Genome analysis and biogeographic distribution of the earliest divergent *Frankia* clade in the southern hemisphere

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Abstract

Coriariaceae are a small plant family of 14–17 species and subspecies that currently have a global but disjunct distribution. All species can form root nodules in symbiosis with diazotrophic *Frankia* cluster-2 strains, which form the earliest divergent symbiotic clade within this bacterial genus. Studies on *Frankia* cluster-2 mostly have focused on strains occurring in the northern hemisphere. Except for one strain from Papua New Guinea, namely *Candidatus* Frankia meridionalis Cppng1, no complete genome of *Frankia* associated with *Coriaria* occurring in the southern hemisphere has been published thus far, yet the majority of the Coriariaceae species occur here. We present field sampling data of novel *Frankia* cluster-2 strains, representing two novel species, which are associated with *Coriaria arborea* and *Coriaria sarmentosa* in New Zealand, and with *Coriaria ruscifolia* in Patagonia (Argentina), in addition to identifying *Ca. F. meridionalis* present in New Zealand. The novel *Frankia* species were found to be closely related to both *Ca. F. meridionalis*, and a *Frankia* species occurring in the Philippines, Taiwan, and Japan. Our data suggest that the different *Frankia* cluster-2 species diverged early after becoming symbiotic circa 100 million years ago.

Keywords: actinorhizal symbiosis; Coriariaceae; *Frankia*; microbiome; New Zealand; Papua New Guinea; Patagonia

Introduction

Coriariaceae are a small plant family within the Cucurbitales, consisting of the single genus *Coriaria* with about 16 plant species (Yokoyama et al. 2000, Renner et al. 2020). They are found worldwide but have a very disjunct distribution (Fig. 1, Data S1). Occupying harsher environments such as riverbeds and volcanic hills, they are considered to be pioneer species (Becking 1977). The origin of the family and their biogeographic history have been discussed in several publications (Good 1930, Skog 1972, Yokoyama et al. 2000, Nouioui et al. 2014), and according to the most recent data, the family evolved around 87 million years ago (Mya), and it split into two lineages between 46 and 57 Mya (Renner et al. 2020). The majority of species belong to the lineage which dispersed in the southern hemisphere and reached America *via* Antarctica (Renner et al. 2020). This includes eight species native to New Zealand, *Coriaria papuana* in Papua New Guinea, and two subspecies of *Coriaria ruscifolia* in America which have dispersed as far north as Mexico. The second lineage is found in the northern hemisphere; spanning from Mediterranean Europe and northern Africa (*Coriaria myrifolia*), Nepal and China (*Coriaria terminalis*, *Coriaria nepalensis,* and *Coriaria duthiei*), Japan (*Coriaria*

japonica), to Taiwan, and the Philippines (*Coriaria intermedia*; Fig. 1; Renner et al. 2020). All *Coriaria* species are described to form root nodules in symbiosis with *Frankia* spp. These specialized organs are formed when plants belonging to the nitrogen-fixing clade engage in symbiosis with soil diazotrophs; i.e. rhizobia for legumes and *Parasponia* spp., or *Frankia* spp. for actinorhizal plants, thereby allowing the host plant to access atmospheric dinitrogen in nitrogen-poor soils.

