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Proposal of '*Candidatus Frankia alpina*', the uncultured symbiont of *Alnus alnobetula* and *A. incana* that forms spore-containing nitrogen-fixing root nodules

Adrien C. Meynier Pozzi, Aude Herrera-Belaroussi, Guillaume Schwob, Hector H. Bautista-Guerrero, Lorine Bethencourt, Pascale Fournier, Audrey Dubost, Danis Abrouk, Philippe Normand* and Maria P. Fernandez

Abstract

The members of the genus *Frankia* are, with a few exceptions, a group of nitrogen-fixing symbiotic actinobacteria that nodulate mostly woody dicotyledonous plants belonging to three orders, eight families and 23 genera of pioneer dicots. These bacteria have been characterized phylogenetically and grouped into four molecular clusters. One of the clusters, cluster 1 contains strains that induce nodules on *Alnus* spp. (*Betulaceae*), *Myrica* spp., *Morella* spp. and *Comptonia* spp. (*Myricaceae*) that have global distributions. Some of these strains produce not only hyphae and vesicles, as other cluster 1 strains do, but also numerous sporangia in their host symbiotic tissues, hence their phenotype being described as spore-positive (Sp+). While Sp+ strains have resisted repeated attempts at cultivation, their genomes have recently been characterized and found to be different from those of all described species, being markedly smaller than their phylogenetic neighbours. We thus hereby propose to create a '*Candidatus Frankia alpina*' species for some strains present in nodules of *Alnus alnobetula* and *A. incana* that grow in alpine environments at high altitudes or in subarctic environments at high latitudes.

INTRODUCTION

The genus *Frankia* [1] comprises nitrogen-fixing actinobacteria that establish root nodule symbioses in association with perennial dicot plants grouped into three orders, eight families and 23 genera of pioneer plants within the nitrogen fixing clade, a phylogenetically coherent group of three orders of host plants tracing to a single evolutionary origin [2]. Members of the genus *Frankia* have been characterized either as isolates or directly from symbiotic nodules, and placed into four 16S rRNA based clusters [3]: cluster 1 contains *Alnus*-, *Myricaceae*- and *Casuarinaceae*-infective strains (except those infective on members of the genus *Gymnostoma*), cluster 2 contains *Coriaria*-, *Datisca*-, *Ceanothus* - and *Rosaceae*-infective strains, cluster 3 contains *Elaeagnaceae*-, *Rhamnaceae*- and *Gymnostoma*-infective strains and cluster 4 contains non nitrogen-fixing or non-symbiotic strains. This grouping has been found with all markers studied, with all tree-reconstruction approaches [4] even with genome-wide approaches [5]. An early report of a nodule isolate with a

morphology characteristic of members of the genus *Frankia* [6] described re-nodulation of aseptically-grown host roots of *Alnus glutinosa*, but the strain was subsequently lost. The first confirmed isolation in 1978 was CpII, a cluster 1 strain [7], later described as the type strain for the species *Frankia torreyi* [8]. Since these works, many strains from the four clusters of the genus *Frankia* have been cultured *in-vitro* with relative ease.

The definition of species in the genus *Frankia* has been a long time in the making. A first proposal was done before isolates became available [9] but it was based on morphology of vesicles inside plant tissues and on cross-inoculation groups that were shown later not to hold with isolates [10]. The standard approach based on DNA–DNA hybridization [11] has identified several genomospecies but since these had few distinguishing physiological features, species were not named, except for *Frankia alni*. One had to wait for genomes to become available [12] and on a concerted international effort to see first species *Frankia alni* emended [13], and

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Keywords: alder; spores; Symbiotic.

Abbreviations: ANI, average nucleotide identity; ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and draft genome sequences reported in this study are MN952610/GCA_902806485 and MN965619/GCA_004803575 for AiOR and AvVan, respectively

One supplementary table is available with the online version of this article.

later, several others proposed: *Frankia casuarinae*, *Frankia elaeagni* [13], *Frankia asymbiotica* [14], *Frankia canadensis* [15], *Frankia coriariae* [16], *Frankia discariae* [17], *Frankia inefficax* [18], *Frankia irregularis* [19], *Frankia saprophytica* [20], *Frankia soli* [21], *Frankia torreyi* [8], and even three unisolated species: 'Candidatus *Frankia californiensis*' [22], 'Candidatus *Frankia datisciae*' [23] and 'Candidatus *Frankia meridionalis*' [24].

