Root Nodule Microsymbionts of Native *Coriaria myrtifolia* in Algeria

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ABSTRACT: *Coriaria myrtifolia* occurs as natural flora of warm temperate climates of northern Algeria which commonly found in hedges, forest and ravine edges. This actinorhizal species was known to establish a mutualistic symbiosis with members of phylogenetic cluster 2 (including strains associated to *Coriaria* spp., *Ceanothus*, *Datiscaceae*, and *Dryadoideae*) within the genus *Frankia*. Attempts to isolate *C. myrtifolia* microsymbionts from native plants growing in 4 locations in Algeria permitted to only recover asymbiotic *Frankia* strains (unable to reestablish nodulation and to fix nitrogen) from phylogenetic cluster 4 and several non-*Frankia* actinobacteria including members of *Micrococcus, Micromonospora, Nocardia, Plantactinospora*, and *Streptomyces* genera. The biodiversity of *Frankia* microsymbionts of *C. myrtifolia* root nodules was assessed using PCR-amplification followed by partial nucleotide sequencing of *gln*A1 (glutamine synthetase type 1) gene. On the 12 different *gln*A1 gene sequences obtained in this study, 9 were detected for the first time, and were mainly closelyrelated to Mediterranean genotypes previously described in the Grand Maghreb countries (Morocco and Tunisia) and in Europe (France) but without clear separations from other cluster 2 genotypes.

KEYWORDS: *Frankia*, *Coriaria myrtifolia*, non-*Frankia*, asymbiotic *Frankia*

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Introduction

Coriaria myrtifolia is endemic to western Mediterranean area including Northern Africa of the Grand Maghreb.[1,2] It is mainly found in hedges, forest, and ravine edges under warm temperate climates of the Rif in Morocco and coastal to tellian atlas sub-sectors of Algeria. As an actinorhizal plant, *C. myrtifolia* forms a symbiotic association with the actinobacterial genus *Frankia*, which results in the development of nitrogen-fixing root nodules.[3-6] The *Frankia* strains[7] associated with *C. myrtifolia* form a distinct lineage[8] which has been grouped in cluster 2 together with other microsymbionts whithin *Coriaria* spp., *Ceanothus, Datiscaceae*, and *Dryadoideae* root nodules.[9-12]

Members of *Frankia* cluster 2 have long been considered as uncultivable actinobacteria. However, several *Frankia* strains which are unable to fulfill Koch's postulates (non-nodulating and/or non nitrogen-fixing) have been established from *Coriaria*, *Datisca*, and *Ceanothus* species.[13-15] These asymbiotic isolates have been affiliated to phylogenetic cluster 4 of the genus *Frankia*.[9-12]

In our previous studies we used a dual approach combining comparative genomics and physiological bioassays to successfully establish axenic cultures of 2 strains from *Frankia* cluster 2 for the first time.[16,17] Here we report results of cultivation attempts of cluster 2 strains from root nodules collected from native *C. myrtifolia* in Algeria and we provided an overview of genetic diversity of the uncultured *Frankia* cluster 2 populations directly in root nodules using *Gln*A1 gene sequence.

Materials and Methods

Root nodules sampling: A total of 44 root nodules were collected from *C. myrtifolia* growing in 4 locations in Algeria (Figure 1) and consisted of 3 nodules from one tree in site GKB: *Les Gorges de Kharrata*, Bejaia (N 36°31′9.379″, E 5°16′45.616″, 314 m), 11 nodules from 2 trees in site CDB: *Les Cascades de Kefrida, Derguina*, Bejaïa (N36°34′14.634″, E5°17′24.175″,550 m), 12 nodules from 3 trees in site PGM: *La Plage de la Grotte Merveilleuse* Jijel (N36°41′4946″ E5°31′45.8322″,15 m) and 18 nodules from 3 trees in site PR: *La Plage Rouge, Ziama mansoria*, Jijel (N36°39′24.289″, E5°26′5.866″,3 m).