Frankia strains are members of the type genus of the Frankiaceae (Actinobacteria). Most of them have the capability of engaging in root nodule symbiosis with a polyphyletic group of so-called actinorhizal host plants that belong to the Fagales, Rosales, and Cucurbitales. Studies have shown that root nodule symbiosis evolved once, in the common ancestor of Fabales, Fagales, Cucurbitales and Rosales; the majority of the descending plant lineages since have lost their symbiotic capability (Griesmann et al. 2018, van Velzen et al. 2018, 2019, Libourel et al. 2023). van Velzen and colleagues et al. (2019) suggest that it is more plausible that *Frankia* rather than a rhizobium was the original symbiont. *Frankia* can be divided into four phylogenetically distinct clades, termed cluster-1 to cluster-4 (Normand et al. 1996, Sen
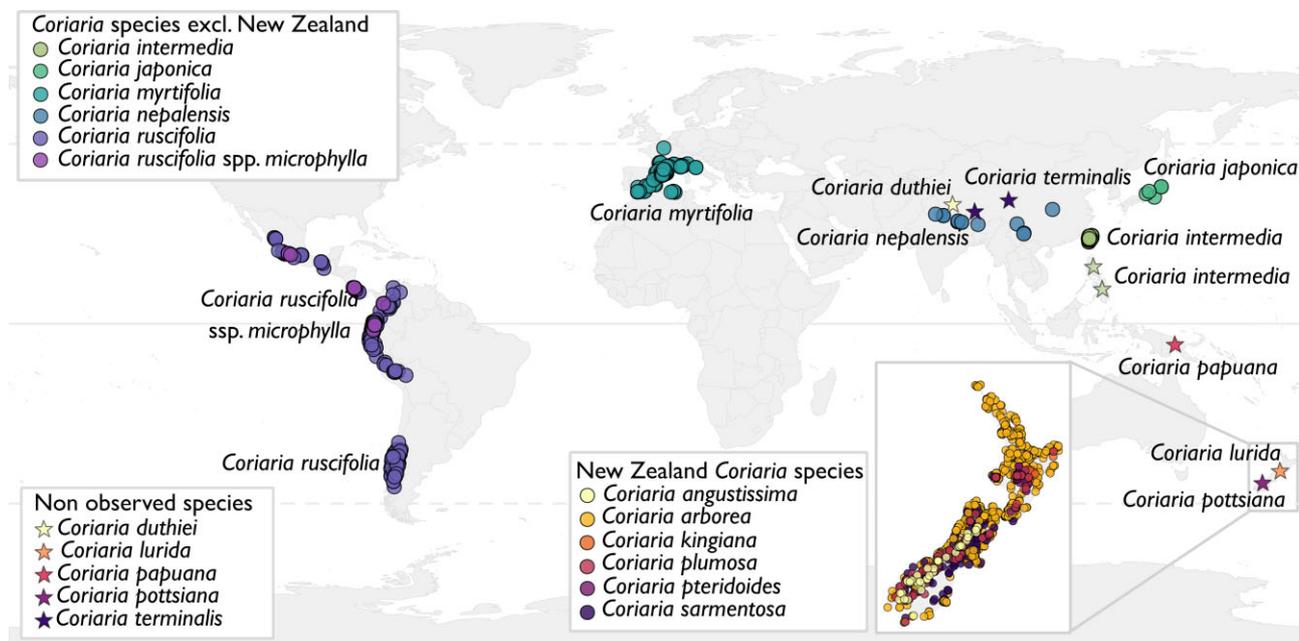
Figure 1. Distribution of Coriariceae. Data were gathered from iNaturalist in June 2023, and only research grade level observations were used. Data from the observations are available in Table S1. The different coloured circles illustrate the different species, and stars indicate the species for which reliable observations were lacking. *C. japonica* is native to Japan, *C. intermedia* to Taiwan and the Philippines, *C. myrtifolia* is native to the Mediterranean, *C. nepalensis* is found in the foothills of the Himalayan and China, *C. ruscifolia* and *C. ruscifolia* spp. *microphylla* are found near the west coast of America. In New Zealand, *C. arborea* is the most common species, followed by *C. plumosa. C. angustissima* and *C. sarmentosa* are found only on the Southern Island, while *C. kingiana* and *C. pteridoides* are mostly observed on the Northern Island. Observations of *C. duthiei* (Nepal), *C. terminalis* (Nepal and southern China), *C. papuana* (Papua New Guinea), *C. lurida,* and *C. pottsiana* (New Zealand) were not recorded. Map was produced in R (RStudio Team 2022).

et al. 2014, Nguyen et al. 2016). Cluster-1, -2, and -3 are symbiotic, while cluster-4 is not. The association of each cluster with its group of host plants has been discussed in several publications (Benson and Dawson 2007, Svistoonoff et al. 2014, Nguyen et al. 2016). *Frankia* cluster-2 is the earliest divergent symbiotic clade within the genus (Nguyen et al. 2016, 2019, Berckx et al. 2022a), and its host plants include *Ceanothus* (Rhamnaceae, Rosales), the Dryadoideae except for *Dryas octopetala* (Rosaceae, Rosales), Datiscaceae (Cucurbitales), and Coriariaceae (Cucurbitales).

Frankia cluster-2 strains are assumed to have a low saprotrophic potential and, to this date despite several unsuccessful and unpublished efforts, only two strains could be cultivated *in vitro: Frankia coriariae* BMG5.1 (Gtari et al. 2015, Nouioui et al. 2017) and *F. coriariae* BMG5.30 (Gueddou et al. 2019). Several more metagenome-assembled genomes (MAGs) of *Frankia* cluster-2 were sequenced from whole nodules obtained from both greenhouse cross-inoculation experiments and field samples. Here it was found that several closely related strains not only occupy a single nodule (Normand et al. 2017, Nguyen et al. 2019, Berckx et al. 2022a), but that some strains can persist as nodule-associated bacteria under unfavourable conditions and become the main symbiont in a more suitable host plant (Berckx et al. 2022a). Based on the analysis of the core genomes of *Frankia* cluster-2 strains from greenhouse samples, it could be concluded that the strains occurring in Eurasia, namely from France to Japan, form a single group with very low diversity (Nguyen et al. 2019). These strains were found to be the direct sister group of the *Frankia* cluster-2 strains occurring in North America, presumably by spreading *via* the Bering Strait (Nguyen et al. 2019). Further analysis of cluster-2 termed the Eurasian and North American strains the 'continental' lineage, to contrast with the 'island' lineage: those *Frankia* cluster-2 strains which occur on islands in the Pacific Ocean, specifically Japan, Taiwan, the Philippines, and Papua New Guinea (Nguyen et

al. 2019, Berckx et al. 2022a). Our previous study highlighted the importance of sequencing field nodules: while studies using cross-inoculation studies or isolation of greenhouse nodules of *D. glomerata* and *C. myrtifolia* found continental *Frankia* cluster-2 strains in Japan (Gtari et al. 2015, Nguyen et al. 2019), sequencing field samples directly identified island lineage *Frankia* to be the main strains present in *C. japonica* nodules (Berckx et al. 2022a).

