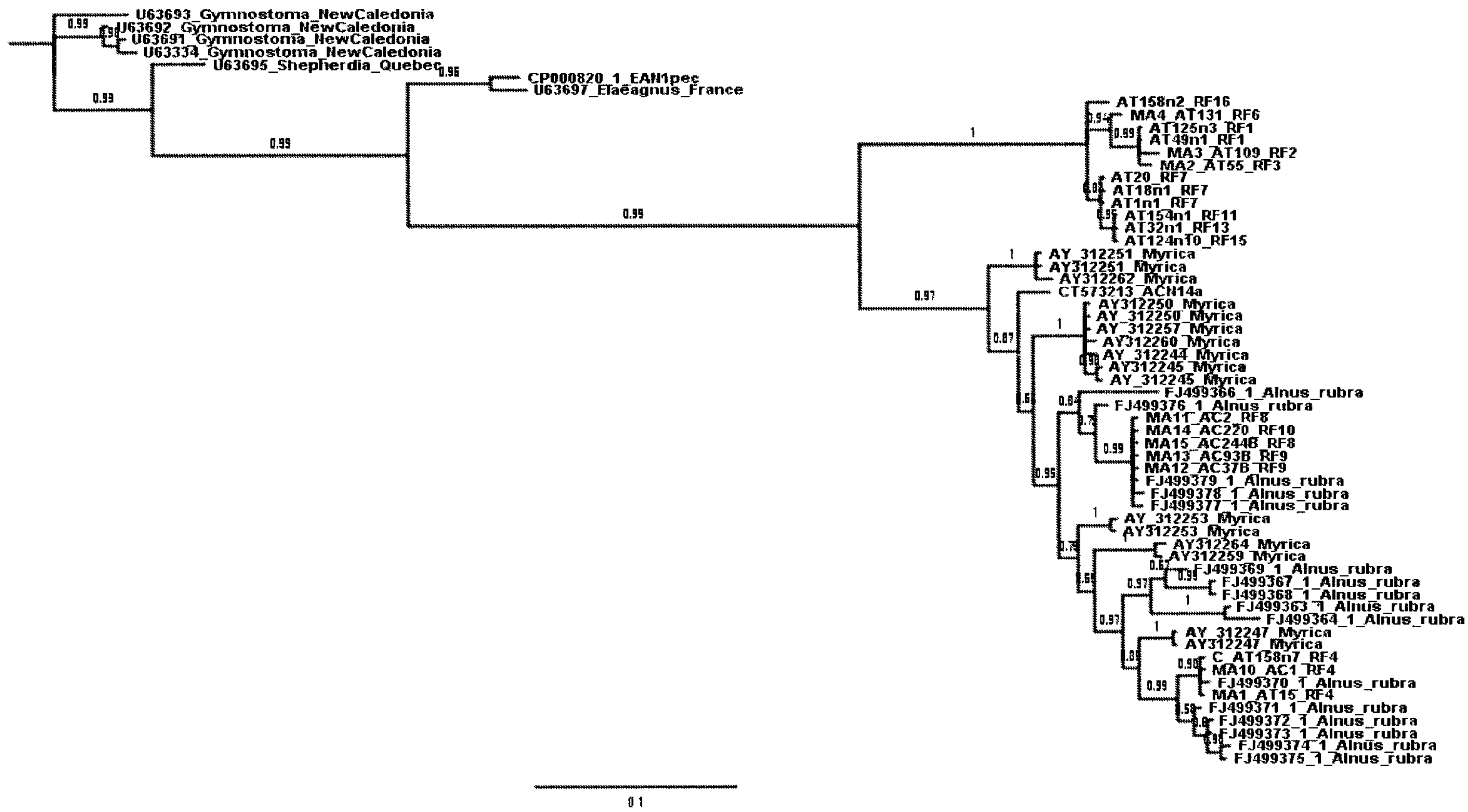

Figure 4.2. *Frankia* phylogeny based on *nif*D-K locus. Bayesian consensus tree based on DNA sequences (757 characters) of the *nif*D-K spacer locus derived from *Alnus tenuifolia* ('AT__') and *A. viridis* ('AC__') nodules collected in early and late succession habitats in the Bonanza Creek Experimental Forest, interior Alaska. 'RF' designations refer to restriction fragment patterns for each sample based on PCR-RFLP of the *nif*D-K locus. Host species and accession numbers for are given in the tree for comparison sequences downloaded from Genbank. Branch labels are Bayesian posterior probabilities.