Microsymbiont isolation: Harvested root nodules were processed as described by Ghodhbane-Gtari et al[18]. After cleaning thoroughly with sterile tap water individual lobes (n = 833) were surface sterilized by shaking in 30% (v/v) H_2O_2 for 30 minutes and aseptically rinsed several times with sterile distilled water. To check the efficiency of nodule disinfection, an aliquot of 300 µl of the last washing solution was inoculated on nutrient agar plates and incubation for several days. Nodules resulting showing contamination-free washing liquid were further considered for nodule microsymbiont isolation by incubation of aseptically crushed individual lobe in BAP medium[19] buffered to range of pH (pH 6.8-pH 9) and supplemented with several organic acids as carbon source including: acetic acid, aspartic acid, butyric acid, citric acid, glutamic acid, malic acid, propionic acid, pyruvic acid, succinic acid, ribose uridine and thymidine and at a final concentration of 10 mM.[16]

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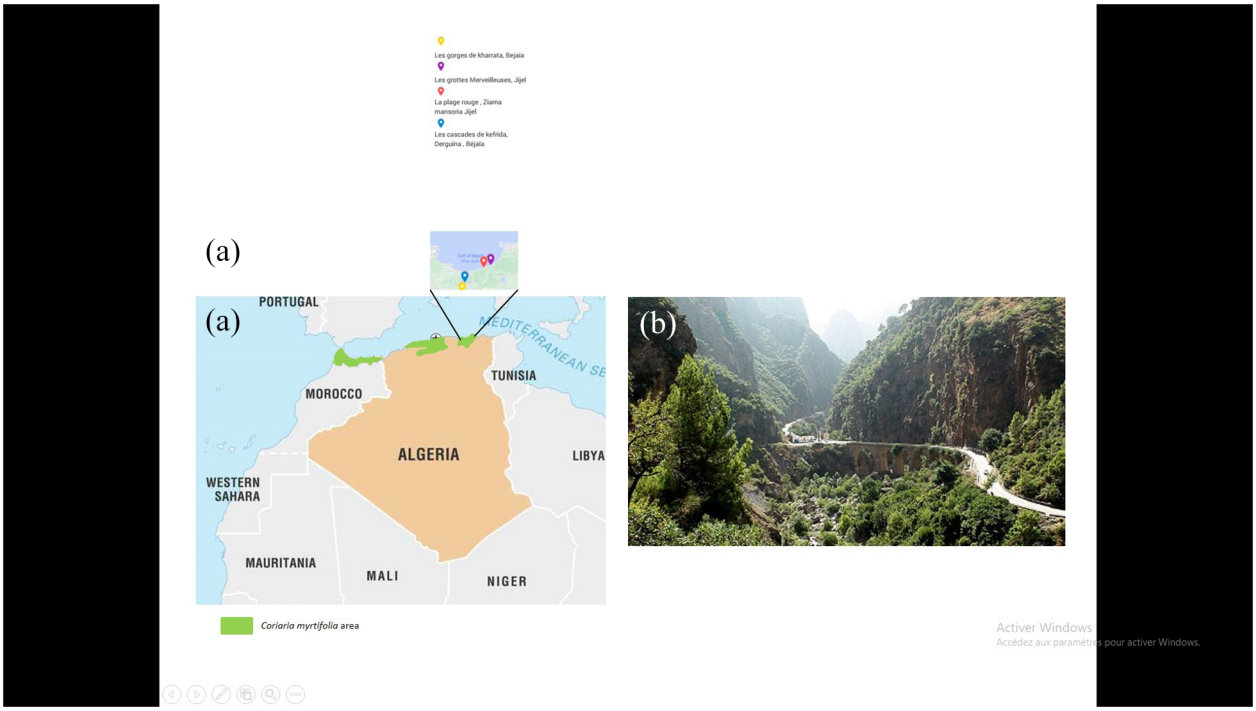

Figure 1. Distribution area of *Coriaria myrtifolia* in Algeria (A), *Coriaria myrtifolia* strand in *Les gorges de kharrata*, Bejaia, Algeria.

