Molecular phylogeny of the actinorhizal Hamamelidae and relationships with host promiscuity towards *Frankia*

L. MAGGIA and J. BOUSQUET
Centre de recherche en biologie forestière, Faculté de foresterie et de géomatique, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4

Abstract

Several of the most studied actinorhizal symbioses involve associations between host plants in the subclass Hamamelidae of the dicots and actinomycetes of the genus *Frankia*. These actinorhizal plants comprise eight genera distributed among three families of 'higher' Hamamelidae, the Betulaceae, Myricaceae, and Casuarinaceae. Contrasting promiscuity towards *Frankia* is encountered among the different actinorhizal members of these families, and a better assessment of the evolutionary history of these actinorhizal taxa could help to understand the observed contrasts and their implications for the ecology and evolution of the actinorhizal symbiosis. Complete DNA sequences of the chloroplast gene coding for the large subunit of ribulose 1,5-bisphosphate carboxylase (*rbcL*) were obtained from taxa representative of these families and the Fagaceae. The phylogenetic relationships among and within these families were estimated using parsimony and distance-matrix approaches. All families appeared monophyletic. The Myricaceae appeared to derive first before the Betulaceae and the Casuarinaceae. In the Casuarinaceae, the genus *Gymnostoma* derived before the genera *Casuarina* and *Allocasuarina*, which were found closely related. The analysis of character-state changes in promiscuity along the consensus tree topology suggested a strong relationship between the evolutionary history of host plants and their promiscuity toward *Frankia*. Indeed, the actinorhizal taxa that diverged more recently in this group of plants were shown to be susceptible to a narrower spectrum of *Frankia* strains. The results also suggest that the ancestor of this group of plant was highly promiscuous, and that evolution has proceeded toward narrower promiscuity and greater specialization. These results imply that a tight relationship between the phylogenies of both symbiotic partners should not be expected, and that host promiscuity is likely to be a key determinant in the establishment of an effective symbiosis.

Keywords: actinorhizal plants, *Frankia*, Hamamelidae, molecular phylogeny, *rbcL*, symbiosis evolution

Received 11 November 1993; revision received 14 February 1994; accepted 18 February 1994

Introduction

The actinorhizal symbiosis derives from an association between the filamentous soil bacteria *Frankia* (Actinomycetales) and an array of host woody dicots plants belonging to more than 20 genera in eight families (Bousquet & Lalonde 1990). The fixation of nitrogen resulting from this symbiosis is central to the dynamics of several ecosystems, many of the host plants being main components of early-successional communities established on poor and marginal sites (Benson & Silvester 1993). When considered all together, there is no close taxonomic affinity among the different actinorhizal plant families (Bousquet & Lalonde 1990). However, actinorhizal genera of the families Casuarinaceae, Myricaceae, and the single actinorhizal genus *Alnus* of the Betulaceae all involve hyphal penetration of deformed root hairs by *Frankia* followed by prenodule development and nodule lobe formation (Callaham *et al.* 1979, Benson & Silvester 1993). These genera are also classified in the same coherent group of 'higher' Hamamelidae (Cronquist 1981,
Various actinorhizal Hamamelidae taxa have been shown to differ drastically in their susceptibility to Frankia. The term ‘promiscuity’ has been coined to describe the plant’s tolerance level to a range of genetically diverse Frankia strains (Baker 1987; Torrey & Racette 1989). For instance, within the Casuarinaceae, Gymnostoma has been shown to be susceptible to a wider array of Frankia strains (hence being more promiscuous) than Allocasuarina or Casuarina (Torrey & Racette 1989). Myrica, the largest genus of Myricaceae, has also been shown to be more promiscuous than either Alnus or Casuarina taxa (Baker 1987; Torrey & Racette 1989). Without a sound phylogenetic framework, it remains difficult to understand the ecological and evolutionary implications of these differences, which are central to a proper understanding of this symbiosis and its optimal utilization in field.

Recently, the chloroplast gene coding for the large subunit of the enzyme ribulose 1,5-bisphosphate carboxylase (rbcL) has been used to estimate the phylogeny of the family Betulaceae, and the results obtained were in complete agreement with the phylogeny estimated from morphological data (Bousquet et al. 1992b) and ribosomal DNA internal transcribed spacer sequences (Savard et al. 1993). rbcL gene sequences have also been used fruitfully to estimate phylogenetic relationships in various other groups of dicots (Soltis et al. 1990, 1993; Bousquet et al. 1992; Giannasi et al. 1992; Chase et al. 1993). Using rbcL gene sequences, the purpose of this study was to clarify the phylogenetic relationships among actinorhizal families of ‘higher’ Hamamelidae in order to help understand the ecological and evolutionary implications of promiscuity differences observed among these taxa.