Unisolated strains represent a high percentage among actinorhizal endophytes [5]. They have been the subject of repeated unsuccessful attempts at isolation in pure culture and were reported mostly from clusters 1 and 2. The reasons for the failures are in general unknown, but it has been claimed that unusual physiological requirements did exist such as that for a very alkaline medium [25]. Cluster 1 unisolated strains mostly display a specific trait, the spore-positive (Sp+) phenotype, which characterizes the strains able to sporulate within plant tissues, during the symbiotic stage.

The existence of spherical cells, then designated as 'bacteroïdes', in *Alnus* nodules was mentioned as early as 1910 [26] and identified as spores contained in multilocular sporangia later, which were described as either abundant or nonexistent depending on the provenance [27]. Actually, it is the morphological resemblance between *in vitro* and *in planta* sporangia that clearly established that *Frankia* was indeed the symbiont present in actinorhizal plant root nodules [5], and not a fungus as previously thought. While *in vitro* sporulation was observed on almost all isolated strains cultivated on artificial media, *in planta* sporulation is only expressed profusely by some endophytes of the genus *Frankia* called spore-positive (Sp+), all refractory to cultivation. In contrast, no or very rare sporangia (less than one per 50 infected plant cells) are present in nodule tissues infected by Sp- *Frankia* strains [28], even if these strains are genetically able to abundantly sporulate in culture. The genetic determination of the ability to sporulate *in-planta* was clearly established in the literature [29–31] and the concept of a Sp+/Sp- dichotomy was based on ecological factors (reviewed by Pozzi et al. [30]), on adaptation to different hosts [27], on differences in infectivity [32], competitiveness [33], adaptation to cold temperatures [30] and recently in terms of phylogeny [34].

The genomes of three unisolated Sp+ *Alnus*-infective strains of members of the genus *Frankia* recently obtained revealed that (1) these strains harbour the smallest genomes of *Alnus*-infective members of the genus *Frankia* so far sequenced (approximately 5 Mbp draft genomes) [35] compared with previously sequenced *Alnus*-infective strains, harbouring approximately 7–8 Mbp genomes [36], (2) belong to several phylogenetic lineages [4, 30] and (3) Sp+ strains infective on the two alpine host species *Alnus alnobetula* and *Alnus incana* are genetically close. This paper aims at describing a species of the genus *Frankia* that encompasses Sp+ strains of members of the genus *Frankia* infective on these two alder species that grow in high altitude or high latitude alpine environments.

METHODS

Nodules were harvested from two alpine alder stands (Vanoise, Savoie, France and Ornon, Isère, France), colonized either by *A. alnobetula* (Ehrh.) K. (also known as *Alnus viridis*, green alder) or *A. incana* ssp. *incana* (L.) Moench (grey alder) and designated AvVan and AiOr, respectively. Microscope observations were done on semi-thin nodule sections stained with lactophenol blue as described previously [30] to determine the phenotype of the nodular strains. Genomes were obtained from nodular DNA and were accessible in GenBank under the accession numbers GCA_902806485 and GCA_004803575, for AiOr and AvVan, respectively [37].

The 16S rRNA sequences were obtained from complete genomes of all species of the genus *Frankia* described to date as well as one outgroup available from GenBank (complete names and accession numbers are given in Fig. 1). 16S rRNA gene sequences were aligned by using CLUSTAL X [38] as implemented on SEAVIEW [39]. Phylogenetic trees were reconstructed by the neighbour-joining [40] method running on SEAVIEW [39]. A bootstrap confidence analysis was performed with 1000 replicates to determine the reliability of the distance tree topologies obtained [41].

Multilocus sequence analysis (MLSA) phylogeny was done using five markers (*atpD*, *dnaA*, *ftsZ*, *pgk* and *rpoB*) defined for members of the genus *Frankia*, on sequences of *Alnus*-infective strains (thus isolated from *Alnus* and Myricaceae) previously published [34]. Sequence alignment and tree reconstructions were done as described above.

Average nucleotide identity (ANI) measurements were computed as described previously [42] to determine the relationship between genomes of members of the genus *Frankia* for all type strains of described species.