Initial studies of the *Frankia* cluster-2 phylogeny, using the marker genes *glnA, dnaA* and 16S rRNA, led to the conclusion that those *Frankia* strains associated with Coriariaceae in New Zealand form a sister group to other *Frankia* cluster-2 strains (Newcomb and Wood 1987, Benson et al. 1996, Nouioui et al. 2014, Nguyen et al. 2016). Interestingly, the study by Nouioui et al. (2014) found that *Frankia* cluster-2 strains in Mexico from *C. ruscifolia* subsp. *microphylla* were most closely related to *Frankia* strains from Nepal. However, it is important to point out that this study did not support a monophyletic position of *C. ruscifolia* in Mexico, and the *Frankia* phylogeny was built on only three phylogenetic marker genes. Several studies since have shown that phylogeny based on a low number of marker genes does not resolve the *Frankia* phylogeny as reliably as core genome or multi-locus sequence analysis does (Nguyen et al. 2016, 2019, Pozzi et al. 2018, Herrera-Belaroussi et al. 2020).

In light of the recent, whole genome analysis of *Frankia* cluster-2 (Berckx et al. 2022a), we were interested to see if *Frankia* strains occurring in New Zealand form a third lineage within cluster-2, or if they group with the strains making up the island lineage. Since *Coriaria* spp. in New Zealand are closely related to *C. ruscifolia* and *C. ruscifolia* subsp. *microphylla* in America (Yokoyama et al. 2000, Renner et al. 2020), we included nodule samples collected in Patagonia (Argentina) as well. We hypothesize that the *Frankia* cluster-2 strains occurring in New Zealand and Patagonia are more closely related to the island lineage than to the continental lineage, such

as strains from Nepal. As New Zealand is the centre of diversity for Coriariaceae, it would be interesting to see if there is a high species diversity of *Frankia* cluster-2 here as well, indicating hosts and microsymbionts have co-evolved together over the last 25 million years. In addition, this would give more insights into the early evolution of root nodule symbiosis as a whole. Further, we were interested in analysing the whole bacterial community of *Coriaria* root nodules. Recent reports have been made of the isolation of non-nodulating bacteria isolated from actinorhizal nodules, such as *Micromonospora* (Trujillo et al. 2006, Carro et al. 2013, Ghodhbane-Gtari et al. 2021) or *Streptomyces* (Allen et al. 1966, Wollum II et al. 1966, Ghodhbane-Gtari et al. 2010, Berckx et al. 2022b). We wanted to investigate the microbiome of *Frankia* cluster-2 nodules and analyse whether differences could be found between sites and species.

Material and methods

Biological material

Nodules and leaves of *Coriaria sp.,* as well as the soil around their root system, were collected along the Waimakariri riverbed near Christchurch (New Zealand), in February and March 2020. Nodules were gently washed in river water to remove excess soil. Nodules and leaves were immediately stored in pure ethanol at room temperature until arriving back in Stockholm where they were stored at −20°C until further analysis. The soil was collected in pouches and kept at 4°C. In the greenhouse, uninoculated seedlings of *C. arborea, C. intermedia, C. myrtifolia,* as well as *Datisca glomerata* were infected with the collected soil. Plants were checked for nodulation status before infection. Plants were watered with MilliQ water two times per week, and with $\frac{1}{4}$ strength Hoaglands without nitrogen one time per week (Hoagland and Arnon 1950). However, plants died six to eight weeks after infection. No nodules could be collected.

Nodules and leaves were collected from *Coriaria ruscifolia*, while the soil was collected from around its root system, near Lago Correntoso in Patagonia, Argentina, in March 2019. DNA isolation failed for freeze-dried nodules. The collected soil was used to infect two *C. ruscifolia* plants, originating from the Nymphenburg Botanical Garden (Munich, Germany), in a greenhouse at LMU Munich, in 2020. Nodules were collected sixteen weeks after infection and stored in 70% EtOH until arriving back in Stockholm where they were stored at −20°C until further analysis. In a second sampling trip, nodules and leaves of *C. ruscifolia* were collected from the same area in Patagonia, in October 2021. Nodules were collected in RNAlater (ThermoFisher Scientific) until arriving back in Stockholm where they were stored at −20°C until further analysis. Leaves were pressed and dried.