### Materials and methods

#### Plant materials

In addition to rbcL gene sequences already published (Table 1), the complete nucleotide sequence of the rbcL ORF was determined for five taxa: Allocasuarina verticillata (EMBL no. X69527), Casuarina cunninghamiana (EMBL no. X69528), Gymnostoma webbianum (EMBL no. X69531), Myrica gale (EMBL no. X69530), and Comptonia peregrina (EMBL no. X69529). Seeds from A. verticillata, C. cunninghamiana, and G. webbianum were germinated. After one month, a single branchlet from one individual of each species was used for DNA extraction. Lyophilized leaf tissues from M. gale and fresh leaves from C. peregrina were also used for DNA extraction. In all cases, DNA extractions were performed using a CTAB procedure (Bousquet et al. 1990).

#### DNA amplification and sequencing of rbcL

rbcL was amplified symmetrically following previously published procedures (Bousquet et al. 1992b). The primers used, including those upstream and downstream of rbcL, are described elsewhere (Frascaria et al. 1993). For each of the two DNA strands, a second asymmetrical amplification was conducted with a primer ratio of 1:50 where the primer in excess was at a concentration of 50 μM and the limited primer at 1 μM. The asymmetrically amplified fragments were purified by ultrafiltration with Centricon-30 (Amicon). Direct sequencing of the two DNA strands was performed by the dideoxy-nucleotide chain-termination procedure using the Sequenase Version 2.0 Kit (USB). Sequencing was conducted using 7% polyacrylamide gels according to the manufacturer’s recommendations (LR). X-ray films were exposed for 24 h to several days.

| Species                  | Family            | Order | Dicot subclass      | References                      |
|--------------------------|-------------------|-------|---------------------|---------------------------------|
| Allocasuarina verticillata* | Casuarinaceae     | Casuarinales | Hamamelidae (higher) | this paper                     |
| Casuarina cunninghamiana* | Casuarinaceae     | Casuarinales | Hamamelidae (higher) | this paper                     |
| Gymnostoma webbianum*    | Myricaceae        | Myricales | Hamamelidae (higher) | this paper                     |
| Myrica gale*             | Myricaceae        | Myricales | Hamamelidae (higher) | this paper                     |
| Comptonia peregrina*     | Myricaceae        | Myricales | Hamamelidae (higher) | this paper                     |
| Liquidambar styraciflua  | Hamamelidaceae    | Hamamelidae | Hamamelidae (lower) | this paper                     |
| Magnolia macrophylla     | Magnoliaceae      | Magnoliaceae | Magnoliidae         | Golenberg et al. (1990)        |

* Families and species are indicated with an asterisk.
**Phylogenetic analysis of rbcL sequences**

Pairwise synonymous ($K_s$) and nonsynonymous ($K_a$) numbers of nucleotide substitutions corrected for multiple hits, and their standard errors, were calculated according to the two-parameter method of Li et al. (1985) modified by Li (1993). This method takes into account transition and transversion rates. Overall numbers of substitutions ($K_o$) were calculated as a weighted average of $K_s$ and $K_a$. Substitution rates were also estimated using the one-parameter method of Jukes and Cantor (1969). One-parameter and $K_o$ pairwise substitution rates were analysed with the neighbour-joining method of phylogenetic tree construction (Saitou & Nei 1987). In addition, parsimony analysis of nucleotide sequences were conducted using the Branch and Bound algorithm of PAUP 3.1 (Swofford 1993). Bootstrap confidence intervals (Felsenstein 1985) were calculated from 1000 replications for both types of analysis, parsimony (with PAUP), as well as for neighbour-joining using Jukes and Cantor’s substitution rates (software NJBOOT2 from T. S. Whittam and M. Nei, Institute of Molecular Evolutionary Genetics, Pennsylvania State University).

**Evolutionary study of promiscuity differences**

Information regarding the promiscuity of actinorhizal Hamamelidaceae genera towards *Frankia* were regrouped in Table 2. Height groups of *Frankia* strains were delineated on the basis of their host genus of origin. Only the *Frankia* strains shown to reinfest their host of origin were considered. A host genus was considered nodulated by a particular *Frankia* strain if one nodulation was induced on at least one species within the genus. The genus *Comptonia* of Myricaceae was not considered because of insufficient data available on promiscuity. From Table 2, a cladistic character-state matrix was constructed where for each actinorhizal genus analysed, promiscuity towards each group of *Frankia* strains was scored as positive (coded 1, all strains tested led to infection), or negative (coded 0, no infection), or polymorphic (coded 1/0, some strains tested led to infection). For each group of *Frankia* strains (the cladistic characters), character-state changes were mapped on the consensus phylogenetic tree derived from the analysis of rbcL sequences of actinorhizal Hamamelidaceae taxa, using MACCLADE 3.1 (Maddison & Maddison 1992). Changes were assumed irreversible and two different analyses were performed, whether promiscuity toward each group of *Frankia* strains was assumed derived or ancestral. Total number of steps and consistency index were monitored for each scenario.

