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Distribution of *Gymnostoma* spp. microsymbiotic *Frankia* strains in New Caledonia is related to soil type and to host-plant species

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Abstract

The diversity of the *Frankia* strains that are naturally in symbiosis with plants belonging to the *Gymnostoma* genus in New Caledonia was investigated. A direct molecular characterization of DNA extracted from nodules was performed, followed by characterization by restriction fragment length polymorphism (RFLP) of the ribosomal *rrs–rrl* (16S–23S) intergenic spacer (IGS) polymerase chain reaction (PCR)-amplified region. Seventeen different patterns were identified among the 358 microsymbiotic strains studied in the eight species of host plant present in New Caledonia. This genotypical approach permitted us to show that a large diversity existed among the patterns and that these did not exhibit a strict specificity to any host-plant species comparable with that previously found in the *Casuarina* and *Allocasuarina* symbioses in Australia. Despite this lack of specificity, a correspondence analysis nevertheless showed that the distribution of these patterns was related to soil type and to host-plant species. Furthermore, several *Frankia* strains were exclusively associated with the ultramafic soils.

Keywords: diversity, *Frankia*, *Gymnostoma*, New Caledonia, nickel, ultramafic soil

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Introduction

The family Casuarinaceae is composed of the four genera of plants *Allocasuarina*, *Casuarina*, *Ceuthostoma* and *Gymnostoma*, of which *Gymnostoma* is considered the most primitive based on morphology (Johnson & Wilson 1989) and on both molecular and host range data (Maggia & Bousquet 1994). These plants are naturally confined to the Malesian–Australian Melanesian region, with *Gymnostoma* endemic on New Caledonia and other neighbouring islands. *Casuarina* and *Allocasuarina* are present mostly in Australia, but some species and particularly *C. equisetifolia* have been exported extensively to other tropical areas, to be used as windbreaks or to stabilize sand dunes (Diem *et al.* 1988; Diem & Dommergues 1990). These uses are due to the efficient nitrogen-fixing symbiosis that most of the 96 extant species belonging to this family have established with the actinomycete *Frankia*, permitting the plants to develop on nitrogen-poor soils.

In spite of its small surface area (19 000 km²), New Caledonia has an abundance of endemic Casuarinaceae species, as eight of the 18 *Gymnostoma* extant species and one *Casuarina* species have been described as belonging exclusively to the island flora. This may be explained by the extent in New Caledonia of ultramafic rocks, including peridotites and serpentinites, that make up one-third of the main island area, on which these species are endemic. In contrast, it can be noted that similar rocks compose only 1% of the Earth's surface-emerged lands (Brooks 1984). In New Caledonia, six *Gymnostoma* species are found only in soils derived from ultramafic rocks: *G. chamaecyparis*, *G. deplancheanum*, *G. glaucescens*, *G. intermedium*, *G. leucodon* and *G. poissonianum*. *G. webbianum* is found on both ultramafic and nonultramafic substrates. Finally, the last species *G. nodiflorum* grows mainly on nonultramafic substrates (Jaffré *et al.* 1994a).

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Various *Gymnostoma* species have been used successfully in experimental plantations on unbalanced soils, abnormally rich in nickel or manganese and deficient in major elements except magnesium which is sometimes found in excess. This demonstrates that these actinorhizal species could be major components of reforestation efforts in recreational zones to counteract damage due to nickel ore mining (Jaffré *et al.* 1994b). Planted and naturally growing New Caledonian *Gymnostoma* spp. trees have been found to be well nodulated revealing the presence of adapted *Frankia* strains in the soil and nitrogen-fixing symbiosis could play a role in the adaptation of the plants to such adverse soil conditions.

However, little is known about the symbiotic association between *Frankia* and plants belonging to the genus *Gymnostoma* (Racette & Torrey 1989; Savouré & Lim 1991). Recently, a phylogenetic study was carried out on the indigenous microbial partners of the New Caledonian endemic symbioses and other *Frankia* strains (Navarro *et al.* 1997). This study showed, based on both phenotypic and genotypic approaches, that *Gymnostoma*-infective strains were *Elaeagnus*-infective but not *Casuarina*-infective. These strains present in the *Gymnostoma* nodules studied are phylogenetically close to one another but nevertheless have different DNA sequences. Thus, it was of interest to evaluate the level of diversity in natural populations of *Gymnostoma* microsymbionts in New Caledonia. In this work, the molecular diversity of *Gymnostoma* strains was analysed together with their symbiotic properties in relation to the soil on which they grow. Furthermore, revegetation works would benefit from assessment of the natural distribution, which could designate strains or groups of strains most appropriate to given types of soil.