Vouchers of the *Coriaria* samples were deposited in the herbarium of the Swedish Museum of Natural History (Stockholm, Sweden), except for the field material of *C. ruscifolia,* which was deposited at the herbarium of Centro Regional Universitario Bariloche (San Carlos de Bariloche, Argentina). An overview of the collected specimens, geographic coordinates, and voucher numbers is given in Table S2A.

DNA isolation

Ethanol was removed from all plant parts using a speed-vac for six to eight hours, after which samples were shock-frozen and kept at −20°C until handling further. To remove RNAlater, samples were washed three times in sterile MilliQ water, patted dry to remove excess water, and immediately used for DNA isolation. Samples

were ground in liquid nitrogen using a sterile mortar and pestle. DNA from leaves and nodules was extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Germany). Polyclar AT (Serva, Germany) was added to the lysis buffer in all samples to remove excess polysaccharides. Ground nodules were sonicated in the lysis buffer as described by Nguyen et al. (2019). DNA of nodules was cleaned using the NucleoSpin gDNA Clean-up kit (Macherey-Nagel, Germany), and concentration was determined using Nanodrop (Thermo Scientific, Sweden) and Qubit (ThermoFisher, Sweden).

Frankia genome sequencing, assembly, phylogeny

Sequencing of the DNA from nodules was performed as previously described (Nguyen et al. 2019, Berckx et al. 2022a). The raw reads were assembled into *Frankia* metagenome-assembled genomes as described before (Berckx et al. 2022a). For the phylogenetic analysis and calculation of the average nucleotide identity (ANI), the EDGAR 3.0 platform was used (Blom et al. 2009, 2016, Dieckmann et al. 2021). The ANI is calculated between two genomes for consecutive 1020 nt fragments of the core genome, after which the average is taken. For the identification of *nod* gene operons, BLAST searches based on AA sequence were used in GenDB platform (Meyer et al. 2003). Sequences were aligned using ClustalO algoritm in SeaView 5.0 (Galtier et al. 1996, Gouy et al. 2021). Sequences were concatenated, and the phylogenetic tree was built using maximum likelihood phylogenies (PhyML) available through SeaView, with 100 iterations.

Coriaria species determination

The species determination of *Coriaria* was based on the *trnL* and ITS intergenic regions, as well as the maturase K (*matK*) sequence of the chloroplast as described by Renner et al. (2020), with an added mixture of trehalose/bovine serum albumin (BSA)/Tween-20 (TBT-PAR; Samarakoon et al. 2013) to enhance PCR efficiency. Primer sequences, amplified gene regions, and accession numbers of used references, are given in Table S2B. In brief, PCR conditions were as followed: 94°C for 3 min, 15 cycles of 94°C for 1 min 30 sec, 45°C for 2 min, 60°C for 3 min, 15 cycles of 94°C for 1 min 30 sec, 45°C for 2 min, 72°C for 3 min, and final elongation cycle of 72°C for 15 min. PCR products were digested using EcoRI and BamHI (Thermo Scientific, *trnL* and ITS) or KpnI and XhoI (Thermo Scientific, *matK*), and ligated pBluescript plasmids (Thermo Scientific) were used to transform One Shot™ TOP10 Chemically Competent *E. coli* cells (Invitrogen).

Analysis of the regions was performed using SeaView 5.0 (Galtier et al. 1996, Gouy et al. 2021), by aligning the obtained sequences to all reference sequences (Renner et al. 2020) using MUSCLE. Next, phylogenetic trees for individual regions and concatenated regions were built, using maximum likelihood phylogenies (PhyML) available through SeaView, with 100 iterations.

Microbiome analysis

The microbiomes were analysed as follows: 16S metabarcoding was performed on the same DNA extracts as presented here, as well as on previous extracts (Berckx et al. 2022a), by amplifying and sequencing the V3-V4 region. PCR amplification, sequencing and initial analysis were performed by Novogene. In brief, DNA quality was assessed by gel electrophoresis and photometrically using a NanoDrop ND-2000c UV–Vis spectrophotometer (NanoDrop Technologies). The V3-V4 region of the 16 s rRNA gene was amplified using barcoded versions of the universal primer set 341F

Table 1a. Overview of collected samples, the origin of inoculum, and accession number of bacterial MAGs. The genome names were based on the method established before (Nguyen et al. 2019, Berckx et al. 2022b). The coordinates; herbarium voucher information, and plant gene sequences for species delineation are presented in Table S2. The full accession numbers of the assembly and the contigs are presented in Table S4a. *This nodule sample was induced in a greenhouse using inoculum from Patagonian soil, whilst all other genomes came directly from field material.