RESULTS

The distance between 16S rRNA genes of AiOr and AvVan was 0.29% (substitutions per 100 nucleotides) or three mismatches, while those with the closest *Alnus*-infective species of the genus *Frankia*, *F. alni*, were both 0.58%. Within cluster one that encompasses *Alnus*-, *Casuarinaceae*- and *Myricaceae*-infective species of the genus *Frankia*, distances were below 1.8%. Distances between AiOR and AvVan and other clusters were up to 3.4% (Table S1 available in the online version of this article).

The 16S rRNA phylogeny of all described species of the genus *Frankia* grouped AiOr and AvVan close to *F. alni* ACN14a with a bootstrap value of 98% (Fig. 1). As previously noted, 16S rRNA phylogeny positions cluster 2 close to cluster 1 strains contrary to most other markers [34].

MLSA phylogeny grouped together AiOr, AvVan and two other *A. alnobetula* Sp+ strains from the Alps (branch support 0.98) (Fig. 2). The three *A. alnobetula* strains grouped very tightly. The closest strain is Av 59.7, a cultured Sp- *Frankia* strain also isolated from *A. alnobetula* nodules.

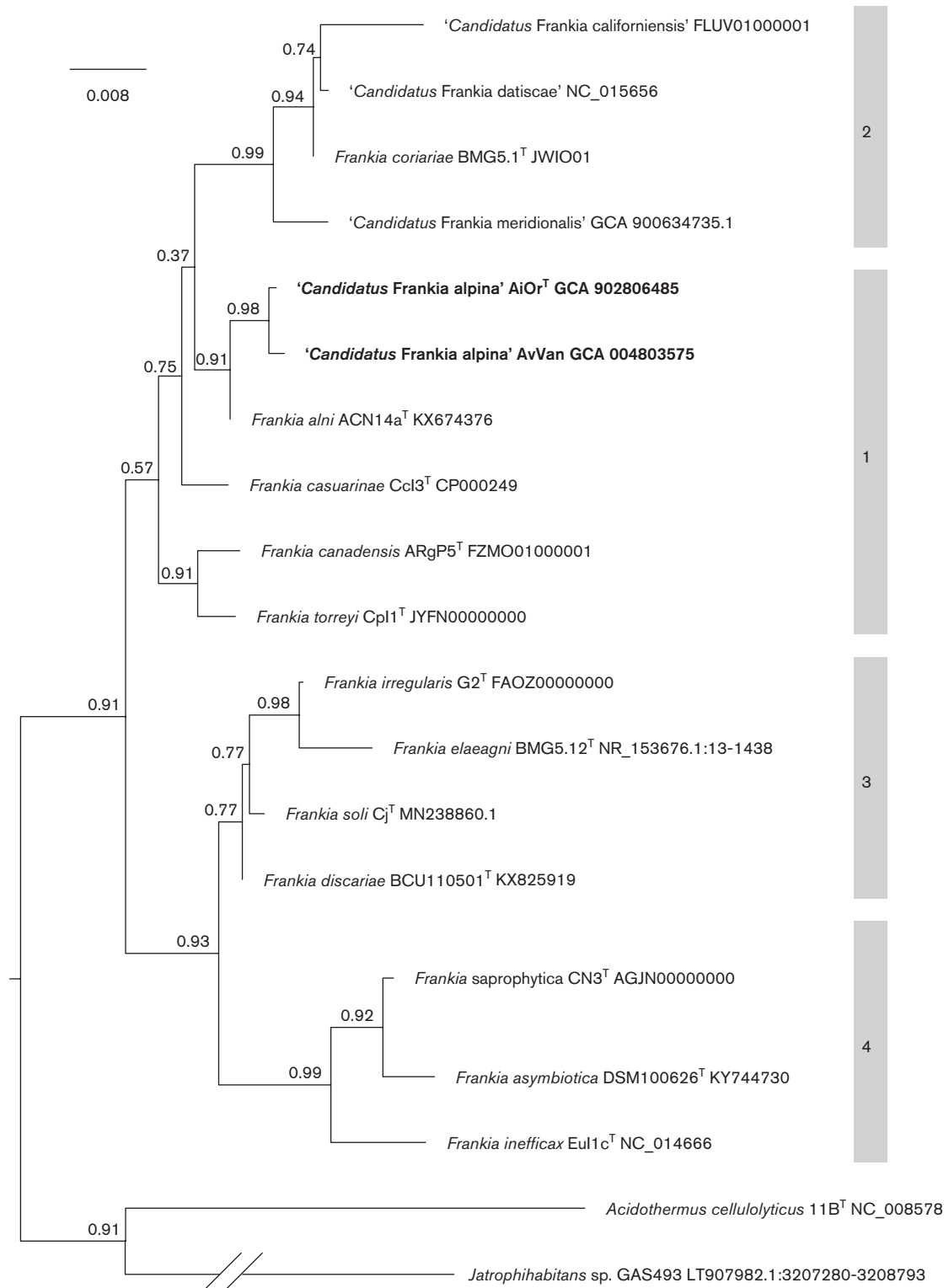


Fig. 1. NJ phylogenetic tree of members of the genus *Frankia* based on the 16S rRNA gene. The scale represents the average number of substitutions per nucleotide. Numbers reported at nodes are the bootstrap branch support. Numbers in shaded boxes on the right correspond to *Frankia* clusters [3].