Materials and methods

Geographical parameters and soil characteristics

For the study, New Caledonia was divided into five geographical sectors in which *Gymnostoma* spp. stands were delineated for the harvesting of nodules (Fig. 1). In each sector, two or three areas were defined (Table 1). The soil

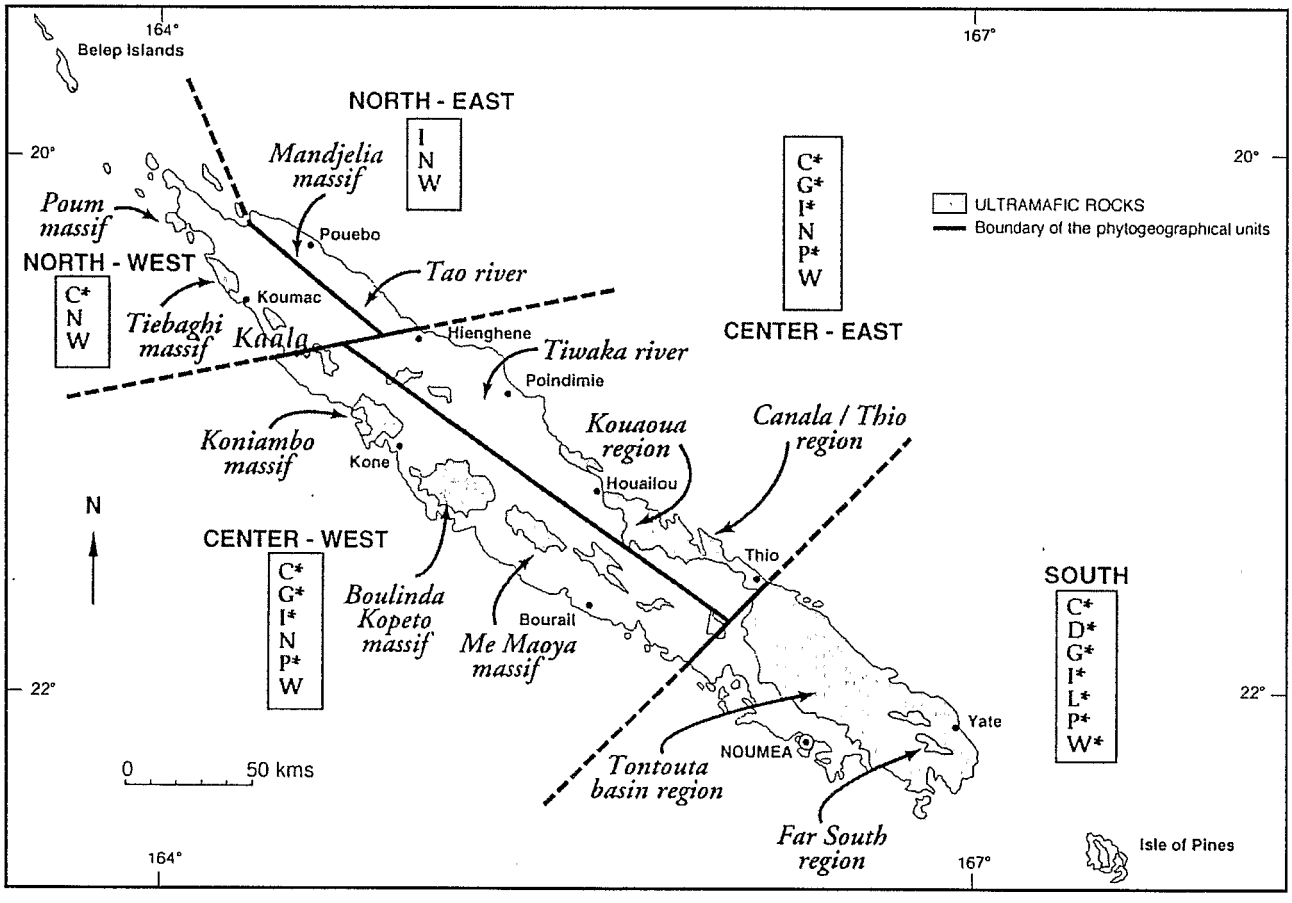

Fig. 1 Map of New Caledonia with the different regions, showing the *Gymnostoma* spp. distribution and the nodule sampling areas. C, *G. chamaecyparis*; D, *G. deplancheanum*; G, *G. glaucescens*; I, *G. intermedium*; L, *G. leucodon*; N, *G. nodiflorum*; P, *G. poissonianum*; W, *G. webbianum*; * indicates ultramafic rocks.