Inoculum	Host plant	Country of origin	Genome	Accession number
Cas1	*Coriaria* sp.	New Zealand	Cas1_Cas_nod	GCA_963555895
Cas2	*Coriaria* sp.	New Zealand	Cas2_Cas_nod	GCA_963555855
Cas3	*Coriaria* sp.	New Zealand	Cas3_Cas_nod	GCA_963555955
Cas4	*Coriaria* sp.	New Zealand	Cas4_Cas_nod	GCA_963555915
Cas5	*Coriaria* sp.	New Zealand	Cas5_Cas_nod	GCA_963555945
Cas7	*Coriaria* sp.	New Zealand	Cas7_Cas_nod	GCA_963555905
Cas8	*Coriaria* sp.	New Zealand	Cas8_Cas_nod	GCA_963555845
Cr1*	*Coriaria ruscifolia*	Argentina	Cr1_Cr_nod	GCA_963555865
Cr2	*Coriaria ruscifolia*	Argentina	Cr2_Cr_nod	GCA_963555885
Cr3	*Coriaria ruscifolia*	Argentina	Cr3_Cr_nod	GCA_963555925
Cr4	*Coriaria ruscifolia*	Argentina	Cr4_Cr_nod	GCA_963555875
Cr5	*Coriaria ruscifolia*	Argentina	Cr5_Cr_nod	GCA_963555935

(5′- CCT AYG GGR BGC ASC AG-3′) and 806R (5′-GGA CTA CNN GGG TAT CTA AT-3′). Amplicon quality was assessed by agarose gel electrophoresis before library preparation. Library quality was controlled using a Qubit 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Illumina NovaSeq 6000 platform employing 250 bp paired-end reads at Novogene Europe (Cambridge, UK). Demultiplexed reads delivered by Novogene were then trimmed using Trimmomatic version 0.38 (Bolger et al. 2014), accessed via the Galaxy portal (Jalili et al. 2020). Analysis of the reads was performed using DADA2 (Callahan et al. 2016), phyloseq (McMurdie and Holmes 2012), and metacoder packages in R. The number of initial, filtered, denoised, and non-chimaera reads are given in Table S3. The R script used is available upon request to the first author.

Results and discussion
Identification of the *Coriaria* species

The phylogenetic tree built on three marker genes (*matK, trnL,* and ITS) for the *Coriaria* samples are given in Fig. S1. Our samples of *Coriaria ruscifolia*, collected in Patagonia and obtained from the Nymphenburg Botanical Garden, are included in a clade that also comprises species of *Coriaria* from New Zealand. It should, however, be noted that the species delimitation of *Coriaria ruscifolia* appears unclear and may need further research. A previous phylogenetic study has indicated that the species is not monophyletic (Figs S3 and S4 of Renner et al. 2020). A sample of *Coriaria ruscifolia* included in that study was shown to be more closely related to the Mexican *Coriaria ruscifolia* subsp. *microphylla,* while two other accessions were found to be more closely related to different New Zealand (NZ) *Coriaria* species (Renner et al. 2020). Our analyses support this conclusion. This is interesting as due to the distance and the fact that *Coriaria* spp. seeds are spread by birds which do not migrate long distances (Burrows 1995), there should not have been exchange of genetic material between South American and NZ *Coriaria* species within the last 30 million years. Further investigation of the South American *Coriaria* speciation is needed, and beyond the scope of this study. As a consequence, we refer to the Patagonian sample as *Coriaria ruscifolia,* the *Coriaria* species in South America.

The New Zealand samples were unidentifiable at the growth stage, as no flowers or fruits were produced. At the time of col-

lection, the *Coriaria* plants from Christchurch, New Zealand (NZ), were assumed to be *Coriaria arborea* or *Coriaria sarmentosa,* the two most likely species, based on distribution and occurrence. The phylogenetic analysis did not yield conclusive results (Fig. S1), and the species identities remain unclear.

Identification of novel *Frankia* cluster-2 strains

The *Frankia* metagenome-assembled genomes (MAGs) obtained in this study were named according to the nomenclature established before (Nguyen et al. 2019, Berckx et al. 2022a): [name of inoculum]_[initials of host plant from which DNA was isolated]_["nod" for direct isolation of mixed plant and bacterial DNA from nodules, "vc" for isolation of DNA from vesicle clusters isolated from nodules]. The NZ samples and their associated inoculum were abbreviated to Cas, with continuous numbering. In total eight different sites were sampled, of which seven MAGs could be assembled (Tables 1a and 1b). Cas1_Cas_nod was found to be a chimeric MAG of at least two *Frankia* strains, which could not be separated bioinformatically, explaining the larger genome size (Table 1b). The *Coriaria ruscifolia* greenhouse MAG, originating from collected soil from Patagonia, was termed Cr1_Cr_nod, referring to *C. ruscifolia,* the only endemic *Coriaria* species. The MAGs Cr2_Cr_nod, Cr3_Cr_nod, Cr4_Cr_nod, and Cr5_Cr_nod originate from nodule field samples taken from root systems of *C. ruscifolia* growing in Patagonia.