**Results**

**rbcL sequences**

The rbcL ORF sequence determined here for the three species of Casuarinaceae and the two species of Myricaceae was 1428 bp long. The degree of DNA homology was greater than 98% within families of ‘higher’ Hamamelidaceae (Table 3). Between the families, the DNA homology was about 97%, whether the families belonged to the same order or to different orders. The synonymous rate was 10–20 times larger than the nonsynonymous rate, except for the Myricaceae (Table 3). Within ‘higher’ Hamamelidaceae, Jukes and Cantor’s one-parameter rates were calculated as weighted averages of $K_s$ and $K_a$ (Table 3).

**Estimated phylogenies**

Using two outgroups, *Magnolia macrophylla* and *Liquidambar styraciflua*, standard parsimony led to one most parsimonious tree with a total length of 273 steps (Fig. 1A). Of these, 153 were accounted for by the internal network linking the ‘higher’ Hamamelidaceae taxa. The consistency index (CI) excluding uninformative characters was 0.778 when considering the most parsimonious network linking the ‘higher’ Hamamelidaceae taxa. The topology obtained from neighbour-joining analysis of one-parameter substitution rates (Fig. 1B) was identical to the one obtained using two-parameter overall numbers of

| Groups        | $n$ | Homology | $K_s$   | $K_a$   | $K_o$   | J.C.     |
|---------------|-----|----------|---------|---------|---------|----------|
| Myricaceae    | 1   | 99.6     | 0.009 ± 0.005 | 0.003 ± 0.002 | 0.004 ± 0.002 | 0.004 ± 0.002 |
| Casuarinaceae | 3   | 98.7     | 0.044 ± 0.020 | 0.004 ± 0.001 | 0.013 ± 0.006 | 0.013 ± 0.005 |
| Betulaceae    | 10  | 98.9     | 0.038 ± 0.015 | 0.004 ± 0.001 | 0.012 ± 0.004 | 0.012 ± 0.004 |
| Fagaceae      | 1   | 99.2     | 0.028 ± 0.010 | 0.003 ± 0.002 | 0.009 ± 0.002 | 0.008 ± 0.002 |
| Between families | 51 | 96.9     | 0.115 ± 0.027 | 0.009 ± 0.003 | 0.033 ± 0.006 | 0.032 ± 0.005 |

Abbreviations used: $n$, number of pairwise sequence comparisons involved; $K_s$, $K_a$, and $K_o$, synonymous, nonsynonymous, and overall numbers of substitutions per site, respectively; J.C., Jukes and Cantor’s number of substitutions per site.
Table 2 Promiscuity of actinorhizal Hamamelidae genera towards Frankia