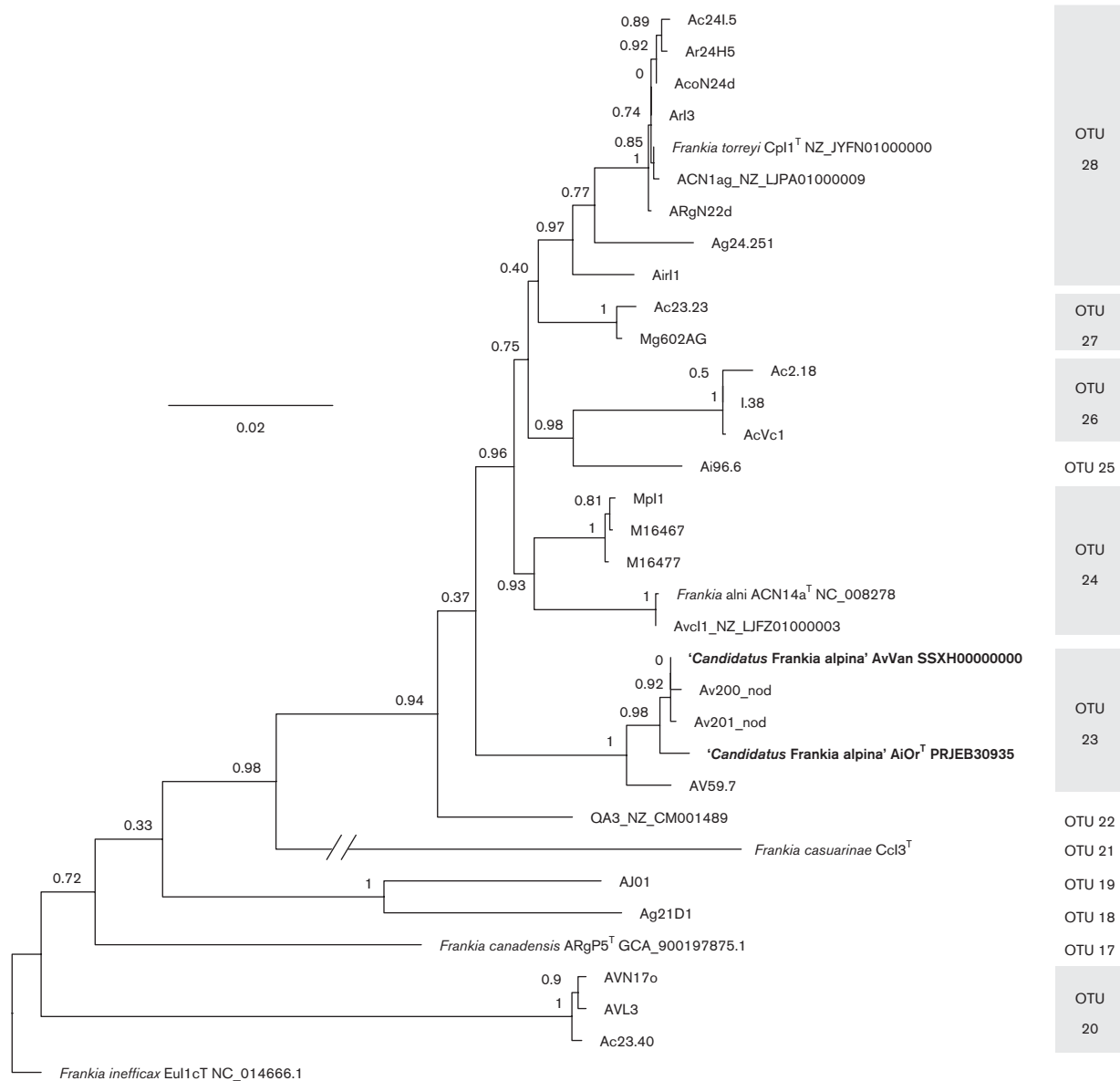


Fig. 2. *Alnus*-infective *Frankia* multilocus sequence analysis (MLSA) phylogeny. A maximum likelihood tree was inferred from the concatenation of five markers (*atpD*, *dnaA*, *ftsZ*, *pgk* and *rpoB*) into a super-alignment. The scale represents the average number of substitutions per nucleotide. Numbers at nodes are the aLRT SH-like branch support. Numbers in shaded boxes on the right correspond to recognized or predicted *Frankia* genomes that are the same as previously described [34].

ANI values are given in Table 1. AiOr and AvVan had values around 99% with one another and values equal to or below 90% with other *Alnus*-infective species of the genus *Frankia*. ANI values with *F. casuarinae*, the most closely related non *Alnus*-infective species of the genus *Frankia*, are about 80%.

Both AiOr and AvVan expressed the Sp+ phenotype in the nodules [37]: besides the presence of branched hyphae and vesicles present within *Alnus* nodule cells whatever the sporulation phenotype, they differentiated numerous spores contained in sporangia that filled the whole volume of the nodule cells (Fig. 3).

DISCUSSION

Both genomic and genetic data (16S, MLSA and ANI) and phenotypic traits grouped together AiOr and AvVan in the same species.