A core genome tree was constructed for the *Frankia* cluster-2 sequences available thus far (Fig. 2). Previous work has shown that cluster-2 can be split into two groups: the continental lineage and the island lineage (Berckx et al. 2022a). We found that the *Frankia* strains in this study also belonged to the island lineage, forming a direct sister group of those *Frankia* strains found in the Philippines, Taiwan, and Japan (Fig. 2). The novel MAGs were grouped into two sister groups based on their geographic origin, except for Cr1_Cr_nod (highlighted in Fig. 2 in orange) which grouped with the NZ MAGs, and Cas1_Cas_nod (highlighted in Fig. 2 in yellow) which grouped with *Candidatus* Frankia meridionalis Cppng1, which originates from Papua New Guinea (Nguyen et al. 2019).

We were interested to see which species the *Frankia* MAGs we sequenced represented, or if they belonged to a novel species. The average nucleotide identity (ANI) was calculated for each MAG of this study compared to other cluster-2 species belonging to both the island and the continental lineage (Fig. 3 for median values;

Table 1b. Overview of genome size and completeness, N50 values, coverage, and the number of reads from the genomes of collected samples. The complete BUSCO values for the genomes are given in Table S4B. *This nodule sample was induced in a greenhouse using inoculum from Patagonia, whilst all other genomes came directly from field material. **This MAG consists of two *Frankia* strains which cannot be separated.

| Inoculum	Genome	Genome size	BUSCO	N50	Coverage	Reads
Cas1	Cas1_Cas_nod	9.9 Mbp**	92%	14560	21	694491
Cas2	Cas2_Cas_nod	4.8 Mbp	84%	13339	22	352012
Cas3	Cas3_Cas_nod	6.1 Mbp	90%	30570	45	915241
Cas4	Cas4_Cas_nod	5.4 Mbp	90%	27728	31	558310
Cas5	Cas5_Cas_nod	4.4 Mbp	73%	9506	11	161312
Cas7	Cas7_Cas_nod	5.6 Mbp	90%	23190	41	779152
Cas8	Cas8_Cas_nod	4.8 Mbp	71%	9560	10	163218
Cr1*	Cr1_Cr_nod	5.8 Mbp	90.5%	12065	18	348716
Cr2	Cr2_Cr_nod	5.3 Mbp	92.60%	83965	137	2431428
Cr3	Cr3_Cr_nod	5.6 Mbp	94.60%	80581	150	2766817
Cr4	Cr4_Cr_nod	5.4 Mbp	84.50%	48191	62	1116218
Cr5	Cr5_Cr_nod	5.2 Mbp	71.60%	28167	52	866514
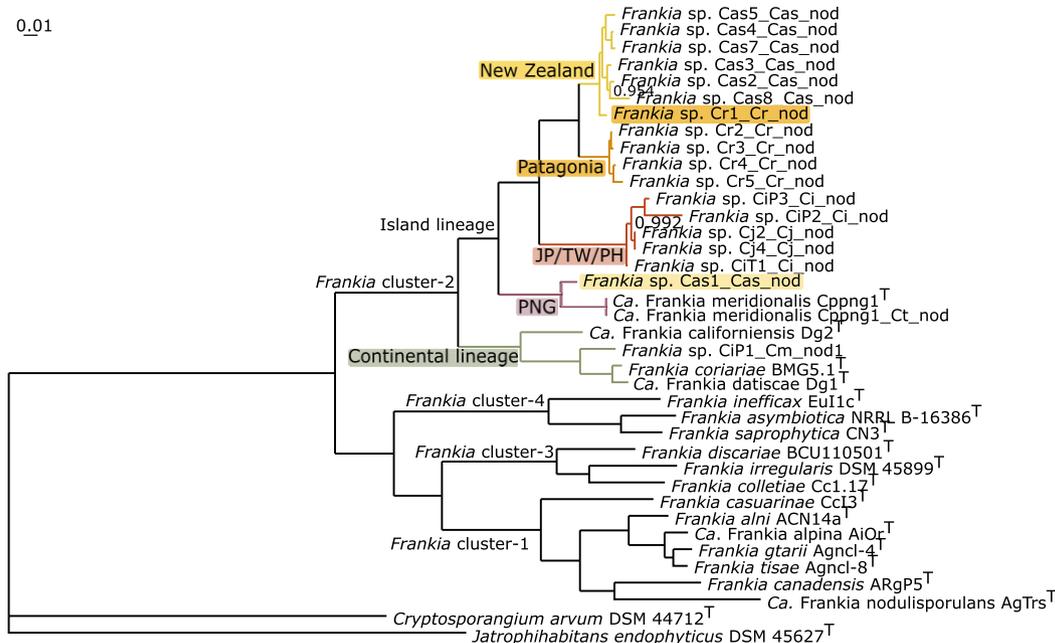
Figure 2. Core genome tree of *Frankia* cluster-2. Tree for 38 genomes, built out of a core of 359 genes per genome, 13642 in total. The core gene sets were aligned using MUSCLE (Edgar 2004) and subsequently concatenated, resuling in an alignment of 150104 AA residues per genome, 5703952 in total. The tree was calculated using FastTree 2 inference of maximum-likelihood phylogeny (Price et al. 2010), available through the EDGAR 3.0 platform (Dieckmann et al. 2021). The scale represents AA substitutions, and the branch support values were computed using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999). As the vast majority of the branches shows a perfect support value of 1.00, only lower values are presented in the tree. The different groups within cluster-2 are annotated in different colours and with text. Abbreviations: Ca.: *Candidatus*; JP/TW/PH: Japan/Taiwan/the Philippines; PNG: Papua New Guinea. The *Frankia* MAGs from New Zealand are depicted in yellow. *Frankia* sp. Cr1_Cr_nod, the greenhouse sample going back to Patagonia, is highlighted in orange and was found to group with the New Zealand MAGs. The MAGs from Patagonia are depicted in orange. The *Frankia* MAGs from the JP/TW/PH (Berckx et al. 2022b) are depicted in red. *Candidatus* Frankia meridionalis Cppng1[T] and *Ca.* F. meridionalis Cppng1_Ct_nod, from PNG, are illustrated in purple (Nguyen et al. 2019). The genome of *Frankia* sp. Cas1_Cas_nod is highlighted in yellow and was found to group with *Ca.* F. meridionalis. All these genomes comprise the island lineage. The continental lineage is given in green, represented by *Ca.* Frankia californiensis Dg2[T] (Nguyen et al. 2016), *Frankia* sp. CiP1_Cm_nod1 (Berckx et al. 2022b), *Frankia coriariae* BMG5.1[T] (Gtari et al. 2015), and *Ca.* Frankia datiscae Dg1[T] (Persson et al. 2015). *Frankia* cluster-1, cluster-3, and cluster-4 are illustrated in black. Cluster-4 is represented by *Frankia inefficax* Eul1c[T] (Nouioui et al. 2017b), *Frankia asymbiotica* NRRL B-16386[T] (Nouioui et al. 2017c), and *Frankia saprophytica* CN3[T] (Nouioui et al. 2018a). Cluster-3 is represented by *Frankia discariae* BCU110501[T] (Nouioui et al. 2017a), *Frankia irregularis* DSM 45899[T] (Nouioui et al. 2018b), and *Frankia colletiae* Cc1.17[T] (Nouioui et al. 2023a). Cluster-1 is represented by *Frankia casuarinae* CcI3[T] (Nouioui et al. 2016), *Frankia alni* ACN14a[T] (Nouioui et al. 2016), *Ca.* Frankia alpina AiOr[T] (Pozzi et al. 2020), *Frankia gtarii* Agncl-4[T] (Nouioui et al. 2023b), *Frankia tisai* Agncl-8[T] (Nouioui et al. 2023b), *Frankia canadensis* ArgP5[T] (Normand et al. 2018), and *Ca.* Frankia nodulisporulans AgTrs[T] (Herrera-Belaroussi et al. 2020). The tree is rooted by *Cryptosporangium arvum* DSM 44712[T] (Tamura et al. 1998) and *Jatrophihabitans endophyticus* DSM 45627[T] (Madhaiyan et al. 2013).

