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# Isolation, characterisation (PCR-RFLP) and specificity of *Frankia* from eight *Gymnostoma* species endemic to New Caledonia

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**Abstract** – Eight *Frankia-Gymnostoma* nitrogen-fixing symbioses endemic to New Caledonia were studied using a range of techniques including molecular typing of *Frankia*, infectivity/effectivity and host spectrum of the isolates. The study was conducted on 128 *Frankia* isolates from the eight *Gymnostoma* species. The RFLP analysis of the rRNA 16S-23S ITS allowed us to cluster the strains in four groups. Symbiotic characterization of the four groups failed to reveal any host specificity both in term of infectivity or effectivity. The characteristics of the strains were those of *Eleagnaceae*-infective *Frankia* strains. Co-inoculation of two *Gymnostoma* species with mixtures of strains evidenced 11 % of mixed RFLP profiles. In such controlled conditions, this was interpreted as co-infections. This work evidenced original features of these *Gymnostoma* isolates: (i) they were closer to *Eleagnaceae*-infective *Frankia* than *Casuarina*-infective *Frankia*; (ii) closer to atypical *Frankia* from *Casuarina*; (iii) poorly host-specific; and (iv) they had a tendency of co-infection. They could represent a primitive group of *Frankia* able to survive in a wide range of habitats. © 2000 Éditions scientifiques et médicales Elsevier SAS

***Gymnostoma* / *Casuarina* / *Eleagnaceae* / infectivity / effectivity / nitrogen fixation / actinorhizal plants / ITS 16S-23S / co-infection**

**Résumé – Isolation, caractérisation (PCR-RFLP) et spécificité de *Frankia* provenant de huit espèces de *Gymnostoma* endémique de Nouvelle-Calédonie.** Une étude a été menée sur huit symbioses fixatrices d'azote *Frankia-Gymnostoma* endémiques à la Nouvelle-Calédonie. Cent vingt-huit souches de *Frankia* ont été isolées et étudiées par diverses techniques, dont le spectre d'hôtes, l'infectivité, l'effectivité et la PCR/RFLP de l'ITS 16S-23S. L'analyse par PCR/RFLP de l'ITS 16S-23S a permis de classer ces *Frankia* en quatre groupes qui n'ont pas montré de différences en ce qui concerne leurs capacités symbiotiques. La co-inoculation de deux espèces de *Gymnostoma* par des mélanges des quatre types de *Frankia* isolés a mis en évidence l'apparition de mélanges de profils RFLP dans les nodules ainsi obtenus. Dans de telles conditions contrôlées, ce résultat a été interprété comme une co-infection. Ce travail a mis en évidence chez les *Frankia* de *Gymnostoma* des caractéristiques originales : (i) ils sont plus proches des *Frankia* des *Elaeagnaceae* que de ceux des *Casuarina* spp. ; (ii) ils sont proches des *Frankia* atypiques des *Casuarina* ; (iii) ils sont peu spécifiques ; et (iv) ils sont capables de co-infection. Ils pourraient représenter un type de *Frankia* primitif capable de survivre dans des habitats très variés. © 2000 Éditions scientifiques et médicales Elsevier SAS

***Gymnostoma* / *Casuarina* / *Elaeagnaceae* / infectivité / effectivité / fixation d'azote / plantes actinorhiziennes / ITS 16S-23S / co-infection**

## 1. INTRODUCTION

Actinorhizal plants can fix atmospheric nitrogen through a symbiotic association with the actinomycete *Frankia*. Among the eight botanical families, *Casuarinaceae* comprise four genera: *Ceuthostoma*, *Gymnostoma*, *Allocasuarina* and *Casuarina* [10]. These genera are essentially tropical and subtropical, originating from the Australian, Melanesian and Malaysian regions.

The flora of New Caledonia is characterized by a very high level of endemicity (75 to 80 % at the species level) [8]. *Casuarinaceae* are the only actinorhizal plants native to New Caledonia, with one *Casuarina* and eight *Gymnostoma* species (among the eighteen existing), all endemic. Nitrogen fixation makes these species good candidates for the rehabilitation of degraded land as well as mine sites in New Caledonia.