Table 4.1. Occurrence of *Frankia* in *A. tenuifolia* nodules. Number of nodules collected from *Alnus tenuifolia* plants that were found to contain each genotype ('RF'= *nif*D-K RFLP pattern) of *Frankia* in replicate sites representing early and late successional habitats in the Bonanza Creek Experimental Forest, interior Alaska.

STAGE	SITE	RF1	RF2	RF3	RF4	RF6	RF7	RF8	RF9	RF11	RF12	RF13	RF14	RF15	RF16
EARLY	FPE1	6	5	7	0	1	35	0	0	0	0	0	0	0	0
	FPE2	4	0	0	1	3	47	0	0	0	0	1	0	0	1
	FPE3	2	1	0	0	0	58	0	0	0	0	0	0	0	0
LATE	FPL1	7	2	3	9	3	7	2	3	3	1	0	4	1	11
	FPL2	13	12	3	1	6	1	0	0	5	0	0	0	0	13
	FPL3	12	8	2	9	3	0	0	1	1	0	0	0	0	24

Table 4.2. Study site characteristics. *Alnus tenuifolia* density (stems/ha) and means (± 1 standard error) of soil variables and first nearest-neighbor distances between sampled *A. tenuifolia* plants in replicate sites representing early and late successional habitats in the Bonanza Creek Experimental Forest, interior Alaska. Total C, N and P values represent percent by mass.

STAGE	SITE	Total C		Total N		Total P		pH	
		Min	Org	Min	Org	Min	Org	Min	Org
Early	FPE1	1.4±0.05	12.3±0.7	0.10±0.003	0.71±0.04	0.08±0.001	0.09±0.002	8.3±0.03	6.9±0.06
	FPE2	1.4±0.05	8.8±0.3	0.09±0.004	0.56±0.02	0.07±0.001	0.08±0.00`	8.2±0.02	7.5±0.06
	FPE3	1.2±0.03	16.1±0.6	0.07±0.002	0.98±0.03	0.08±0.001	0.09±0.001	8.2±0.04	7.9±0.02
Late	FPL1	1.5±0.14	23.1±2.8	0.09±0.006	0.91±0.10	0.07±0.001	0.08±0.001	7.4±0.23	5.7±0.05
	FPL2	5.5±0.47	30.7±1.1	0.23±0.014	1.08±0.03	0.07±0.001	0.08±0.003	5.7±0.16	5.6±0.08
	FPL3	2.1±0.22	21.6±	0.11±0.010	0.83±0.03	0.06±0.001	0.07±0.002	6.1±0.18	5.2±0.04

C:N		N:P		C:P		Plant Density	1st NN
Min	Org	Min	Org	Min	Org		
14.6±0.2	17.4±0.3	1.3±0.04	8.2±0.3	18.6±0.6	142±6	2827	8.8±3.2
16.6±0.2	15.7±0.1	1.2±0.05	6.8±0.2	20.4±0.8	107±4	4853	9.9±2.7
18.2±0.2	16.4±0.2	0.8±0.04	10.4±0.4	14.8±0.5	167±7	2015	8.4±3.9
17.1±0.5	25.3±0.3	1.2±0.09	11.8±1.4	20.8±2.1	299±37	132	12.0±3.5
23.7±0.8	28.4±0.5	3.0±0.19	13.0±0.7	73.9±6.2	351±19	537	9.9±3.5
18.1±0.4	26.1±0.3	1.8±0.18	11.9±0.5	34.2±4.0	308±12	56	11.3±10.6

Table 4.3. Spatial structure of *Frankia* genotypes. Results of Mantel correlogram analysis of the distribution of *Frankia nif*D-K restriction fragment (RF) patterns in *Alnus tenuifolia* nodules collected in three late succession sites in the Bonanza Creek Experimental Forest in interior Alaska. Separate analyses were performed for all RF types and for the most frequent RF types observed in each site. Numbers are correlation coefficients from Mantel tests for each distance class between a geographic distance matrix and a binary distance matrix indicating shared RF types. All distance classes were designated to contain an equal number of pair-wise distances except the first, which was set to 0-1 m to encompass nodules collected within the same plant. Negative coefficients indicate positive autocorrelation. Asterisks indicate Bonferroni adjusted P-values from permutation tests (999 permutations) for each Mantel test as follows: **P<0.01, *P<0.05.