The genome size of strains proposed as '*Candidatus Frankia alpina*' is significantly lower at 5Mb than those of other *Alnus*-infective *Frankia* strains so far sequenced (about 7Mb) [5]. The only species of the genus *Frankia* with similarly small genome sizes belong to cluster 2 and to *F. casuarinae*, a sister species of *Alnus*-infective species of the

Table 1. Average nucleotide identity (ANI) between genomes of AiOr, AvVan and *Alnus*-infective cluster 1 genomes

Strain name	AiOr	AvVan	ACN14a	AvcI1	ACN1ag	CpI1	QA3	ARgP5	CcI3
' <i>Can. F. alpina</i> ' AiOr	100	98.6	88.1	88.0	88.9	88.9	89.7	81.2	80.3
' <i>Can. F. alpina</i> ' AvVan	98.4	100	88.6	88.6	89.3	89.4	90.1	81.9	81.0
<i>F. alni</i> ACN14a	87.6	88.2	100	99.3	90.9	90.9	89.0	81.2	80.6
<i>F. alni</i> AvcI1	87.5	87.9	99.3	100	90.7	90.8	88.9	81.2	80.5
<i>F. torreyi</i> ACN1ag	88.4	88.8	90.8	90.8	100	99.3	89.7	81.2	80.6
<i>F. torreyi</i> CpI1	88.4	88.8	90.8	90.8	99.1	100	89.7	81.0	80.8
<i>Frankia</i> sp. QA3	89.2	89.7	89.1	89.0	90.0	90.0	100	81.6	80.9
<i>F. canadensis</i> ARgP5	80.9	81.5	81.1	81.2	81.2	81.2	81.3	100	79.3
<i>F. casuarinae</i> CcI3	80.5	81.0	80.9	80.9	80.9	81.0	81.2	79.7	100

Bold type indicates values above 95%, the threshold used to consider two genomes as representing the same species.