DNA extraction, PCR amplification, and sequencing

DNA extraction from root nodules was performed as described by Gtari et al[20]. Individual lobe from the 44 root nodules were surface sterilized as described for microsymbiont isolation protocol, ground in nitrogen liquid and taken up in the extracting buffer (100 mmol l^{-1} Tris-HCl [pH 8]; 20 mmol l^{-1} EDTA [pH8.2]; 1.4 M NaCl, and 2% w/v cetyl trimethyl ammonium bromide; CTAB). For cultivated strains, DNA was extracted from 1 month-old liquid cultures for *Frankia* strains and for non-*Frankia* actinobacteria from 3 to 5 well-grown colonies on solid Luedemann medium. After washing 3 times in sterile saline buffer, bacterial cells were taken up in the same extracting buffer used for nodules and forced several time through a 0.7 × 30 mm sterile needle to homogenize the mycelium. The resulting pellets, from grinded nodules or bacterial cells collected through centrifugation (3000 rpm for 30 minutes), were used for further DNA extraction steps. After incubation for 30 minutes in extracting buffer, DNA was chloroform extracted and ethanol precipitated through centrifugation (12 000 rpm, 30 minutes at 4°C), the DNA pellet was dissolved in 50 µl TE (10 mmol l^{-1} Tris-HCl [pH 8]; 20 mmol l^{-1} EDTA [pH 8.2]). PCR amplifications were performed using universal primers S-D-Bact0008-a-S- 20 (5′- AGAGTTTGATCCTGGCTCAG-3′) and S-DBact-1495-a-A-20 (5′-CTACGGCTACCTTGTTACGA-3′)[21] for almost full 16S rRNA gene and primers DB41 (5′-TTCTTCATCCACGACCCG-3′) and DB44 (5′-GGCTTCGGCATGAAGGT-3′)[22] for 477 bp fragment of *gln*A1 gene, in 50 µl final reaction volume containing 10 ng genomic DNA, 1x Taq polymerase buffer (Promega, Madison, WI, USA), 1.5 mmol l^{-1} MgCl2, 0.1 µM each dNTP, 0.2 µM each primers and 2 U Taq DNA polymerase. The thermal program consisted of 3 minutes at 95°C followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. PCR-products were purified using the QIAquick Wizard PCR purification Kit (Promega, Madison, VVI, USA) and then cycle-sequenced in both directions using an ABI cycle sequencing kit (Applied Biosystem 3130) as described by Ghodhbane-Gtari et al[18]. The nucleotide sequences of *gln*A1 and 16S rRNA genes were aligned using ClustalW[23] and compared to corresponding homologous sequences retrieved from GenBank. Phylogenetic trees were constructed using MEGA version 11.[24] Bootstrap values were determined from 1000 replicates.[25]

C. myrtifolia *inoculation test*

To fulfill Koch's postulates, plant inoculation experiments were performed as previously described.[16] Seeds of *C. myrtifolia*, locally collected from Algeria were surface sterilized by shaking in 30% (v/v) H_2O_2 for 30 minutes and aseptically rinsed several times with sterile distilled water, germinated on moistened filter paper under daylight illumination at 28°C and then transferred to sterilized moist sand. Collected cells from 4 weeks old *Frankia* cultures were washed several times with sterile distilled, water crushed in sterile glass homogenizer and used to inoculate 3 week-old *C. myrtifolia* seedlings. Seedlings were weekly checked for nodulation for 3 months. Seedlings inoculated with *Frankia coriaria* strain BMG5.1 and sterile water were used as positive and negative controls respectively.

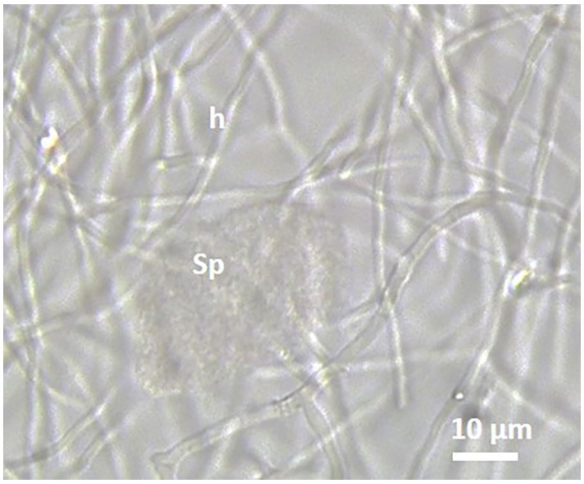

Figure 2. Phase-contrast microscopy of strain BMG5.36 grown for 4 weeks at 28°C on liquid BAP medium showing the 2 morphological structures; hyphae (h) and sporangia (sp) but never produces vesicle.