Table 1 Geographical, soil types and host-plant species origins of samples used in this study

Region	Localization	No. of sites sampled	Soil type*	*Gymnostoma* species†
South	Far south	11	O–U	D–G–I–L–P–W
	Tontouta	2	B–O	C–G–I–P
Centre-east	Canala/Thio	7	A–O	N–P–W
	Kouaoua	5	B–O	C–G–I
	Tiwaka	1	A	N
Centre-west	Me Maoya	2	B–O	P–G
	Boulinda Kopeto	4	B–O–U	C–G–P–W
	Koniambo	1	B	C
Northwest	Kaala	1	B	C
	Tiebaghi	4	A–B–U	C–N–W
	Poum	1	B	C
Northeast	Tao	2	A	N–W
	Mandjelia	1	A	W

*A, acidic alluvium; B, brown hypermagnesian inceptisol; O, oxisol; U, ultramafic alluvium.
†C, *G. chamaecyparis*; D, *G. deplancheanum*; G, *G. glaucescens*; I, *G. intermedium*; L, *G. leucodon*; N, *G. nodiflorum*; P, *G. poissonianum*; W, *G. webbianum*.

from the sampling stands belonged to four classes, based on parent rock nature, soil origin, evolution degree, structure and chemical composition (Latham *et al.* 1978). Soil from acidic rocks was represented by alluvial soils (A), essentially with an acidic pH and a low nickel concentration (0.016%; Jaffré *et al.* 1994a). Although poor in phosphorus, calcium and potassium, and abnormally rich in nickel (from 0.2 to 0.87%; Jaffré *et al.* 1994a), soil from ultramafic rocks appeared to be more diversified than soil from acidic rocks. According to their degree of evolution, three soils were identified: brown hypermagnesian inceptisols (B), oxisols (O) and ultramafic alluvial soils (U).

DNA extraction

DNA from nonisolated strains present in nodules was extracted and purified according to a modification of the protocol described initially by Simonet *et al.* (1994) and used for *Casuarina* and *Gymnostoma* nodules (Rouvier *et al.* 1996; Navarro *et al.* 1997). Each rinsed nodule lobe was peeled off to eliminate potential contaminants from the periderm, rinsed with sterile distilled water and crushed in 500 µL of TCP buffer (100 mM Tris–HCl, pH 8, 1.4 M NaCl; 20 mM ethylenediamine tetraacetic acid (EDTA), pH 8, 2% (w/v) CTAB (Sigma) and 3% (w/v) PVPP (Sigma). The endophyte suspension was then incubated at 65 °C for 1 h and centrifuged (3000 g for 5 min at 20 °C). The supernatant was chloroform extracted and ethanol precipitated before the DNA pellet was dissolved in 10 µL of TE buffer (Tris 10 mM, EDTA 1 mM, pH 7.5).

DNA amplifications

The polymerase chain reaction (PCR) amplifications were performed using primers FGPS989e (5′-GGGGTCCTTAGGGGCT-3′) (Bosco *et al.* 1992) and FGPL73′ (5′-ATCGGCTCGAGGTGCCAAGGGTC-3′) (Navarro *et al.* 1992) targeting the ribosomal 16S–23S intergenic spacer (IGS) with the standard conditions described by Simonet *et al.* (1991) including the use of the *Taq* DNA polymerase from Gibco BRL and a PTC-100TM programmable thermal controller (MJ Research). To verify the efficiency of the amplification, 1/10 of the amplification reaction was analysed by electrophoresis on a 2% (w/v) agarose gel.

PCR product restriction analysis

Restriction endonuclease digestions were carried out by using 15 µL of a positive control PCR reaction for each enzyme. The endonucleases, *Msp*I, *Hae*III and *Cfo*I, found to be the most resolutive in a preliminary study (data not shown) were used as specified by the manufacturer (Boehringer). Electrophoresis in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) was carried out in a horizontal slab gel on a 4% (w/v) Nusieve (FMC) agarose gel containing 0.5 µg/mL ethidium bromide with gels running at 4 V/cm for 3 h.