	Cas1	Cas2	Ca81	Cas5	Cas3	Cr1	Cas4	Cas7	Cr3	Cr2	Cr4	Cr5	CiT1	Cj2	Cj4	CiP2	Cppng1	CiP1	Dg1	BMG5.1	Dg2
Ca. Frankia californiensis Dg2[T]	80.3	80	79.6	80.1	79.5	80	79.8	79.8	79.6	79.7	79.5	79.5	79.1	79.1	79	78	80.2	86	86	86	100
Frankia coriariae BMG5.1[T]	79.7	79.7	79.2	79.5	79.1	79.5	79.2	79.1	79.2	79.2	78.9	79.2	78.9	78.9	78.8	77.7	79.5	91.4	96.8	100	86.2
Ca. Frankia datiscae Dg1[T]	80	80	79.7	80	79.3	79.9	79.5	79.5	79.3	79.3	79.1	79.3	79.1	79	79	78.5	80	91.3	100	96.8	86.7
Frankia sp. CiP1_Cm_nod1	80.4	80.4	80	80.2	79.4	80	80	79.8	79.4	79.3	79.1	79.3	79.4	79.4	79.3	78.5	80.3	100	91.1	91.6	86.4
Ca. Frankia meridionalis Cppng1[T]	99.3	81.2	81	80.9	80.7	80.9	80.6	80.5	81	80.6	80.5	80.6	80.5	80.2	80.1	79.6	100	80.4	80.1	80	80.6
Frankia sp. CiP2_Ci_nod	83.2	84.3	83.9	84.1	83.4	83.9	83.3	83.4	83.5	83.6	83.6	83.5	96.2	96.3	96.3	100	79.8	78.6	78.5	78.1	78.6
Frankia sp. Cj4_Cj_nod	83.9	85	84.9	84.9	84.1	84.6	84.1	84.1	84.1	84.1	84.1	84	99.6	99.8	100	96.2	80.2	79.4	79.2	78.9	79.5
Frankia sp. Cj2_Cj_nod	84.1	85	84.8	84.9	84.2	84.5	84.2	84.2	84.1	84.1	84.1	84.1	99.6	100	99.8	96.3	80.4	79.3	79.2	79.2	79.6
Frankia sp. CiT1_Ci_nod	83.9	85	84.8	84.9	84	84.7	84.2	84.1	84.4	84.1	84.1	84.3	100	99.6	99.6	96.2	80.6	79.2	79	79	79.3
Frankia sp. Cr5_Cr_nod	92.4	93.1	93.1	92.8	92.8	92.9	92.7	92.6	99.8	99.8	99.8	100	83.9	83.9	83.8	83.2	80.3	79.6	79.2	79.3	80.1
Frankia sp. Cr4_Cr_nod	92.4	93.3	93	92.9	92.8	93	92.7	92.7	99.9	99.8	100	99.8	84	83.9	83.9	83.4	80.3	79.1	79.1	79.1	79.5
Frankia sp. Cr2_Cr_nod	92.9	93.5	93.3	93.1	93	93.2	92.8	92.8	99.9	100	99.8	99.8	84	84	84	83.2	80.3	79.2	79.1	79.1	79.5
Frankia sp. Cr3_Cr_nod	92.8	93.5	93.3	93.1	93	93.2	92.9	92.9	100	99.9	99.9	99.8	84	83.9	83.9	83.3	80.9	79.5	79.1	79.1	79.8
Frankia sp. Cas7_Cas_nod	98.8	98.8	98.6	98.4	98.3	98.7	99.1	100	92.8	92.8	92.6	92.5	83.9	84	83.9	83.2	80.3	79.4	79.2	78.9	79.7
Frankia sp. Cas4_Cas_nod	98.9	98.8	98.7	98.5	98.4	98.8	100	99.1	92.9	92.8	92.8	92.6	84	83.9	84	83.3	80.2	79.8	79.2	79.2	79.8
Frankia sp. Cr1_Cr_nod	98.8	98.8	98.6	98.6	98.5	100	98.8	98.7	93.2	93.2	92.9	92.9	84.4	84.3	84.3	83.7	80.7	79.7	79.2	79.3	79.9
Frankia sp. Cas3_Cas_nod	98.9	99.8	99.7	99	100	98.4	98.4	98.3	92.9	92.9	92.8	92.7	83.9	83.8	83.8	82.9	80.4	79.1	78.8	78.6	79.6
Frankia sp. Cas5_Cas_nod	99.1	99.2	99	100	99	98.6	98.5	98.4	93.1	93	92.8	92.8	84.8	84.8	84.8	83.8	81.1	80.2	79.6	79.7	80.1
Frankia sp. Cas8_Cas_nod	99	99.7	100	99	99.7	98.6	98.7	98.7	93.3	93.2	93	92.9	84.6	84.7	84.7	83.5	81.1	79.9	79.4	79.2	80
Frankia sp. Cas2_Cas_nod	99.2	100	99.7	99.2	99.8	98.9	98.9	98.8	93.5	93.4	93.2	93	84.9	84.9	84.9	84	81.2	80.3	80	79.7	80.4
Frankia sp. Cas1_Cas_nod	100	96.6	96	95.9	96.2	95.8	95.7	96.2	88.7	88.5	88.5	88.1	82.5	82.5	82.5	81.7	97.9	80.2	80	79.8	80.4