	SITE												
	FPL1						FPL2						
Distance Class	Range (m)	ALL RFs	RF1	RF4	RF7	RF16	Range (m)	ALL RFs	RF1	RF2	RF6	RF11	RF16
1	0-1	-0.25**	-0.18**	-0.17*	-0.12*		0 -1						-0.17*
2	1-15						1 - 13						
3	15-19						13 - 24	-0.09**					-0.14*
4	19-30						24 - 31				-0.09*		
5	30-50	-0.06*				-0.12*	31 - 39						
6							39 - 52						
7							52 - 58						
8							58 - 75						
9							75 - 84						
10							84 - 94						

Table 4.3, cont'd.

SITE					
FPL3					
Range (m)	ALL RFs	RF1	RF2	RF4	RF16
0 - 1	-0.22**		-0.17*		
1 - 19					
19 - 27					
27 - 46					
46 - 56					
56 - 63	-0.07*				-0.09**
63 - 72					
72 - 77					
77 -91					
91 - 93					

Table 4.4. Correlations between *Frankia* genotypes and soil variables. Summary of multiple logistic regressions examining the response of *Frankia* genotype (*nif*D-K restriction fragment (RF) pattern) in *Alnus tenuifolia* nodules to variation in several measured soil variables in mineral and organic horizons from early (A) and late (B) succession habitats (n=3 sites each). Separate models were examined for each habitat and for all RF types as well as the most common individual RF types in each habitat. P-values for individual RF types are unadjusted, but significance after Bonferroni adjustment is indicated with an asterisk. 'Dir' indicates the direction of the observed correlation between individual RF type frequency and the indicated soil variable ('pos'=positive, 'neg'=negative). Late succession data yielded two equally good models.

A)

EARLY SUCCESSION	ALL RF TYPES					RF1		RF3		RF7	
	-logL	r^2	χ^2	df	P	P	Dir	P	Dir	P	Dir
whole model	31.5	0.52	62.9	20	<0.0001	0.014*		0.0003*		<0.0001*	
mineral N			13.1	5	0.022	ns		0.023	pos	ns	
mineral C			13.7	5	0.018	ns		0.022	pos	ns	
mineral pH			11.7	5	0.038	ns		0.003*	pos	ns	
mineral C:N			15.0	5	0.010	0.014*	neg	0.013*	neg	<0.0001*	pos

Table 4.4. B)

LATE SUCCESSION	ALL RF TYPES					RF1		RF2		RF3		RF4		RF6		RF7		RF16	
	-logL	r^2	χ^2	df	P	P	Dir	P	Dir	P	Dir	P	Dir	P	Dir	P	Dir	P	Dir
MODEL 1																			
whole model	35.8	0.38	71.6	27	<0.0001	ns		0.043		0.012		0.0059*		0.0009*		0.05		0.001*	
organic N			22.3	9	0.008	ns		0.043	pos	ns		ns				0.007*	neg	ns	
organic C			18.1	9	0.034	ns		ns		0.021	pos	ns				0.008*	neg	ns	
mineral pH			21.4	9	0.011	ns		0.026	pos	ns		ns						0.047	neg
organic pH			ns					ns		ns		ns		0.001*	pos			0.007*	neg
MODEL 2	-logL	r^2	χ^2	df	P	P	Dir	P	Dir	P	Dir	P	Dir	P	Dir	P	Dir	P	Dir
whole model	35.8	0.38	71.6	27	<0.0001														
organic N			19.9	9	0.019														
organic C			19.9	9	0.019														
organic C:N			21.4	9	0.011	ns		ns		0.031	neg	0.0059*	neg	0.003*	pos	0.01	pos		

REFERENCES

Anderson MD (2011) Two in the far north: the alder-*Frankia* symbiosis, with an Alaskan case study. In: *Ecological Aspects of Nitrogen Metabolism in Plants* (eds Polacco JC, Todd CD). pp. 138-166, Wiley & Sons, West Sussex UK.