The eight species of *Gymnostoma* grow on soils originating from ultramafic rocks, with the exception of *G. nodiflorum* which grows on alluvial soils of volcano-sedimentary origin. Ultramafic soils are characterized by very low levels of P, K and Ca, high levels of Ni and Mn, a slow mineralization rate of organic matter and a poor level of available N. They can be either little transformed and rich in Mg (hypermagnesian inceptisol) or highly transformed in indurated oxisols. *G. chamaecyparis* is restricted to hypermagnesian soils and *G. deplancheanum* to indurated oxisols. The others species colonize a wider range of habitats such as forest (*G. glaucescens*, *G. intermedium* and *G. poissonianum* ) or riparian ecosystems (*G. leucodon*, *G. nodiflorum* and *G. webbianum*) [9]. Their relative specificity towards adverse soils together with their nitrogen fixing capacity make these tree species almost obligate candidates in the revegetation processes of mine sites in New Caledonia.

Probably due to their high endemicity, these *Gymnostoma* species and their symbiotic characteristics have been little studied. In 1989, Racette and Torrey [15] first isolated a *Frankia* strain from *G. papuanum*, then Savouré and Lim [18] isolated four strains from *G. sumatranum*. These strains were mostly studied for their symbiotic characteristics and their current availability can be seriously questioned. More recently, Navarro et al. [11] sequenced the *nif* DK ITS of six unisolated strains from six *Gymnostoma* species from New Caledonia and compared them to reference *Frankia* strains. These studies showed that *Frankia* infecting *Gymnostoma* nodules were indistinguishable from *Eleagnaceae* infective strains. However, there was still a need for a study (i) made on isolated *Frankia* strains, available as inoculum in field experiments, and (ii) on a significative number of strains susceptible to represent a wide genetic pool. The aim of this work was thus to isolate a large number of *Frankia* strains from a wide range of *Gymnostoma* species, and to characterize the isolates with molecular tools and by their symbiotic properties, i.e. infectivity, effectivity and host spectrum.

## 2. MATERIALS AND METHODS

### 2.1. Nodules harvest

Nodules were harvested in the natural stands of the host-plants, mainly in the Province Sud, within a radius of about 100 km around Nouméa (*table I*).

### 2.2. *Frankia* strains isolation

Nodules were directly harvested from the roots of the *Gymnostoma* plants. Each lobe was individually rinsed under tap water, disinfected for 10 to 20 min in 30 % (wt/v) $H_2O_2$ and rinsed with sterile distilled water before being cut into small fragments. All the fragments from one individual lobe were incubated at 30 °C, in one tube containing 5 mL Nitrogen Free Medium (NFM) or Complex Medium (CM) [11], for 1 to 3 months. This method allowed to ascertain that each strain originated from one single original lobe. The isolated strains, identified microscopically by the presence of the *Frankia* characteristic morphological structures including hyphae, vesicles, sporangia and spores were then subsequently cultured in the CM medium.

### 2.3. Plant material

Seeds were sterilised by incubation in concentrated $H_2SO_4$ for 2 min. The seeds were rinsed out with sterile distilled water and germinated in sterile sand, in a shade house. We tested only seven species of *Gymnostoma*, *G. glaucescens* seeds being not available. The other actinorhizal plants tested were: *Allocasuarina littoralis*, *A. stricta*, *A. torulosa*, *Casuarina collina*, *C. cunninghamiana*, *C. equisetifolia* and *Elaeagnus angustifolia*. After 1 month, the seedlings were transferred into pots containing one of the three sterile soil (see below) from New Caledonia and watered every 12 h. Two-month-old plants were fertilized with 1 $g \cdot L^{-1}$ standard Welgro nutrient solution (Ulvir limited, Barcelona, Spain).

### 2.4. Soils

The three soils used in this study were from New Caledonia. They were all derived from ultramafic rocks, with low P, K and Ca and high Ni levels. The C/N ratio was about 15 with a low organic decomposition and a poor N availability. The soils were sterilized by autoclaving at 120 °C for 2 h twice at 24-h interval.