Results

Isolation of Frankia from C. myrtifolia root nodules

On the 833 lobes derived from the 44 sampled root nodules and used for isolating *Frankia* micosymbionts, only 3 isolates have been obtained in BAP medium from *La Plage de la Grotte Merveilleuse* Jijel site. These 3 isolates showed 2 of the 3 typical morphological structures of the genus *Frankia*; hyphae and sporangia but never produce vesicles (Figure 2). The strains were unable to grow on nitrogen free BAP medium and to reinduce root nodules on *C. myrtifolia* seedlings. Based on partial *gln*A1 gene and 16S rRNA gene sequences the 3 isolates represent the almost the same strain which clustered within phylogenetic cluster 4 (Figure 3).

Isolation of non-Frankia actinobacteria from C. myrtifolia *root nodules*

After incubation of 833 lobes in isolating BAP medium, 160 showed contaminations of fast growing fungi and bacteria within 2 to 5 days of incubation that have been discarded. These fast growing contaminants may have resisted the sterilization procedure or have happened during the rest of manipulation. Other vials showed slowly outgrowing mycelia which shown obvious substantial improvement of growth rate after transfer on solid BAP medium containing nitrogen source. Based on 16S rRNA gene sequence analysis, these isolates were assigned to members of *Micrococcus, Micromonospora, Nocardia, Plantactinospora*, and *Streptomyces* genera (Table 1).

Overview of genetic diversity of cluster 2 microsymbionts

PCR-sequencing of the partial *gln*A1 gene was performed on DNA directly extracted from single lobes representing each *C. myrtifolia* tree growing in the 4 locations in Algeria. BLAST (Basic local alignment search tool) analysis[26] indicated that all obtained 24 sequences have the best BLAST hit with *gln*A1 sequences of *Frankia* from phylogenetic cluster 2 and none of them was affiliated to the asymbiotic phylogenetic cluster 4. Further multiple alignment of the total sequences showed that there are 11 different *glnA1* gene sequences from Algeria. Nine of them differed from all previously described among *C. mytiolia* micosymbionts. Three subclusters may be defined (Figure 4) occurring in 4 sampling locations. A large subcluster (62.5% of obtained *gln*A*1* gene sequences) includes *C. mytifolia* micosymbionts in 3 sites (*Les Cascades de Kefrida, Derguina, Béjaïa, La Plage de la Grotte Merveilleuse Jijel and La Plage Rouge, Ziama mansoria, Jijel*) which were homologous to those detected in Morocco[11] and in Tunisia[27] and France[11] together with microsymbionts detected in *Datisca glomerata*, *Dryadoideae* (*Purshia tridentata*, *Cercocarpus betuloides, Cercocarpus ledifolius*, *C. foliolosa*) and *Ceanothus* (*C. cuneatus, C. prostratus* and *C. integerrimus*) root nodules sampled from North American Midwest in California, USA.[28] A second subcluster representing 16.6% of obtained *gln*A*1* gene sequences in 2 sites (*La Plage de la Grotte Merveilleuse Jijel* and *La Plage Rouge, Ziama mansoria, Jijel*), together with microsymbionts from *C. myrtifolia* from France and Morocco, *C. micophylla* in Mexico and *C. nepalensis* in Pakistan[11] together with microsymbionts of *Cercocarpus betuloides* and *Datisca glomerata* in California.[28] A third subcluster representing 16.6% of analyzed sequences from 2 sites (*Les Gorges de Kharrata*, Bejaia and *Les Cascades de Kefrida*, *Derguina, Béjaïa*) were associated with *C. myrtifolia* microsymbionts from France and Morocco, *C. japonica* micosymbionts from Japan, and *C. microphylla* micosymbionts from Mexico.[11]