| Frankia strains* | Host of origin | Catalog no.§ | Promiscuity of host genera† | Alnus | Myrica |
|------------------|----------------|--------------|-----------------------------|-------|-------|
|                   | Allosuarauna | HFP022801    | (4,11,13)                  | (4,11,13,14) | (9,11) | (13,14) |
|                   | TA            | ORS022602    | (13)                       | (13)  | (13)  |
|                   | LRU/2         | CFN022802    | (13)                       | (13)  | (13)  |
|                   | Dec           | CNF022901    | (13)                       | (13)  | (13)  |
|                   | Ce3           | HFP020203    | (11)                       | (5,11) | (9,11,12) | (5,11) |
|                   | CeD           | ORS020606    | (5,11)                     | (5)   | (5)   |
|                   | CeF           | ORS020607    | (5,11)                     | (5)   | (5)   |
|                   | CI-82         | ORS021001    | (11)                       | (5,11,14) | (5,14) | (5,14) |
|                   | Ce5           | UFG026605    | (11)                       | (5,11,12) | (5,11) | (11) |
|                   | CeG           | UFG028501    | (11)                       | (5,11,12) | (5,11) | (11) |
|                   | Ce3           | IAE02020001  | (14)                       | (14)  | (14)  |
|                   | Ce24          | IAE02030023  | (14)                       | (14)  | (14)  |
|                   | 1995          | DAB020603    | (14)                       | (14)  | (14)  |
|                   | 1960          | DAB021004    | (14)                       | (14)  | (14)  |
|                   | Ce4           | HFP020604    | (14)                       | (14)  | (14)  |
|                   | Cpl           | HFP021801    | (9,11)                     | (9,10,11,12) | (9)   | (9)   |
|                   | Art1          | LLB01321     | (9,12)                     | (9,12) | (9)   | (9)   |
|                   | Art3          | HFP013003    | (9,12)                     | (9,12) | (9)   | (9)   |
|                   | Avcl          | DDB01020110  | (5,14)                     | (5,14) | (5,14) |
|                   | Avrs3         | DDB01060610  | (5,14)                     | (5,14) | (5,14) |
|                   | S4012         | DDB01362210  | (5)                        | (5)   | (5)   |
|                   | ACN1          | ULQ010201007 | (14)                       | (14)  | (14)  |
|                   | Ace8207       | IA01040002   | (14)                       | (14)  | (14)  |
|                   | Agc8204       | IAE01782004  | (14)                       | (14)  | (14)  |
|                   | Ahe8201       | IAE01982001  | (14)                       | (14)  | (14)  |
|                   | Cpl1          | HFP070101    | (5)                        | (5,14) | (5,14) | (5,14) |
|                   | Cpl3          | DDB07013010  | (5)                        | (5)   | (5)   |
|                   | Mp3           | DDB16201010  | (5)                        | (5)   | (5)   |
|                   | Mg25          | HFP161105    | (14)                       | (14)  | (14)  |
|                   | MGX35a        |                |                             |       |       |
|                   | MGX35b        |                |                             |       |       |
|                   | MGX39b        |                |                             |       |       |
|                   | MGX39c        |                |                             |       |       |
|                   | MGX40a        |                |                             |       |       |
|                   | MGX40b        |                |                             |       |       |
|                   | MGX40c        |                |                             |       |       |
|                   | MGX31a        |                |                             |       |       |
|                   | MGX34a        |                |                             |       |       |
|                   | MGX34b        |                |                             |       |       |
|                   | MGX34c        |                |                             |       |       |
|                   | MGX34d        |                |                             |       |       |
|                   | MGX34e        |                |                             |       |       |
|                   | 33051         | DDB16060200  | (5)                        | (5)   | (5)   |
|                   | K2115         | IMB0692115   | (14)                       | (14)  | (14)  |
|                   | Mrc8302       | IAE16248302  | (14)                       | (14)  | (14)  |
|                   | TX31eFHR      | ULQ0231058   | (14)                       | (14)  | (14)  |
|                   | EUN1f         | ULQ132500106 | (14)                       | (14)  | (14)  |
|                   | Ea118         | SIB13010111  | (14)                       | (14)  | (14)  |
|                   | Egcl07        | IAE13131017  | (14)                       | (14)  | (14)  |
|                   | K2061         | IMB1312061   | (14)                       | (14)  | (14)  |
|                   | K1510         | IMB13271510  | (14)                       | (14)  | (14)  |
|                   | Em273         | SIB13320723  | (14)                       | (14)  | (14)  |
|                   | Em373         | SIB13320737  | (14)                       | (14)  | (14)  |
|                   | Emoc1211      | IAE13131231  | (14)                       | (14)  | (14)  |
|                   | Eoec85        | IAE13360085  | (14)                       | (14)  | (14)  |
|                   | Eu131         | SIB13250131  | (14)                       | (14)  | (14)  |
|                   | Hr16          | IAE14010016  | (14)                       | (14)  | (14)  |
|                   | Hr21          | IAE14010021  | (14)                       | (14)  | (14)  |
|                   | Hr34          | IAE14010034  | (14)                       | (14)  | (14)  |
|                   | Hr37          | IAE14010037  | (14)                       | (14)  | (14)  |
|                   | Hr104         | SIB14010104  | (14)                       | (14)  | (14)  |
|                   | Ea11          | ULQ130100144 | (14)                       | (14)  | (14)  |
|                   | E1            | ULI13720110  | (15)                       | (15)  | (15)  |
|                   | E2            | ULI13720237  | (15)                       | (15)  | (15)  |
|                   | E3            | ULI13720250  | (15)                       | (15)  | (15)  |
|                   | E4            | ULI13720257  | (15)                       | (15)  | (15)  |
|                   | WgCc17        |                | (14)                       | (14)  | (14)  |

* Excluding Frankia strains unable to reinfect their host of origin.
† Excluding Comptonia because of insufficient data.
‡ = nodulation on at least one species tested within the genus; = no nodulation. In parentheses, reference numbers as follow: (1): Lechevalier et al. (1983); (2): Sougoufara (1983); (3): Zhang et al. (1984); (4): Zhang & Torrey (1985); (5): Baker (1987); (6): St-Laurent & Lalonde (1987); (7): Rosbrook & Bowen (1987); (8): St-Laurent et al. (1987); (9): Racette & Torrey (1989a); (10): Racette & Torrey (1989b); (11): Torrey & Racette (1989); (12): Racette et al. (1990); (13): Sougoufara (1990); (14): Du & Baker (1992); (15): Bosco et al. (1992).
§ Catalog numbers as defined by Lechevalier (1985).
substitutions (K_s), and highly congruent with results from parsimony analysis.

In both parsimony and neighbour-joining trees, the different families were supported by high bootstraps (Fig. 1A,B). The order Fagales, which contains the families Betulaceae and Fagaceae, did not appear to form a coherent group from the different phylogenetic analyses conducted. The neighbour-joining analysis of substitution rates led to a regrouping of the families Casuarinaceae and Betulaceae, which was supported by a bootstrap value higher than 50% (Fig. 1B) (the alternative bootstrap for the Fagales using this method was only 5%). Standard parsimony analysis led to a regrouping of the families Casuarinaceae and Fagaceae with a bootstrap value (43%) higher than that obtained for the Fagales (15%). Overall, the divergence between the Fagaceae, the Betulaceae, and the Casuarinaceae appeared essentially as a trichotomy.