genus *Frankia* in cluster 1. In both cases it was suggested that the reduced genome size had led to a higher dependence on the host plant [12]. In the extreme case of cluster 2 strains, genomes were found to be smaller than most (5.1 Mb) and to have lost several genes associated with saprophytic life such as *gvp*, *dpp* and *xdh* [24]. Those uncultured cluster 2 strains are thus considered obligate symbionts [5]. Like cluster 2 and *F. casuarinae* strains, alpine *Alnus* Sp+ strains showed a very low diversity in MLSA studies that included a large variety of species and sub-species of the genus *Alnus* and geographical origins [29, 30]. However even if Sp+ strains associated with alpine *Alnus* species are very close genetically, large-scale sampling highlighted two well supported clades corresponding to *A. alnobetula* and *A. incana* symbionts respectively [29, 30] and within *A. alnobetula* strains a division between North American–East Asian and European strains. The host-specificity observed in cross-inoculation experiments was narrower for Sp+ than that of Sp– strains and was different for *A. alnobetula* and *A. incana* Sp+ strains [33, 43]. All these results indicate the influence of

host biogeography and genetics on the symbiont phylogeny and ecology and are consistent with the possible obligate symbiont hypothesis proposed for these strains [34]. Using comparative genomics would allow determination of a core genome for these strains and better prediction of the physiological and ecological consequences of the Sp+ genotype.

There are now 13 species named [5] plus the very recently named *F. soli* [21] and there is every reason to think there should be other species named soon. Indeed several other phylogenetic clades of the genus *Frankia* have been identified through large-scale MLSA as distinct from those described species [34], these should soon be proposed as novel species. Among these potential novel species, several are *Alnus*-infective and correspond to different Sp+ lineages within cluster 1. The presence of Sp+ genotype has been described in the nodules of at least nine actinorhizal plant species whose isolated endophytes belong to cluster 2 and 3. Nothing is known about the diversity, the grouping of these strains in the *Frankia* phylogeny and their divergence from the Sp– strains isolated from the same host plant, they could thus represent novel species. Finally the comparison of all these Sp+ species should lead to the determination of a core genome of the Sp+ strains and the understanding of their evolutionary history.

The European *Alnus alnobetula* and *Alnus incana* have overlapping ranges that straddle the Alps and the Northern plains, with a difference in altitude with *Alnus alnobetula* growing at 500–2300 m and *Alnus incana* at 100–1800 m (www.florealpes.com). The two are quite distant phylogenetically [44], yet they share similar wet and nitrogen-poor biotopes.

For these reasons, we propose '*Candidatus Frankia alpina*' the uncultured symbionts in spore-containing nitrogen-fixing nodules of both *Alnus alnobetula* and *A. incana*.

DESCRIPTION OF '*CANDIDATUS FRANKIA ALPINA*'

'*Candidatus Frankia alpina*' (al.pi'na. L. fem. adj. *alpina*, of or pertaining to the Alps).

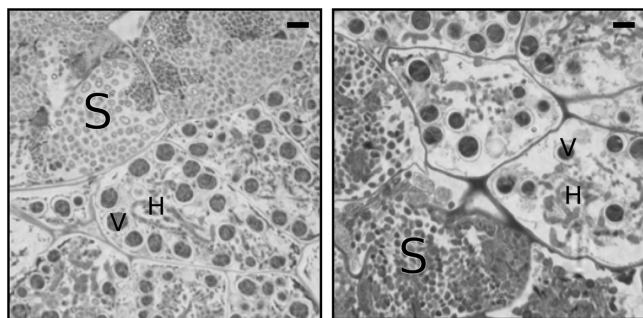


Fig. 3. Microscopic structures of Sp+ *Frankia* (capable of *in-planta* sporulation) within root nodules of *Alnus incana* (left) and *Alnus alnobetula* (right). Semi-thin nodule sections were stained with lactophenol blue. Some of the colonized cells mostly contain hyphae (h) and vesicles (v) while others are full of spores gathered in sporangia (s). Mature spores are unstained by the lactophenol blue (left) while immature spores appear darker (right). The scale bars represent 10 μm.

The species nodulates and fixes nitrogen in the root nodules of *Alnus alnobetula* (formerly *A. viridis*) and *A. incana* that both grow in alpine and subarctic regions [45]. It produces in symbiotic host tissues branched and septate hyphae, nitrogen-fixing vesicles and a large number of sporangia.

The proposed type strain is AiOr. Based on a draft-quality metagenome enriched for strain AiOr, the DNA G+C content is 71.60% and its genome size is 5.01 Mb. The GenBank/EMBL/DDJ accession number of the whole genome sequence of strain AiOr is GCA_902806485. A second strain of '*Candidatus* *Frankia alpina*' described here is AvVan, for which genome accession number is GCA_004803575.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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