Discussion

*Gln*A1 sequencing has been chosen to assess diversity of *C. myrtifolia* microsymbionts because it has been shown to be more variable than other genetic markers including 16S rRNA and *nif*H genes.[22] Our study of the native *Frankia* microsymbionts from this actinorhizal species using PCR-amplification and sequencing of *gln*A*1* gene sequence showed a heterogeneity among the 4 sampling provenances in Algeria confirming previous reports for cluster 2 microsymbionts. By analyzing a large collection of cultivated strains and nodules, Vanden Heuvel et al[28] reported a low genetic diversity among *Frankia* microsymbionts in sympatric populations of actinorhizal species of *Dryadoideae*, *Ceanothus*, and *Datisca glomerata* in North American Midwest. Nouioui et al[11] also showed a low diversity of micosymbionts from 5 disjunct *Coriaria* species: *C. myrtifolia*, *C. arborea*, *C. nepalensis*, C. *japonica*, and *C. microphylla*, growing in the Mediterranean area (Morocco and France), New Zealand, Pakistan, Japan, and Mexico. These authors also reported the absence of cospeciation between the microsymbionts and their respective *Coriaria* species. Here we confirmed that the biogeography of sampling sites in Algeria does not affect the microsymbiont genotypes. Moreover, *gln*A1 gene sequences detected