Anderson MD, Ruess RW, Myrold DD, Taylor DL (2009) Host species and habitat affect nodulation by specific *Frankia* genotypes in two species of *Alnus* in interior Alaska. *Oecologia,* **160**, 619-630.

Béna G, Lyet A, Huguet T, Olivieri I (2005) *Medicago-Sinorhizobium* symbiotic specificity evolution and the geographic expansion of *Medicago*. *Journal of Evolutionary Biology,* **18**, 1547-1558.

Bousquet J, Lalonde M (1990) The genetics of actinorhizal Betulaceae. In *The Biology of* Frankia *and Actinorhizal Plants* (eds. Schwintzer CR, Tjepkema JD). pp. 239-261, Academic Press, Inc., San Diego, CA.

Bruns TD (1995) Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant and Soil,* **170**, 63-73.

Burdon JJ, Gibson AH, Searle SD, Woods MJ, Brockwell J (1999) Variation in effectiveness of symbiotic associations between native rhizobia and temperate Australian *Acacia*: within-species interactions. *Journal of Applied Ecology,* **36**, 398-408.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, **17**, 540-552.

Chaia EE, Wall LG, Huss-Danell K (2010) Life in soil by the actinorhizal root nodule endophyte *Frankia*: a review. *Symbiosis,* **51**, 201-226.

Chao A (1984) Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics,* **11**, 265-270.

Chapin III FS, Viereck LA, Adams PC, Van Cleve K, Fastie CL, Ott RA, Mann D, Johnstone JF (2006) Successional processes in the Alaskan boreal forest. In *Alaska's Changing Boreal Forest.* (eds. F.S. Chapin III, M.W. Oswood, K. Van Cleve, L.A. Viereck, D.L. Verbyla) Oxford University Press, New York.

Clawson ML, Bourret A, Benson DR (2004) Assessing the phylogeny of *Frankia*-actinorhizal plant nitrogen-fixing root nodule symbioses with *Frankia* 16S rRNA and glutamine synthetase gene sequences. *Molecular Phylogenetics and Evolution,* **31**, 131-138.

Colwell RK (2009) EstimateS: Statistical estimation of species richness and shared species from samples. Version 8.2.

Colwell RK, Mao CX, Chang J (2004) Interpolating, extrapolating, and comparing incidence-based species accumulation curves. *Ecology,* **85**, 2717-2727.

Dawson JO (2008) Ecology of actinorhizal plants. In: *Nitrogen Fixation: Origin, Applications, and Research Progress, Vol. 6 Nitrogen-Fixing Actinorhizal Symbioses* (eds Pawlowski K, Newton WE) Springer, Dordrecht, The Netherlands.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47-50.

Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479-491.

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Computer Applications in the Biosciences,* **12**, 543-548.

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. *Plant Physiology*, **131**, 872-877.

Hahn D (2008) Polyphasic taxonomy of the genus *Frankia*. In *Nitrogen Fixation: Origins, Applications, and Research Progress,* Vol. 6 *Nitrogen-fixing Actinorhizal Symbioses*. (eds. K. Pawlowski & W.E. Newton) Springer, Dordrecht, The Netherlands.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

Heath KD (2010) Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. *Evolution,* **64**, 1446-1458.

Heath KD, Lau JA (2011) Herbivores alter the fitness benefits of a plant-rhizobium mutualism. *Acta Oecologica,* **37**, 87-92.

Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial mutualists. *Proceedings of the Royal Society B,* **274**, 1905-1912.

Heath KD, Tiffin P (2009) Stabilizing mechanisms in a legume-rhizobium mutualism. *Evolution,* **63**, 652-662.

Heath KD, Stock AJ, Stinchcombe JR (2010) Mutualism variation in the nodulation response to nitrate. *Journal of Evolutionary Biology,* **23**, 2494-2500.

Hollingsworth TN, Lloyd AH, Nossov D, Ruess RW, Charlton BA, Kielland K (2010) Twenty-five years of vegetation change along a putative successional chronosequence on the Tanana River, Alaska. *Canadian Journal of Forest Research,* **40**, 1273-1287.

Jackson ML (1958) Soil *Chemical Analysis*. Prentice-Hall, Englewood Cliffs, NJ.