Neighbour-joining and parsimony analyses both indicated the Myricaceae to have diverged first among the families of 'higher' Hamamelidae analysed (Fig. 1A,B). Any other topologies placing the Fagaceae, Betulaceae, or Casuarinaceae as first group to diverge among the 'higher' Hamamelidae received bootstraps lower than 50%. In the family Casuarinaceae, both neighbour-joining and parsimony analyses indicated a subclade which contained Allocasuarina and Casuarina (the Casuarineae) (Fig. 1A,B). As previously reported, the Betulaceae were divided into two subclades, the tribe Betuleae containing the genera Alnus and Betula and the tribe Coryleae containing the remaining genera (Bousquet et al. 1992b).

**Evolutionary analysis of promiscuity**

The promiscuity of the each actinorhizal genus of the 'higher' Hamamelidae was scored successively for each group of *Frankia* strains (Fig. 2), based on available data presented in Table 2. Character-state changes, that is, the promiscuity differences among host genera relative to the various groups of *Frankia* strains, were then monitored over the *rbcL* consensus tree of actinorhizal plant taxa. *Comptonia* could not be included in the analysis because of insufficient data on promiscuity (see Table 2). A strong relationship could be observed between the phylogeny of actinorhizal Hamamelidae and their spectrum of promiscuity towards the various groups of *Frankia* strains (Fig. 2). *Myrica*, assumed to diverge first, is equally or more promiscuous than the other genera. Within the Casuarinaceae, *Gymnostoma*, assumed to diverge first, is more promiscuous than the other genera of the family.
Moreover, the Casuarinae group (Allocasuarina and Casuarina) appears to be restricted in its symbiotic association to a group of Frankia strains not uninfective on Alnus roots, while Alnus can only be infected by strains not infective on Casuarinae roots (Table 2 and Fig. 2). When promiscuity was assumed ancestral, the number of steps necessary to explain the observed character-state variation among actinorhizal Hamamelidae was much smaller than when promiscuity was assumed derived (Fig. 2).

Discussion

Molecular phylogeny of actinorhizal Hamamelidae taxa

The different families analysed appeared as natural entities through the different phylogenetic analyses conducted, but the order Fagales was not observed as a coherent group. This observation is congruent with those of Nixon (1989), who suggested a paraphyletic or polyphyletic origin of the order Fagales, based on morphological characters. However, the closer relationship we observed between the Fagales (Betulaceae and Fagaceae) and the Casuarinales (Casuarinaceae), than with the Myricales (Myricaceae), is in agreement with views proposed by Takhtajan (1980) and Conquist (1988). This is in contrast to other views considering the Casuarinaeae more primitive and classifying the family as evolutionary intermediate between the ‘lower’ Hamamelidae and other more specialized ‘higher’ Hamamelidae such as the Betulaceae, Fagaceae, and Myricaceae (Barabé et al. 1982). Part of the disagreement could be explained by the interpretation of morphological variation. As indicated by Cronquist (1988), Casuarinaeae flowers are reduced rather than primitively simple, and such a reduced aspect in morphological characters should not necessarily be linked to a primitive state, because of potential adaptation to environmental conditions.

The likely divergence of the Myricaceae before the Betulaceae (Alnus) and Casuarinaeae is in agreement with the fossil record where the family Myricaceae appeared in the Cenomanian stage of the Upper Cretaceous (Gladskov 1962; MacDonald 1977), before the Betulaceae and Casuarinaeae, which appeared later in the Cretaceous-Santonian stage of the Upper Cretaceous (Cronquist 1988; Crane (1984) and at the limit between the Cretaceous and the Tertiary (Johnson & Wilson 1989), respectively. At lower taxonomic level, the topology of the Casuarinaeae obtained from rbcL sequences is in complete agreement with the treatment of the family by Johnson & Wilson (1989), which was based on morphological and chromosomal characters. Gymnostoma would have first diverged, followed by Casuarina and Allocasuarina which, in our analyses, were regrouped (Casuarinae clade).

This topology is also confirmed by the fossil record, where Gymnostoma is much postdated by the other genera of the family (Barlow 1983).

The evolution of promiscuity

Our survey of infectivity tests showed that Myrica and Gymnostoma can be infected by all host groups of Frankia strains tested, unlike other actinorhizal genera of 'higher' Hamamelidae which were found to be more specific in their Frankia symbiotic association. Because both molecular and traditional approaches indicate an earlier divergence of the family Myricaceae relative to the Betulaceae and Casuarinaceae, and an earlier divergence of Gymnostoma relative to Casuarina and Allocasuarina within the Casuarinaceae, it appears that evolution has proceeded in the direction of narrower promiscuity. This is also supported by the smaller number of state changes in promiscuity along the consensus topology of actinorhizal taxa, when promiscuity was assumed to be ancestral. Therefore, a narrower promiscuity could be interpreted as a more specialized feature, with possible evolution towards total resistance to Frankia infection, such as observed quite frequently for Allocasuarina under field conditions (Johnson & Wilson 1989).