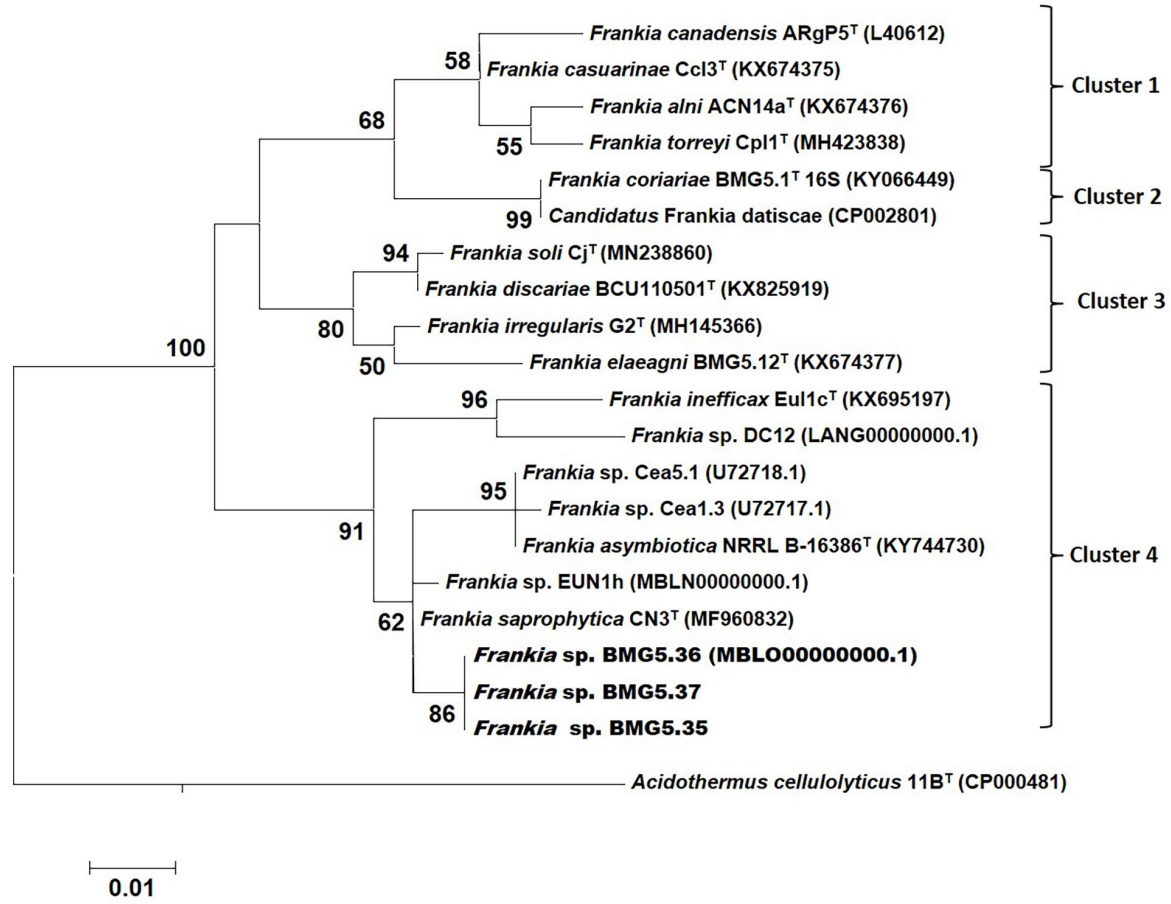

Figure 3. Maximum-likelihood tree based on partial 16S rRNA gene showing the phylogenetic position of *Frankia* isolated from *C. myrtifolia* root nodules sampled in Algeria (in bold). *Acidothermus cellulolyticus* strain 11BT (CP000481) was used as out-group. Bootstrap values (in %) determined from 1000 replicates. Only values above 50% were shown.

Table 1. Identification based on 16S rRNA gene partial sequencing of the non-*Frankia* strains isolated from *C. myrtifolia* root nodules in this study.

STRAIN DESIGNATION	ORIGIN	GENBANK ACCESSION NUMBER	TOP HIT (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION SEARCH DATABASE)
Micrococcus sp. BMG8.4	Les grottes merveilleuses jijel	MH295634	*Micrococcus endophyticus* strain YIM 56238 (NR_044365.1)
Micromonospora sp. strain BMG8.18	Les grottes merveilleuses jijel	MH295123	*Micromonospora coriariae* NAR01 (NR_042314.1)
Micromonospora sp. strain BMG8.1	Les grottes merveilleuses jijel	MH294801	*Micromonospora pisi* strain GUI 15 (NR_104523.1)
Micromonospora sp. strain BMG8.3	Plage les grottes merveilleuses jijel	MH298660	*Micromonospora pisi* strain GUI 15 (NR_104523.1)
Micromonospora sp. strain BMG8.19	Les grottes merveilleuses jijel	MH295124	*Micromonospora vinacea strain* GUI63T (NR_151946.1)
Nocardia sp. BMG8.12	Les grottes merveilleuses jijel	MH295125	*Nocardia jiangxiensis* strain *ATCC 33726* (NR_117329.1)
Nocardia sp. BMG8.4	Cascade de derguina bejaia	MH236149	*Nocardia rhamnosiphila* 202GMO (NR_116015.1)
Nocardia sp. BMG8.13	Les grottes merveilleuses jijel	MH295308	*Nocardia rhamnosiphila* NBRC 108938 (NR_116015.1)
Plantactinospora sp. BMG8.20	Plage rouge Jijel,	MH295637	*Plantactinospora mayteni* strain YIM 61359T (NR_044592.1)
Streptomyces sp. strain BMG8.9	Cascade de derguina bejaia	MH298646	*Streptomyces glomeroaurantiacus* strain NBRC 15418 (NR_041436.1)

Figure 4. Phylogenetic tree based on nearly 477-bp of *glnA1* and inferred by neighbor-joining treeing method. *GlnA1* sequences obtained in this study are in bold. Acronym of each sequence indicates site of collection; *Cascade de kefrida, Derguina*, Bejaïa (CDB), *Plage Rouge, Ziama Mansoria*, Jijel (PR), *Plage de la Grotte Merveilleuse* Jijel (PGM), and *Les Gorges de Kharrata*, Bejaia (GKB) and the plant number (1, 2, and 3) and the range of nodule (-, A, and B). *Frankia saprophytica* strain CN3 (NZ_KI912267.1) as out-group. Bootstrap values (in %) determined from 1000 replicates. Only values above 50% were shown.

in this study were homologous to those retrieved from *C. myrtifolia* nodules collected in neighbor countries (Tunisia and Morocco) or in Europe (France) and even in other *Coriaria* species; *C. nepalensis* (Pakistan), *C. japonica* (Japan), and *C. microphylla* (Mexico)[11] and *Dryadoideae*, *Ceanothus* and *Datisca glomerata* in North American Midwest.[28] However, *C. arborea* micosymbionts still form a distinct sublineage of phylogenetic cluster 2. Nguyen et al[29] indicated that even at a global scale and for almost associate host species, the *gln*A*1*-based phylogeny did not result in an unambiguous separation of African, Eurasian and North American Cluster 2 strains.