Kennedy PG, Weber MG, Bluhm AA (2010) *Frankia* bacteria in *Alnus rubra* forests: genetic diversity and determinants of assemblage structure. *Plant and Soil,* **335**, 479-492.

Khan A, Myrold DD, Misra AK (2007) Distribution of *Frankia* genotypes occupying *Alnus nepalensis* nodules with respect to altitude and soil characteristics in the Sikkim Himalayas. *Physiologia Plantarum,* **130**, 364-371.

Kiers ET, Denison RF (2008) Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual Review of Ecology, Evolution, and Systematics,* **39**, 215-236.

Kiers ET, Rousseau RA, Denison RF (2006) Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly. *Evolutionary Ecology Research,* **8**, 1077-1086.

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. *Nature,* **425**, 78-81.

Kimura M (1981) Estimation of evolutionary distances between homologous nucleotide sequences. *Proceedings of the National Academy of Sciences USA*, **78**, 454-458.

Larson JL, Siemann E (1998) Legumes may be symbiont-limited during old-field succession. *American Midland Naturalist,* **140**, 90-95.

Legendre P, Fortin M-J (1989) Spatial pattern and ecological analysis. *Vegetatio,* **80**, 107-138.

Lipus A, Kennedy PG (2011) *Frankia* assemblages associated with *Alnus rubra* and *Alnus viridis* are strongly influenced by host species identity. *International Journal of Plant Sciences,* **172**, 403-410.

McInnes A, Thies JE, Abbott LK, Howieson JG (2004) Structure and diversity among rhizobial strains, populations and communities – a review. *Soil Biology and Biochemistry,* **36**, 1295-1308.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research,* **27**, 209-220.

Marco DE, Carbajal JP, Cannas S, Pérez-Arnedo R, Hidalgo-Perea A, Olivares J, Ruiz-Sainz J, Sanjuán J (2009) An experimental and modelling exploration of the host-sanctions hypothesis in legume-rhizobia mutualism. *Journal of Theoretical Biology,* **259**, 423-433.

Markham JH (2008) Variability of nitrogen-fixing *Frankia* on *Alnus* species. *Botany,* **86**, 501-510.

Miller MA, Pfeiffer W, Schwartz, T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *Proceedings of the Gateway Computing Environments Workshop (GCE),* 14 Nov. 2010, New Orleans, LA pp. 1-8.

Mirza BS, Welsh A, Rasul G, Rieder JP, Paschke MW, Hahn D (2009) Variation in *Frankia* populations of the *Elaeagnus* host infection group in nodules of six host plant species after inoculation with soil. *Microbial Ecology,* **58**, 384-393.

Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H (1986) Specific enzyme amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symposium in Quantitative Biology,* **51**, 263-273.

Navarro E, Jaffre T., Gauthier D, Gourbiere F, Rinaudo G, Simonet P, Normand P (1999) Distribution of *Gymnostoma* spp. microsymbiotic *Frankia* strains in New Caledonia is related to soil type and to host-plant species. *Molecular Ecology,* **8**, 1781-1788.

Nossov DR, Hollingsworth TN, Ruess RW, Kielland K (2011) Development of *Alnus tenuifolia* stands on an Alaskan floodplain: patterns of recruitment, disease and succession. *Journal of Ecology,* **99**, 621-633.

Parker MA (1995) Plant fitness variation caused by different mutualist genotypes. *Ecology,* **76**, 1525-1535.

Parker MA, Spoerke JM (1998) Geographic structure of lineage associations in a plant-bacterial mutualism. *Journal of Evolutionary Biology,* **11**, 549-562.

Parker MA, Malek W, Parker IM (2006) Growth of an invasive legume is symbiont limited in newly occupied habitats. *Diversity and Distributions,* **12**, 563-571.

Pokharel A, Mirza BS, Dawson JO, Hahn D (2010) *Frankia* populations in soil and root nodules of sympatrically grown *Alnus* taxa. *Microbial Ecology,* **61**, 92-100.

Posada D (2003) Using Modeltest and PAUP* to select a model of nucleotide substitution. In *Current Protocols in Bioinformatics* (eds. Baxevanis AD, Davison DB, Page RDM, Petsko GA, Stein LD, Stormo GD) John Wiley & Sons, New York.

Posada D (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253-1256.