This evolutionary trend is also observed in the Betulaceae, even though it contains only a single actinorhizal genus, Alnus. The Betuleae clade, which contains the actinorhizal genus Alnus and the non-actinorhizal genus Betula, has been shown to be less morphologically advanced than the Coryleae clade (Crane 1989; Bousquet et al. 1992b), which contains only nonactinorhizal genera. This suggests the actinorhizal state typical of Alnus to be less advanced than the nonactinorhizal state. Furthermore, the most ancient Betulaceae fossils were pollen grains typical of the actinorhizal Alnus (Crane 1989), with pollen typical of other genera, particularly from the Coryleae clade, appearing later in the fossil record. Moreover, the non-actinorhizal genus Betula is found more closely related to Alnus than to other non-actinorhizal genera of the Coryleae clade (Bousquet et al. 1992b; Savard et al. 1993), and this is paralleled by evidence that Betula is more dependent on Frankia than taxa from the morphologically advanced Coryleae clade, again suggesting the association with Frankia to be less advanced. Indeed, higher densities of Frankia of more than one order of magnitude have been observed in soils under stands of different Betula species, as compared to stands of spruce, oak (a Fagaceae), or even Corylus (Coryleae clade) (Van Dijk 1984; Smolander 1990; Paschke & Dawson 1992). Under such Betula stands, it has further been shown that Frankia could survive as an associative nitrogen fixer in a loose, unspecific but beneficial relationship with the non-host plant Betula roots (Rönkkö et al. 1993).
Ecological and evolutionary implications for the actinorhizal symbiosis

This evolutionary trend toward a narrower promiscuity of the host plants, suggests that the host plant root system plays a key role in the specific relationships established with *Frankia*. Not only the array of *Frankia* strains capable of infecting a host root system seems to be controlled by the plant, but also other types of characters involved in the symbiosis such as vesicle formation and morphology (Lalonde 1979; Tjepkema et al. 1980; St-Laurent & Lalonde 1987; Benson & Silvester 1993), or the mechanism of *Frankia* penetration into host roots: in this case, *Frankia* strains able to infect both Elaeagnaceae (subclass Rosidae of the dicots) and some actinorhizal Hamamelidaceae species show differential type of root penetration depending of the host, either by root hair infection for the Hamamelidaceae taxa, or by intracellular penetration for the Elaeagnaceae (Miller & Baker 1986; Racette & Torrey 1989b).

Furthermore, variation in the efficiency of the symbiosis has been shown to be much more controlled by plant clonal effects than *Frankia* strain effects (Simon et al. 1985; Mackay et al. 1987; Prat 1989; Souguoufara et al. 1992). This might simply derive from differences in plant genotypes with respect to promiscuity towards compatible *Frankia* strains, which would result in efficiency differences. Such larger control of the symbiotic efficiency by the host plant has also been reported in the legumes (see Galiana et al. 1991) and in free nitrogen-fixation associations involving various bacteria and nonhost plants (Rönnkö et al. 1993). Therefore, for the practical use of the actinorhizal symbiosis in the field where an efficient system is required, selection of the optimal host genotype–*Frankia* combination should exploit knowledge of the promiscuity of the plant genotypes used. This would imply a larger emphasis on the number of host genotypes tested rather than the number of *Frankia* strains involved.

It is now generally agreed that taxonomic grouping of *Frankia* strains from root nodules is much more dictated by the host plant from which the strains were isolated, rather than by the spectrum of infectivity of the strains (Lalonde et al. 1988; Fernandez et al. 1989; Beyazova & Lechevalier 1992). While this observation should be considered as a direct effect of host promiscuity, it is also likely that *Frankia* strains naturally found on promiscuous plant taxa or genera would exhibit more genetic or taxonomic diversity than strains naturally found on less promiscuous plant taxa or genera. Indeed, for *Frankia* strains naturally found on actinorhizal Hamamelidaceae taxa and which could reinfect their host of origin, this relationship between host promiscuity and strain diversity seems to hold. It is supported by the apparently narrower genetic diversity of *Frankia* strains isolated from *Casuarina* and *Allocasuarina*, as compared to strains isolated from the more promiscuous *Alnus* (Fernandez et al. 1989; Nazaret et al. 1991). It is also supported by the apparently much larger biochemical and genetic diversity of *Frankia* strains isolated from the largely promiscuous genus *Myrica*, which usually fail to form coherent taxonomic groups (Gardes et al. 1987; St-Laurent et al. 1987; Bloom et al. 1989; Simon et al. 1989).