Statistical analysis

A multiple correspondence analysis (Benzécri 1973; Greenacre 1984; Tenenhaus & Young 1985) using MACMUL4 software (Thioulouse 1990) was carried out on the PCR/restriction fragment length polymorphism (RFLP) patterns as a function of the host-plant species and of the soil on which the host plants were found. A graphical representation of the resulting analysis was made using GRAPHMU software (Thioulouse 1990). Chi-square analysis

Table 2 *Frankia* pattern (%) distribution in the *Gymnostoma* species

Plant species*	Soil types†	Pattern 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Absolute no. of samples
C	B	0	2.1	0	86.3	4.2	0	2.1	0	0	4.2	0	0	1.1	0	0	0	0	95
D	O	0	7.1	17.1	35.7	31.4	7.1	0	0	0	1.4	0	0	0	0	0	0	0	70
G	O, U	0	5.9	5.9	71	2.9	8.8	0	0	2.9	2.9	0	0	0	0	0	0	0	34
I	O	0	0	4	80	8	4	0	0	0	0	0	0	0	0	4	0	0	25
L	U	0	25	12.5	56.2	6.3	0	0	0	0	0	0	0	0	0	0	0	0	16
N	A	26.7	20	0	0	6.7	0	0	20	0	0	3.3	0	6.7	0	0	3.3	13.3	30
P	B, O	0	3.9	7.8	68.6	15.7	0	0	0	0	0	0	1.9	0	1.9	0	0	0	51
W	A, U	2.7	32.4	2.7	18.9	32.4	0	0	10.1	0	0	0	0	0	0	0	0	0	37
Absolute no. of samples		9	33	22	202	52	9	2	10	1	6	1	1	3	1	1	1	4	358

*A, acidic alluvium; B, brown hypermagnesian inceptisol; O, oxisol; U, ultramafic alluvium.
†C, *G. chamaecyparis*; D, *G. deplancheanum*; G, *G. glaucescens*; I, *G. intermedium*; L, *G. leucodon*; N, *G. nodiflorum*; P, *G. poissonianum*; W, *G. webbianum*.

Table 3 *Frankia* pattern (%) distribution in the soils

Soil type*	Pattern 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Absolute no. of samples
Acidic soil																		
A	17.8	26.7	0	0	15.6	0	0	22.2	0	0	2.2	0	4.4	0	0	2.2	8.9	45
Ultramafic soils																		
B	0	2	0	83	8	0	2	0	0	4	0	0	1	0	0	0	0	100
O	0	4.7	10.6	59.2	17.2	4.1	0	0	0.6	1.2	0	0.6	0	0.6	0.6	0	0	169
U	2.3	25	9.1	40.9	18.2	4.5	0	0	0	0	0	0	0	0	0	0	0	44
Absolute no. of samples	9	33	22	202	52	9	2	10	1	6	1	1	3	1	1	1	4	358

*A, acidic alluvium; B, brown hypermagnesian inceptisol; O, oxisol; U, ultramafic alluvium.

was carried out on dominant patterns after grouping the less-abundant patterns.

Results

Characterization of Gymnostoma *microsymbionts*

The method used for extracting DNA from *Gymnostoma* nodule lobes yielded endophyte DNA that was pure enough to be efficiently amplified with the set of primers tested (data not shown). After direct DNA extraction and purification, a 960-bp DNA fragment corresponding to the ribosomal region comprising part of the 16S gene, the 16S–23S IGS and a small part of the 23S gene was obtained for all nodule templates (data not shown). Different fragments were obtained when DNAs were digested with *Msp*I and *Hae*III, yielding patterns that were subsequently used for group or 'pattern' delineation. The electrophoresis conditions on 4% (w/v) Nusieve agarose gels permitted resolution of DNA fragments larger than 75 bp.

The number of nodules studied was 358 (Table 2), a majority of which were on *G. chamaecyparis* (95; 27%) and on *G. deplancheanum* (70; 20%). The naturally less-abundant species (*G. leucodon* and *G. intermedium*) were present as 16 and 25 samples, corresponding to 4.5 and 7% of the whole, respectively. The restriction patterns found comprised one dominant (no. 4 for 56%), six subdominants (from 3 to 15%) and 10 rare patterns (from 1.2 to 0.3%) that were not considered further. The majority of the nodule samples were from the oxisol and from the brown hypermagnesian inceptisol, while the ultramafic alluvium and the acidic soil were less represented (Table 3). The dominant pattern, no. 4, was present in all species and soil types, except *G. nodiflorum*, which grows exclusively in acidic soil. Patterns 2 and 5 were present in all soil types, the latter being the only one present in all *Gymnostoma* host-plant species (Table 2). Subdominant patterns 3 and 6 were absent from the acidic alluvium and hypermagnesian inceptisol, conversely subdominant pattern 8 was present only in the acidic alluvium (Table 3).