Reed SC, Cleveland CC, Townsend AR (2011) Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annual Review of Ecology, Evolution, and Systematics*, **42**, 489-512.

Rosenberg MS, Anderson CD (2011) PASSaGE: pattern analysis, spatial statistics and geographic exegesis. Version 2. *Methods in Ecology and Evolution*, **2**, 229-232.

Sawada H, Kuykendall LD, Young JM (2003) Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. *Journal of General and Applied Microbiology,* **49**, 155-179.

Schimel JP, Chapin III FS (2006) Microbial processes in the Alaskan boreal forest. In: *Alaska's Changing Boreal Forest* (eds Chapin III FS, Oswood MW, Van Cleve K Viereck LA, Verbyla DL). pp. 227-240. Oxford University Press, NY.

Simms EL, Taylor DL (2002) Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and Comparative Biology,* **42**, 369-380.

Simms EL, Taylor DL, Povich J, Shefferson RP, Sachs JL, Urbina M, Tausczic Y (2006) An empirical test of partner-choice mechanisms in a wild legume-rhizobium interaction. *Proceedings of the Royal Society B,* **273**, 77-81.

Simonet P, Navarro E, Rouvier C, Reddell P, Zimpfer J, Dommergues Y, Bardin R, Combarro P, Hamelin J, Domenach A-M, Gourbiére F, Prin Y, Dawson JO, Normand P (1999) Coevolution between *Frankia* population and host plants in the family Casuarinaceae and consequent patterns of global dispersal. *Environmental Microbiology,* **1**, 525-533.

Spoerke JM, Wilkinson HH, Parker MA (1996) Nonrandom genotypic associations in a legume-*Bradyrhizobium* mutualism. *Evolution,* **50**, 146-154.

Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models., *Bioinformatics*, **22**, 2688–2690.

Swensen SM, Benson DR (2008) Evolution of actinorhizal host plants and *Frankia* endosymbionts. In:*Nitrogen Fixation: Origin, Applications, and Research Progress, Vol. 6 Nitrogen-Fixing Actinorhizal Symbioses* (eds Pawlowski K, Newton WE) Springer, Dordrecht, The Netherlands.

Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. In *Some Mathematical Questions in Biology – DNA Sequence Analysis* (ed. M. Miura) American Mathematical Society, Providence, RI.

Thrall PH, Burdon JJ, Woods MJ (2000) Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian legumes: interactions within and between genera. *Journal of Applied Ecology,* **37**, 52-65.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research,* **25**, 4876-4882.

Thompson JN (1994) *The Coevolutionary Process.* University of Chicago Press, Chicago, Il.

Thompson JN (2005) *The Geographic Mosaic of Coevolution.* University of Chicago Press, Chicago Il.

Wall LG (2000) The actinorhizal symbiosis. *Journal of Plant Growth Regulation,* **19**, 167–182.

Wang H, Man CX, Wang ET, Chen WX (2009) Diversity of rhizobia and interactions among the host legumes and rhizobial genotypes in an agricultural-forestry ecosystem. *Plant and Soil,* **314**, 169-182.

Welsh A, Mirza BS, Reider JP, Paschke MW, Hahn D (2009a) Diversity of frankiae in root nodules of *Morella pensylvanica* grown in soils from five continents. *Systematic and Applied Microbiology,* **32**, 201-210.

Welsh AK, Dawson JO, Gottfried GJ, Hahn D (2009b) Diversity of *Frankia* populations in root nodules of geographically isolated Arizona alder trees in central Arizona (United States). *Applied and Environmental Microbiology,* **75**, 6913-6918.

Wheeler CT, Miller IM (1990) Current and poterntial uses of actinorhizal plants in Europe. In *The Biology of* Frankia *and Actinorhizal Plants*. (eds. C.R. Schwintzer & J.D. Tjepkema) Academic Press, San Diego, CA.

Wilkinson HH, Parker MA (1996) Symbiotic specialization and the potential for genotypic coexistence in a plant-bacterial mutualism. *Oecologia,* **108**, 361-367.

Wilkinson HH, Spoerke JM, Parker MA (1996) Divergence in symbiotic compatibility in a legume-*Bradyrhizobium* mutualism. *Evolution,* **50**, 1470-1477.