As a consequence of these promiscuity differences among actinorhizal plants, the stringency of coevolutionary relationships between *Frankia* and the plant host should vary extensively, and a tight relationship between the phylogenies of both partners involved in the symbiosis should not be expected. Such a scenario is also observed in the legume–*Rhizobium* symbiosis, where little correspondence is observed between the phylogenies of the symbiont and the host (Young & Johnston 1989). This is in contrast with other mutualistic or parasitic systems where much tighter relationships are often observed between the phylogenies of both partners (Mitter et al. 1991; Moran & Baumann 1994). As for the legume–*Rhizobium* symbiosis, differences in promiscuity among actinorhizal Hamamelidaceae and total resistance of closely related taxa to root invasion by *Frankia* should reflect various levels of long-term mutualistic and antagonistic interactions between the two partners (Young & Johnston 1989). Because *Frankia* is not an obligate symbiont, being able like *Rhizobium* to survive as a free-living organism in the soil, the lack of coevolutionary relationships should even be more apparent with rhizospheric *Frankia*, where the plant host effects on *Frankia* taxonomic and genetic diversity would be much relaxed, if not negligible.

Acknowledgements

We thank D. Gauthier (ORSTOM, Nouméa, Nouvelle Calédonie) and the seed centre of CIFT-CIRAD (Nogent sur Marne, France) for providing Casuarinaceae seed materials. Also, thanks to P. Li (CRBF, Univ. Laval) for reading previous drafts of this manuscript, M. Michaud (CRBF, Univ. Laval) for his assistance in the laboratory and data analysis. This work was supported by AUPELF-UREF association and Natural Sciences and Engineering Research Council (NSERC) of Canada postdoctoral fellowships to L.M., and by Canadian NSERC grant (OGP0046273) and Québec FCAR grants (ER-0693 and NC-0642) to J.B.

References

Baker D (1987) Relationships among pure cultured strains of *Frankia* based on host specificity. *Physiologia Plantarum*, 70, 245–248.
Barabe D, Bergeron Y, Vincent G (1982) Étude quantitative de la classification des Hamamelidaceae. *Taxon*, 31, 619–645.
Barlow BA (1985) *Casuarina* – A taxonomic and biogeographic review. In: *Casuarina Ecology, Management and Utilization* (eds
Middley SJ, Turnbull JW, Johnston RD (1993) Biology of Frankia strains, actinomycyte symbionts of actinorhizal plants. Microbiological Reviews, 57, 293–319.

Beyazova M, Lechevalier MP (1992) Low-frequency restriction fragment analysis of Frankia strains (Actinomycetales). International Journal of Systematic Bacteriology, 42, 422-433.

Bloom RA, Mullin BC, Tate III L (1989) DNA restriction patterns and DNA–DNA solution hybridization studies of Frankia isolates from Myrica pectinifera (Bayberry). Applied and Environmental Microbiology, 55, 2155-2160.

Bousquet J, Lalonde M (1990) The genetics of actinorhizal Betulaceae. In: The Biology of Frankia and Actinorhizal Plants (eds Schwinzer CR, Tjepkema JD), pp. 239–261. Academic Press, New York.

Bousquet J, Simon L, Lalonde M (1990) DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. Canadian Journal of Forest Research, 20, 254–257.

Bousquet J, Strauss SH, Price RA (1992) Extensive variation in evolutionary rate of rbcL gene sequences among seed plants. Proceedings of the National Academy of Sciences of the USA, 89, 7844–7848.

Bousquet J, Strauss SH, Li P (1992) Complete congruence between morphological and rbcL-based on molecular phylogenies in birches and related species (Betulaceae). Molecular Biology and Evolution, 9, 1076–1088.

Callaham D, Newcomb W, Torrey IG, Peterson RL (1979) Root hair infection in actinomycete-induced root nodule initiation in Casuarina, Myrica and Comptonia. Botanical Gazette, 140, 51–59.

Chase MW, Soltis DE, Olmstead RG et al. (1993) Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene rbcL. Annals of the Missouri Botanical Garden, 80, 529–550.

Crane PR (1989) Early fossil history and evolution of the Betulaceae. In: Evolution, Systematics and Fossil History of the Hamamelidae, Vol. 2. Higher Hamamelidae (eds Crane PR, Blackmore S), Systematics Association Special Volume NO. 40B, pp. 87–116. Clarendon Press, Oxford.

Cronquist A (1981) A Integral System of Classification of Flowering Plants. Columbia University Press, New York.

Cronquist A (1988) The Evolution and Classification of Flowering Plants, 2nd edn. The New York Botanical Garden, New York.

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

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39, 783–791.

Fernandez MP, Meehner G, Grimont AD, Bardin R (1989) Deoxyribonucleic acid relatedness among members of genus Frankia. International Journal of Bacteriology, 39, 424–429.

Frascaria N, Maglia L, Michaud M, Bousquet J (1993) rbcL gene sequence from chestnut indicates a slow rate of evolution in the Fagaceae. Genome, 36, 668–671.

Galana A, Tibo A, Duboux E (1991) Nitrogen-fixing potential of micropropagated clones of Acacia mangium inoculated with different Bradyrhizobium spp. strains. Plant and Soil, 135, 161–166.

Gardes M, Bousquet J, Lalonde M (1987) Isozyme variation among 40 Frankia strains. Applied and Environmental Microbiology, 53, 1596–1603.