### 2.4.1. Tontouta soil

Tontouta soil is a river sand (pH 6.6) from southern New Caledonia. It was considered as a control soil where all species can grow.

### 2.4.2. Nepoui soil

Nepoui soil is an hypermagnesian inceptisol (pH 6.7) toxic for most of *Gymnostoma* species where *G. chamaecyparis* can grow.

### 2.4.3. Prony soil

Prony soil is an indurated oxisol (pH 4.7) with high Fe, Ni and Mn levels, toxic for most of *Gymnostoma* species but where *G. deplancheanum* can grow.

## 2.5. DNA extraction

### 2.5.1. From cultures

DNA was extracted from 1-month-old *Frankia* strains according to Brenner et al. [2] and Simonet et al. [19].

### 2.5.2. From nodules

Nodule lobes were disinfected with 30 % w/v $H_2O_2$ for 5 min, rinsed with sterile distilled water and kept at 20 °C. One nodule lobe was crushed in 500 mL TCP buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, pH 8, 2 % w/v CTAB (Sigma, St Louis, MO, USA) and 3 % w/v PVPP (Sigma)). The mixture was incubated at 65 °C for 1 h and centrifuged at 3 000 × *g* for 5 min (20 °C). The supernatant was chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 10 mL TE buffer (pH 7.5).

## 2.6. PCR amplification

The 16S-23S InterTranscribed Spacer (ITS) rRNA fragment were amplified using primers FGPL2054' (5'-CCG-GGT-TTC-CCC-ATT-CGG-3') (beginning of 23S, universal) [14] and FGPS989e (5'-GGGGTCCT-TAGGGGCT-3') (end of 16S, *Eleagnaceae*-specific) [1] or FGPS989ac (5'-GGGGTCCGTAAGGGTC-3') (end of 16S, *Casuarina*-specific) [1] as described by Simonet et al. [20] and Nazaret et al. [12]. PCR were run on Perkin-Elmer 2400 GeneAmp PCR Systems.

## 2.7. RFLP

Restriction analyses of the PCR products were performed with three enzymes: *Hae*III, *Msp*I and *Cfo*I. Digestion was performed on 10 µL PCR products at 37 °C for 2 h. Electrophoreses were made on 3 % agarose gels (Sigma) at 140 mV for 1 h.

## 2.8. Cross inoculation studies

The seven *Gymnostoma*, three *Casuarina*, three *Allocasuarina* species and *Eleagnus angustifolia* were grown as already described, on the control sandy soil (Tontouta). They were inoculated 4 months after germination with 10 mg packed cell volume [13] of an 1-month-old *Frankia* culture. For each ITS group, one representative strain was arbitrarily selected and used for the experiment. The four selected strains were: Gd03 (ITS Group 1), Gg40 (ITS Group 2), Gl03 (ITS Group 3) and Gc18 (ITS Group 4). Each treatment was repeated four times and two non-inoculated controls were used for each plant species. Because of very poor growth, *Gymnostoma* species were measured 11 months after inoculation. The other species were measured 6 months after inoculation. Infectivity was qualitatively estimated by the presence/absence of nodules for each plant, and effectivity was determined through the acetylene reduction method on the entire root system under standard procedures. Ethylene production was determined on a Carlo Erba GC6000 gas chromatograph. Results were expressed as µM $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodule dry weight.

## 2.9. Incidence of soil and host plant combination on symbiotic properties of the four ITS groups of Frankia

This study was conducted on two *Gymnostoma* species, *G. chamaecyparis* and *G. deplancheanum*, selected for their extremely opposite soil properties requirements. Moreover, these two species were the only ones to correspond to exclusive ITS group of *Frankia*. The three soils (Tontouta, Nepoui and Prony) were used as growth substrate. The growth and shade house conditions were as described above.

## 2.10. Infectivity and effectivity tests

Inoculations were performed with each ITS group using the same four selected strain as above, by delivering 10 mg (packed cell volume) of an 1-month-old culture. Four inoculated plants and two non-inoculated controls were used per treatment. Infectivity and effectivity were estimated as described above, 11 months after inoculation.