Yamakura T, Sahunalu P (1990) Soil carbon/nitrogen ratio as a site quality index for some southeast Asian forests. *Journal of Tropical Ecology,* **6**, 371-377.

Young JPW, Johnston AWB (1989) The evolution of specificity in the legume-rhizobium symbiosis. *Trends in Ecology and Evolution*, **4**, 341-349.

Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD Dissertation, University of Texas, Austin.

CONCLUSIONS

The aim of this thesis was to investigate sources of variation in distribution and *in situ* functioning of symbiotic associations in the *Alnus-Frankia* system, and determine whether such patterns are consistent with host-choice by alder. The results of this study indicate: 1) the acetylene reduction assay (ARA) is not an appropriate tool for quantitative comparisons of N-fixation rates in our study system, 2) the host species studied differ widely in genetic structure of *Frankia* assemblages *in situ*, 3) *Frankia* structure can be strongly affected by habitat, but this effect differs between the two host species, 4) differences in N-fixation among *Frankia* genotypes were suggested, but were small compared to variation within genotypes.

Comparison of the ARA with measurements of ^{15}N uptake indicated several shortcomings of the ARA for estimating N-fixation rates in the system studied. The ratio between ARA and N_2 fixation rate based on ^{15}N uptake is often used to estimate values of the latter from the former, which implicitly assumes a stable ratio. To the contrary, this ratio varied over three orders of magnitude among individual measurements in this study, and varied systematically with site/season, which may have been related to high nitrogenase activity. Further, in sites with high ^{15}N uptake rates the values yielded by the two assays were uncorrelated. Finally, a further assumption implicit in use of the ARA for quantitative purposes – that acetylene inhibits N-fixation by nitrogenase at concentrations of 10-20% – also was not supported. Thus, the ARA does not appear to be a reliable estimator of N-fixation activity in this study system.

Host specificity was an important component of *Frankia* distribution in this study. *A. tenuifolia* and *A. viridis* supported very different assemblages of *Frankia*, even in sites where the two hosts co-occurred. Most *A. tenuifolia*-associated *Frankia* appear to belong to a single clade that is relatively diverse and far removed from other described *Frankia*, while *A. viridis* associates were less varied and much more similar to previously described strains. The occurrence on each host of genotypes dominant on the other host, albeit at very low frequency, suggests that host specificity is not absolute, consistent with the possibility of host choice.

Environmental variation showed the potential to affect *Frankia* structure, but this effect differed for the two hosts. In *A. viridis* very little variation was evident across a secondary successional sere, or in late succession in a primary sere. By contrast, *Frankia* structure in *A. tenuifolia* nodules differed strongly between early and late primary successional habitats, and was consistent among replicate sites representing each habitat. These habitat-based differences were paralleled by differences in host leaf N content which, together with differences in total soil N between habitats, suggests the highest N demand in *A. tenuifolia* plants occupying sites with the lowest N availability. Consistent with the hypothesis that host choice is more stringent in early succession as a result of greater reliance on fixed N, nodules in early succession sites were dominated by a single genotype of *Frankia*, while late succession sites supported higher overall symbiont diversity within and among host plants, displayed wider variation among plants in each site, and also displayed spatial structure of genotypes within and among host plants that was correlated with soil chemistry. Contrary to expectations under the host-choice

hypothesis, however, little evidence was observed to suggest physiological differences among *Frankia* genotypes, at least in specific N-fixation rates.

Overall, the results of the field studies are consistent with the hypothesis that host choice occurs in alder and is an important component of the alder-*Frankia* interaction in natural settings. However, each supporting observation is also consistent with several alternative hypotheses. For instance, low frequency of symbiont genotypes on the less common host associate may be due to errors in physiological barrier mechanisms rather than selection of optimal bacterial symbionts, and dominance of a single *Frankia* genotype in early succession sites may reflect higher abundance in soil rather than non-random associations with host plants. Further experiments are needed at this point to determine whether: 1) host plants can select specific *Frankia* genotypes under laboratory conditions, and whether the selected genotypes enhance plant performance, 2) altering edaphic factors such as N and P in field sites changes *Frankia* composition in host nodules, 3) *Frankia* composition in field nodules is non-random compared with composition in soil, and whether this differs between successional stages, and 4) *Frankia* genotypes in the field differ in other potentially relevant parameters such as cost of N-fixation.