Table 4 *Frankia* pattern (%) distribution in the species *Gymnostoma webbianum* as a function of soil type

	Pattern																	
Soil types*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Absolute no. of samples
A	0	40.0	0	0	33.3	0	0	26.7	0	0	0	0	0	0	0	0	0	15
U	4.5	27.3	4.5	31.8	31.8	0	0	0	0	0	0	0	0	0	0	0	0	22
Absolute no. of samples	1	12	1	7	12	0	0	4	0	0	0	0	0	0	0	0	0	37

*A, acidic alluvium; U, ultramafic alluvium.

The multiple correspondence analysis of the *Frankia* pattern distribution as a function of the host-plant species and of the soil type is shown in Fig. 2. The first horizontal axis, which explains 10.3% of the total inertia (average correlation ratio for the first axis 0.90), opposes *G. nodiflorum*, its specific soil (A) and patterns 1 and 8 from all other species and from the three ultramafic soils. The second vertical axis, which explains 8.4% of the total inertia (average correlation ratio for the first axis 0.73), opposes *G. chamaecyparis* and *G. nodiflorum*, their specific soils (B and A, respectively) and patterns 1 and 8 from all other *Gymnostoma* species, soil types and patterns and more specifically, patterns 3 and 6. On the first axis, the intermediate position of *G. webbianum* can be explained by its presence in two soil types (A and U). The patterns associated with *G. webbianum* are distributed between the A and U soil types (Table 4); for instance, pattern 8 associated by the analysis with the acidic alluvium was found only in *G. webbianum* present in this soil. The statistical analyses corroborate these results. The significance of observed differences between dominant patterns was assessed by the χ^2 test after grouping the less-abundant patterns. Differences between ultramafic soils and the acidic soil were highly significant ($\chi^2 = 180$, d.f. = 7, $P = 0.0001$). It was also the case for host-plant species ($\chi^2 = 321$, d.f. = 49, $P = 0.0001$). For these two factors, patterns 1, 4 and 8 show maximal contribution of individual (hoc) cells in Table 4 to the chi-square statistic.

These results can be summarized by two criteria illustrating the soil–plant-pattern relationships (Fig. 3). The first criterion would be the presence or lack of patterns 4/1–8, thus separating the acidic alluvium from the ultramafic soils, and *G. nodiflorum* from the other *Gymnostoma* species in agreement with the statistical analysis. The second criterion would be the presence or lack of pattern 3. It can be noticed that the dominant pattern 4 was specific to the ultramafic soils, as it was not found associated with *G. nodiflorum* and *G. webbianum* on acidic alluvium (Table 4).

Discussion

The mainland of New Caledonia has its origin in the Gondwana supercontinent and is characterized by an important plant endemicity (Jaffré *et al.* 1994a) and a large proportion of soils overlying ultramafic substrata. These soils are rich in nickel and manganese and thus constitute an original environment to study microbial ecology from both fundamental and applied points of view. *Gymnostoma* spp. are the dominant nitrogen-fixing plant species in New Caledonia, where they colonize soils laid bare following fires and landslides. *Gymnostoma* is an endemic genus of major interest, because of its symbiotic association with *Frankia* and thus its capacity to colonize mining waste (Jaffré *et al.* 1994b). However, the improvement of disturbed land by this nitrogen-fixing symbiosis requires a better knowledge of *Frankia* population diversity as a function of soil parameters. One of the major soil factors hypothesized to affect microbial distribution in Neocaledonian soils is nickel. It is a necessary cell constituent at nanomolar concentrations, being a cofactor of such enzymes as hydrogenases, superoxide dismutase, urease and glyoxalase (for a review see Ragsdale (1998)). On the other hand, nickel at millimolar concentrations is toxic for cell processes, presumably by binding to histidine residues (Nies 1992; Rey *et al.* 1994; Silver 1996).