## 2.11. Co-inoculation study

Inoculation was performed by delivering to each soil/plant combination 10 mg inoculum. The inoculum was an equal volume (packed cell volume) mixture of

the four ITS selected strains. Four inoculated plants and two non-inoculated controls were used for each treatment. Nodules were harvested 11 months after inoculation and the infecting Frankia rRNA was characterized as above.

## 3. RESULTS AND DISCUSSION

### 3.1. *Frankia* strain isolation

The isolation method we used (one nodule lobe per tube, liquid medium) has two advantages: (i) it allows to quickly and easily eliminate contaminated lobes; and (ii) it ensures that isolates originate from one lobe.

About 2 100 nodule lobes had to be dissected to allow the obtention of 128 isolates from eight *Gymnostoma* and ten localities (*table I*). This corresponds to about 6 % of success of isolation among *Gymnostoma* species. With different method and different media, Rosbrook and Reddell [17] obtained isolation rates of 50 % for *Casuarina equisetifolia*, 17 % for *C. glauca* and 3 % for *C. cunninghamiana*. The first strain of *Frankia* was isolated 20 years ago [3], however the isolation step is still a considerable bottleneck to obtain pure cultures, and a strong variability is observed among host plants and methods used. Isolation seemed to be more successful on nitrogen-free medium (90 % of the successful isolations). This could be due to the early selection of nitrogen fixers (and the non-proliferation of contaminants) as well as a better compatibility between the culture medium and *Frankia* strains.

**Table I.** Origin of the strains of *Frankia* isolated in this study.

| Host plant | Geographical origin | *Frankia* isolates |
|---|---|---|
| *Gymnostoma chamaecyparis* | Nepoui | Gc01, 06, 10 to Gc16, 18 |
| *G. deplancheanum* | Chute de la Madeleine | Gd33, 41, 51, 103 104, 106 |
| | Creek Pernod | Gd02, 03, 17, 18 |
| | Prony | Gd94, 95 |
| | Rivière bleue | Gd73 |
| | Yaté (col de ) | Gd19, 20, 21, 22, 30, 32, 35, 36, 88, 91, 93, 121, 130, 132 |
| *G. glaucescens* | Dzumac | Gg01 to Gg30 |
| | Étoile filante | Gg40 to Gg51 |
| *G. intermedium* | Dzumac | Gi01 to Gi03 |
| *G. leucodon* | Rivière des pirogues | Gl01 to Gl15 |
| *G. nodiflorum* | Ciu | Gn01 to Gn12 |
| *G. poissonianum* | Dzumac | Gp01 to Gp04 |
| *G. webbianum* | Rivière bleue | Gw01, 02, 07, 09, 12, 13, 20, 33, 34, 36, 40, 41, 51, 52 |

The strains isolated from *Gymnostoma* exhibited all the characteristic morphological structures of the genus *Frankia* including hyphae, sporangia, spores and vesicles. Vesicles were very numerous in the NFM medium and still present after ammonium complementation. All the strains presented an orange to blood-red pigmentation. These characteristics are close to those of (i) the five already isolated *Gymnostoma* strains [15, 18], (ii) atypical strains from *Casuarina* (i.e. strains unable to nodulate the host of origin but which nodulate species belonging to *Eleagnaceae* and *Rhamnaceae* families) [5, 6, 22] and (iii) strains isolated from *Eleagnaceae* or *Rhamnaceae* described by Clawson et al. [4], Gauthier et al. [7] or Nazaret et al. [12]. The 128 isolates represented a wide genetic pool suitable to evaluate diversity of the strains nodulating the eight species of *Gymnostoma*.

### 3.2. Molecular characterization of the bacterial rRNA operon

#### *3.2.1. PCR amplification*

The DNA extraction protocol yielded DNA that was pure enough to obtain amplification with primer FGPS989e, which is *Elaeagnaceae* group-specific. No amplification was obtained with primer FGPS989ac, which is *Casuarina* group-specific. The amplified fragments were about 1 000 bp long. Similar grouping of non-isolated *Frankia* from *Gymnostoma* (in nodule) with those of *Eleagnaceae* was already described by Navarro et al. [11] by using specific primers which confirms the particular position of *Gymnostoma* nodulating *Frankia* among the *Casuarinaceae* family.