Giannasi DE, Zurawski G, Lern G, Clegg MT (1992) Evolutionary relationships of the Caryophyllidae based on comparative rbcL sequences. Systematic Botany, 17, 1–15.

Glazkova AN (1962) Fragments of the history of the Myricaceae family. Polen and Spores, 4, 345.
Racette S, Torrey JG (1989a) The isolation, culture and infectivity of a Frankia strain from Gymnostoma papuorum (Casuarinaceae). Plant and Soil, 118, 165–170.

Racette S, Torrey JG (1989b) Root nodule initiation in Gymnostoma (Casuarinaceae) and Shepherdia (Elaeagnaceae) induced by Frankia strain HFP-CpII. Canadian Journal of Botany, 67, 2875–2879.

Racette S, Torrey JG, Berg RH (1990) Sporulation in root nodules of actinorhizal plants inoculated with pure cultured strains of Frankia. Canadian Journal of Botany, 69, 1471–1476.

Rönnkö R, Smolander A, Nurminaho-Lassila E-L, Haaghtela K (1993) Frankia in the rhizosphere of nonhost plants: A comparison with root-associated N$_2$-fixing Enterobacter, Klebsiella and Pseudomonas. Plant and Soil, 153, 85–95.

Rosbrook PA, Bowen GD (1987) The abilities of three Frankia isolates to nodulate and fix nitrogen with four species of Casuarina. Physiologia Plantarum, 70, 373–377.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406–425.

Savard L, Michaud M, Bousquet J (1993) Genetic diversity and phylogenetic relationships between birches and alders using ITS, 18S rRNA, and rbcL gene sequences. Molecular Phylogenetics and Evolution, 2, 112–118.

Simon L, Stein A, Cote S, Lalonde M (1985) Performance of in vitro propagated Alnus glutinosa (L.) Gaertn: clones inoculated with Frankia. Plant and Soil, 87, 125–133.

Simon L, Jabaji-Hare S, Bousquet J, Lalonde M (1989) Confirmation of Frankia species using cellular fatty acids analysis. Systematic and Applied Microbiology, 11, 229–235.

Smolander A (1990) Frankia population in soils under Betula pendula. Plant and Soil, 121, 1–10.

Soltis DE, Soltis PS, Clegg MT, Durbin M (1990) rbcL sequence divergence and phylogenetic relationships in Saxifragaceae sensu lato. Proceedings of the National Academy of Sciences of the USA, 87, 4640–4644.

Soltis DE, Morgan DR, Grable A, Soltis PS, Kuzoff R (1993) Molecular systematics of Saxifragaceae sensu stricto. American Journal of Botany, 80, 1056–1081.

Sougoufara B (1983) Méthodologie impliquée dans l'étude de la symbiose d'une non-légumineuse forestière tropicale (Casuarina) avec Frankia. Thèse de DEA, Université de Nancy, France.

Sougoufara B (1990) La fixation de N$_2$ par les Casuarinaceae: amélioration de la symbiose d'une non-légumineuse forestière tropicale (Casuarina) avec Frankia. Thèse de doctorat de l'Université de Nancy, ORSTOM (eds), Paris, France.

Sougoufara B, Maggia L, Duhoux E, Dommergues YR (1992) Nodulation and N$_2$ fixation in nine Casuarina clone–Frankia strain combinations. Acta Oecologica, 13, 497–503.

St-Laurent L, Lalonde M (1987) Isolation and characterization of Frankia strains isolated from Myrica gale. Canadian Journal of Botany, 65, 1356–1363.

St-Laurent L, Bousquet J, Simon L, Lalonde M (1987) Separation of various Frankia strains in the Alnus and Elaeagnus host specificity groups using sugar analysis. Canadian Journal of Microbiology, 33, 764–772.

Swoford DL (1993) PAUP—Phylogenetic Analysis Using Parsimony, Version 3.1. Illinois Natural History Survey, Champaign.

Takhtajan A (1980) Outline of the classification of flowering plant (Magnoliophyta). Botanical Review, 46, 225–239.

Tjepkema JD, Ormerod W, Torrey JG (1980) Vesicle formation and acetylene reduction activity in Frankia sp. Cpl1 cultured in defined nutrient media. Nature, 287, 633–635.

Torrey JG, Racette S (1989) Specificity among the Casuarinaceae in root nodulation by Frankia. Plant and Soil, 118, 157–164.

Van Dijk C (1984) Ecological aspect of spore formation in the Frankia–Alnus symbiosis. PhD thesis, University of Leiden, the Netherlands.

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

Zhang Z, Torrey JG (1985) Studies of an effective strain of Frankia from Allocasuarina lehmanniana of the Casuarinaceae. Plant and Soil, 87, 1–16.

Zhang Z, Lopez MF, Torrey JG (1984) A comparison of cultural characteristics and infectivity of Frankia isolates from root nodules of Casuarina species. Plant and Soil, 78, 79–90.