The *Frankia* strains found in symbiosis with Casuarinaceae host-plant species fall into two phylogenetically divergent groups: those infective on *Casuarina* spp. and *Allocasuarina* spp. are phylogenetically close, based on *rrs* gene analysis (Normand *et al.* 1996) to the *Alnus*-infective strains and are in general resistant to isolation attempts (Rouvier *et al.* 1996), while those infective on *Gymnostoma* are in the group infective on *Elaeagnus* and in general relatively easy to grow in pure culture (Navarro *et al.* 1997, 1998). This fundamental dichotomy, presumably resulting from an early allopatric distribution (*Gymnostoma* in the north and east of Gondwana islands, *Casuarina*/*Allocasuarina* in the drier Australia) also means that the *Frankia* strains symbiotic on *Casuarina* and *Allocasuarina* have become progressively dependent on their host-plant species while the strains symbiotic on *Gymnostoma* are more saprophytic and thus must have developed biochemical properties to adapt to the different soil niches, independently of the symbiotic part of their life cycle. This is in accordance with the increase of specificity observed

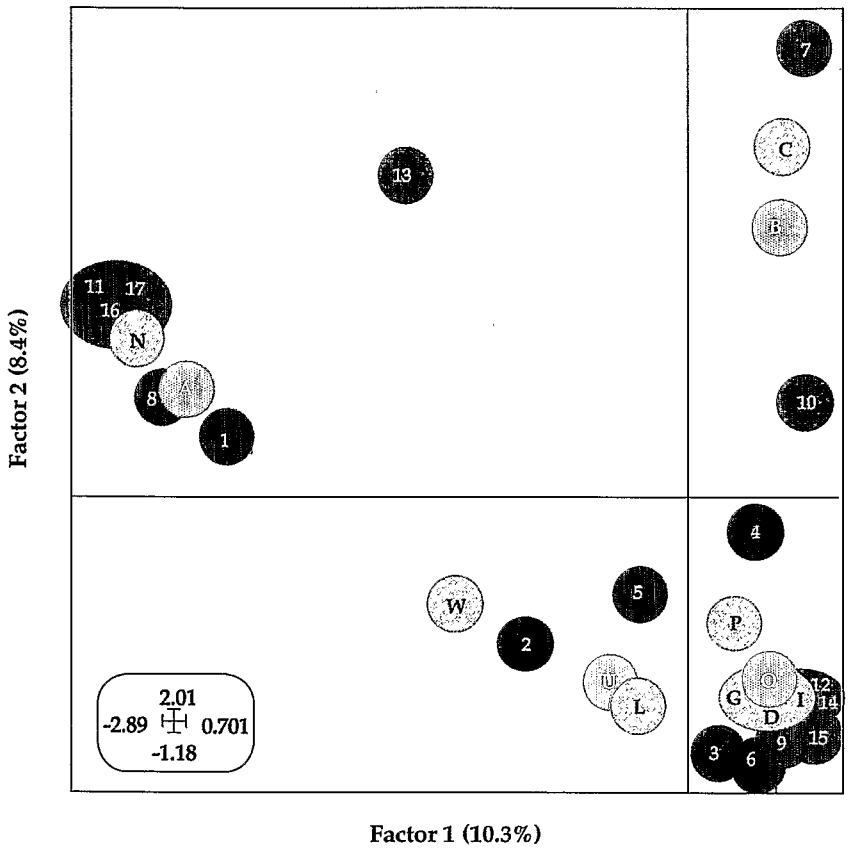

Fig. 2 Multiple correspondence analysis of *Frankia* patterns, soil type and host-plant species. The soil types are in red (A, acidic; B, brown hypermagnesian ultramafic; O, oxysol; U, ultramafic alluvium), the *Gymnostoma* host plants are in green (D, *G. deplancheanum*; C, *G. chamaecyparis*; G, *G. glaucescens*; I, *G. intermedium*; L, *G. leucodon*; N, *G. nodiflorum*; P, *G. poissonianum*; W, *G. webbianum*) and the 17 patterns are in blue (dominants in dark blue). The range of the first two factors is given in the lower left.