#### *3.2.2. RFLP*

Discriminant patterns were observed when PCR products were digested with *Hae*III and *Msp*I. Each enzyme produced three profiles which, in combination, allowed to discriminate four different ITS groups named 1, 2, 3 and 4. Groups 2 and 3 were predominant: 59 and 26 %, respectively. These two groups were widely represented in the different species of *Gymnostoma* in their various ecological habitats. Groups 1 and 4 represented only 8.6 and 6.3 % of the isolates and were found to exclusively occur in *G. deplancheanum* and *G. chamacyparis*, respectively, in their natural stands (*table II*). Working with non-iso-

**Table II.** *Frankia* isolated from *Gymnostoma* nodules.

| *Gymnostoma* | *Frankia*: ITS groups (16S-23S) | | | | |
|---|---|---|---|---|---|
| | Number | 1 | 2 | 3 | 4 |
| *G. chamaecyparis* | 10 | | | 2 | 8 |
| *G. deplancheanum* | 28 | 11 | 17 | | |
| *G. glaucescens* | 42 | | 41 | 1 | |
| *G. intermedium* | 3 | | 2 | 1 | |
| *G. leucodon* | 15 | | | 15 | |
| *G. nodiflorum* | 12 | | 11 | 1 | |
| *G. poissonianum* | 4 | | 4 | | |
| *G. webbianum* | 14 | | 1 | 13 | |
| Total | 128 | 11 | 76 | 33 | 8 |

lated strains of six *Gymnostoma* species, Navarro et al. [11] compared mainly sequences of the *nif* DK intergenic spacer, which did not allow a direct comparison with our results.

### 3.3. Symbiotic characterization of each group

#### *3.3.1. Host spectrum of each group*

The four ITS groups were found to be infective and effective on the seven *Gymnostoma* species tested and on *Eleagnus angustifolia*. They were non-infective on the three *Casuarina* and three *Allocasuarina* species tested. No significant difference was found in either infectivity or effectivity between the different groups. Re-isolation and PCR/RFLP characterization of a representative strain from the four *Frankia* groups allowed to confirm Kochs postulate for each group. Twenty-one arbitrarily chosen strains from the different groups were additionally tested, mainly on *G. chamaecyparis* and *G. deplancheanum*, which allowed us to confirm the lack of specificity between the groups and the species (data not shown).

#### *3.3.2. Incidence of soil and host plant combination on symbiotic properties of the four ITS groups*

Considering the relative exclusivity of groups 1 and 4 on *G. deplancheanum* and *G. chamaecyparis* and the specificity of the soils they colonized (Prony and Nepoui), it seemed particularly interesting to test infectivity and effectivity of the four ITS groups on these two species on their respective soil. A less extreme soil (Tontouta) was also used as control. Despite numerous attempts, it was impossible to maintain heterologous soil-plant combinations such as *G. deplancheanum*/Nepoui and *G. chamaecyparis*/Prony. *Table III* illustrates the results which did not allow to symbiotically differentiate the four groups whatever the tested combination. Even in extreme edaphic conditions, it is thus not possible to establish a clear relationship between the origin of a given strain and the symbiotic performances of the association. In a control sandy soil where both plant species can grow, there is no evidence of a clear advantage for any strain/plant combination.

#### *3.3.3. Co-inoculation of the four groups in the different soil/plant combinations*

Co-inoculation of two *Gymnostoma* species (*table IV*) with mixtures of strains allowed to evidence 11 % of mixed RFLP profiles. In such controlled conditions, this was interpreted as co-infections. Such co-infections have been reported by several authors [16, 21] and were already observed in the *Frankia-Gymnostoma* symbiosis through: (i) isolation of mixtures of strains originates from one nodule lobe (Gauthier, unpubl.); and (ii) observation of more than one ITS pattern in some nodule lobes harvested in the field (data not shown). *Table IV* presents the percentage of the different ITS groups of *Frankia* detected within the nodules in the different soil/plant combinations. A similar number of nodule lobes was analysed for each plant species. All the groups appeared to be equally represented within the nodule for any combination. No comparative advantage could thus be detected for any of the ITS groups in term of competitivity in any of the situations.