Despite the utilization of the most recent protocol for cultivating cluster 2 microsymbionts which previously permitted the isolation of 2 symbiotic strains,[16,17] no *Frankia* isolate able to fulfill Koch's postulates was obtained in this study. While, 3 asymbiotic *Frankia* strains have been successfully isolated and cultivated in axenic conditions. Except few cases,[30-32] almost all cluster 4 members have been established from cluster 2 host plant species; *Coriariaceae*, *Datiscaceae, Dryadoideae*, and *Ceanothus*.[13-15] It is worth noting that no of these asymbiotic strains have been detected using the PCR-amplification and sequencing of *gln*A*1* gene on DNA directly extracted from root nodules. This is coherent with several other studies that never reported asymbiotic *Frankia* strains based on similar molecular methods directly applied on root nodules of cluster 2 host species.[8,11,18,22,28,33] However, Ramírez-Saad et al[34] were the first to report that beside symbiotic *Frankia* within root nodules of *Ceanothus*, asymbiotic stains were also present in the outer layers of the nodules. Recently other studies have confirmed this nodule surface emplacement of *Casuarina* incompatible *Frankia* strains.[35,36]

Alongside with the asymbiotic *Frankia* strains, the protocol used in the present study permitted the isolation of several other actinobacteria which were affiliated to *Micrococcus, Micromonospora, Nocardia, Plantactinospora*, and *Streptomyces* genera. Numerous are the studies which reported the isolation of non-*Frankia* actinobacteria from actinorhizal root nodules[18,20,34,37-45] as it was shown before for legume nodules which contain both rhizobial and non-rhizobial bacteria.[46,47] Three type strains; *Micromonospora coriariae*[42]; *Nocardia casuarinae*[44] and *Nocardia alni*[48] have been isolated respectively from *Coriaria; Casuarina* and *Alnus* root nodules. Interestingly, a *Micromonospora* strain isolated in the present study is very closely related to *M. coriariae* species suggesting a widespread occurrence of members of this species within *C. myrtifolia* root nodules. Plant growth promoting proprieties have been attributed to several of these non-*Frankia* actinobacteria such as nitrogen fixation,[20,40,41,49] phosphorous solubilization[18,50,51] hormone production,[18,44,51] siderophores,[18] or antagonisms against pathogenic fungal and insect predators.[18,50] Stimulation of actinorhizal plant nodulation in co-inoculation condition with *Frankia* was also reported.[41,52] More interesting 2 *Nocardia* strains, which have been isolated from *Casuarina glauca* nodules, were reported to use the *Frankia* infection pathway resulting in the induction of nodule-like structures in original host species and the promotion of its growth.[45]

In conclusion native *C. myrtifolia* in Algeria harbors *Frankia* microsymbionts which are closely related to other microsymbionts of disjunct *Coriaria* species within phylogenetic cluster 2. These *Frankia* strains have resisted isolation and cultivation in axenic condition even with the most recent protocol we previously reported.[16] Instead we obtained strains that failed to reinduce root nodules on *C. mlyrtifolia* seedlings and to fix nitrogen, which have been assigned to phylogenetic cluster 4. The fact that asymbiotic *Frankia* strains were consistently isolated from cluster 2 host plant species merit further investigation to help clarify their ecological role and their evolutionary radiation in actinorhizal symbiosis. This study provided new non-*Frankia* actinobacteria that should enhance our understanding of rhizosphere microbiome associated with actinorhizal nodules.

Author Contributions

MG designed the study. AG, ML and HC-S sampled Coriaria myrtifolia nodules. AG, IM and FG-G performed the experiments and analysis. MG wrote the manuscript. All authors approved the submitted version.

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