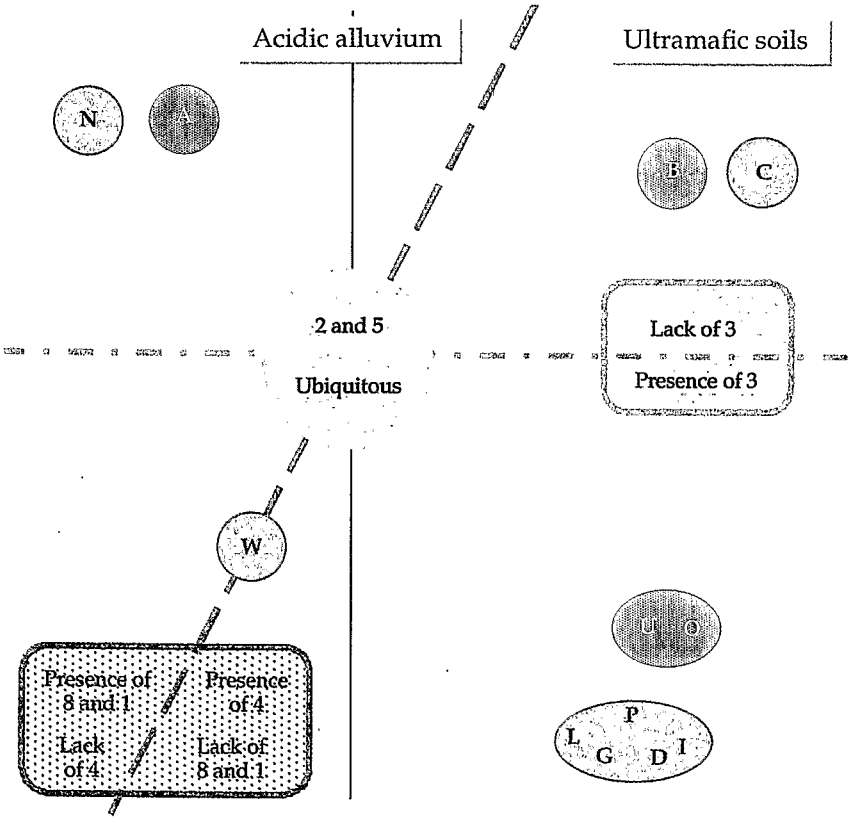

Fig. 3 Schematic representation of the multiple correspondence analysis results. For legend, see Fig. 2.

between *Gymnostoma* and *Casuarina/Allocasuarina* (Maggia & Bousquet 1994).

The study of the genetic diversity of *Gymnostoma* microsymbionts in New Caledonia clearly indicates that a large diversity exists, as the 358 DNAs tested were classified into 17 different patterns. The present factorial analysis shows some associations between *Frankia* patterns, *Gymnostoma* host-plant species and soil types. The major differentiating factor, figured by axis 1, would be soil type, with, on the one hand, the acidic alluvium and on the other hand, the three ultramafic soils. There is only one *Gymnostoma* species, *G. webbianum*, that grows on both of these soils and the present analysis positions it in the centre of Fig. 2, as expected from a species with a large ecological niche. Moreover, pattern 8 associated with both *G. nodiflorum* and *G. webbianum* but only with acidic soil, is positioned close to the acidic alluvium. This would suggest that soil conditions are important factors driving local bacterial adaptation, together with host-plant presence. Such a major role for soil pH on strain selection has been described previously by Jamann *et al.* (1992) for *Frankia*, and by Harrisson *et al.* (1989) and Dughri & Bottomley (1984) for *Rhizobium*. Other factors have also been found to affect microbial distribution, such as depth (Waldon *et al.* 1989; Nalin *et al.* 1997), habitat variability (McArthur *et al.* 1988) and of course the plant root (Mavingui *et al.* 1992; Lemanceau *et al.* 1995).

The present study shows that soil factors select particular *Frankia* genotypes. This finding suggests that some *Frankia* populations would be adapted to a nickel-rich environment, while others would be adapted to soils derived from acidic rocks. This may help in focusing isolation efforts, which so far have not been fruitful for all *Frankia* patterns, on to those areas, soil types and host-plant species most appropriate for revegetation efforts on the nickel-rich mine spoils of New Caledonia.

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Elisabeth Navarro has been working for 4 years in New Caledonia on *Frankia* strains infective on *Gymnostoma*, together with Tanguy Jaffre, a botanist, Daniel Gauthier, a microbiologist, and Gérard Rinaudo, a microbial taxonomist. François Gourbiere, is a statistician interested in microbial successions, Pascal Simonet works on the characterization of microbial strains in complex environments and Philippe Normand works on the genetics and evolutionary history of actinorhizal bacteria–plant interactions.