Groups 1 and 4 are not more adapted to their original plants whatever the soil, and the predominance of groups 2 and 3 cannot be related to any symbiotic ability. One can thus speculate that the relative higher frequency of groups 2 and 3 among isolates is attributable to a better isolability. It can be questioned whether the isolation steps underestimate the biodiversity of the strains by selecting some groups above others. Using the same strategy (PCR/RFLP on 16S-23S ITS) with non-isolated *Frankia* from *Gymnostoma* (in nodule), it

**Table III.** Incidence of soil and *Gymnostoma* spp. combinations on symbiotic properties of the four ITS groups of *Frankia*. For each species, four repetitions were carried out. I, Infectivity (% of nodulation); E, effectivity (as ARA: µmoles $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodule dry weight, extreme values).

| *Frankia* | Soils and plants tested | | | |
|---|---|---|---|---|
| | Tontouta soil | | Nepoui soil | Prony soil |
| | *G. chamaecyparis* | *G. deplancheanum* | *G. chamaecyparis* | *G. deplancheanum* |
| ITS group 1 | | | | |
| Gd03 | I:100<br>E: 13–35 | I: 100<br>E: 15–35 | I: 75<br>E: 11–43 | I:75<br>E: 18–50 |
| ITS group 2 | | | | |
| Gg40 | I: 75<br>E: 15–37 | I: 100<br>E: 15–37 | I: 100<br>E: 13–123 | I: 100<br>E: 18–36 |
| ITS group 3 | | | | |
| Gl03 | I: 75<br>E: 12–52 | I: 75<br>E: 30–36 | I: 75<br>E: 15–51 | I: 75<br>E: 15–37 |
| ITS group 4 | | | | |
| Gc18 | I: 75<br>E: 5–53 | I: 100<br>E: 8–44 | I: 100<br>E: 13–79 | I: 75<br>E: 16–69 |

**Table IV.** Co-inoculation of the four ITS groups in soil/*Gymnostoma* spp. combinations. We used four inoculated plants per treatment. Number in parentheses represent number of lobes analysed.

| Soils and plants tested | Percentages of the four ITS groups within the nodules | | | | |
|---|---|---|---|---|---|
| | ITS group 1 | ITS group 2 | ITS group 3 | ITS group 4 | Mixture of ITS groups |
| Tontouta soil | | | | | |
| *Gymnostoma chamaecyparis* (21) | 14 | 24 | 28.5 | 24 | 9.5 |
| *G. deplancheanum* (20) | 20 | 15 | 20 | 30 | 15 |
| Nepoui soil (hypermagnesian) | | | | | |
| *Gymnostoma chamaecyparis* (24) | 20.9 | 12.5 | 33.3 | 25 | 8.3 |
| Prony soil (oxisol) | | | | | |
| *Gymnostoma deplancheanum* (25) | 24 | 20 | 24 | 20 | 12 |

was possible to determine seventeen ITS groups (Navarro, pers. comm.). However, the isolation of *Frankia* strains is essential for the studies of actinorhizal symbiosis and to produce inoculum for field experimentations.

This work made available a range of *Frankia* strains that have in common certain characteristics: these strains sporulate easily; they are pigmented, they grow rapidly in vitro even on nitrogen-free media; they are poorly host-specific, widely represented, easily isolatable and able to co-infect. They are close to strains of *Eleagnaceae*, and to atypical strains of *Casuarina* spp. Together with strains from *Eleagnaceae* and atypical strains, they could represent a primitive group of *Frankia* able to survive in a wide range of habitats thanks to a high adaptability: (i) they can infect or co-infect a wide range of species; (ii) they have a strong ability to survive in soils both through a high sporulating capacity and a putative ability of saprophytic life (that could be reflected by their high isolability).

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