Figure 3. Median average nucleotide identity (ANI) of core genomes of *Frankia* cluster-2. The query-genomes are on the y-axis. Names of genomes along the x-axis have been reduced. The analysis includes the novel *Frankia* MAGs of this study and those representatives of all *Frankia* cluster-2 species published previously. Seven different groups with high ANI values (95% or more) can be distinguished. Going from left to right: the first group includes novel MAGs of New Zealand (Cas1_Cas_nod, Cas2_Cas_nod, Cas8_Cas_nod, Cas5_Cas_nod, Cas3_Cas_nod, Cas4_Cas_nod, and Cas7_Cas_nod), as well as the greenhouse nodules originating from Patagonia soil (Cr1_Cr_nod). The second group includes MAGs from all Patagonian field samples (Cr3_Cr_nod, Cr2_Cr_nod, Cr4_Cr_nod, and Cr5_Cr_nod). The third group includes MAGs from Taiwan (CiT_Ct_nod), Japan (Cj2_Cj_nod and Cj4_Cj_nod) and the Philippines (CiP2_Ci_nod) (Berckx et al. 2022b). The MAG Cas1_Cas_nod from New Zealand was found to share high ANI with MAGs of *Candidatus* Frankia meridionalis Cppng1 (Nguyen et al. 2019). The fifth group includes the novel species of the continental lineage, namely CiP1_Cm_nod1 (Berckx et al. 2022b). The sixth group is formed by *Candidatus* Frankia datiscae Dg1 (Persson et al. 2011, 2015) and *Frankia coriariae* BMG5.1 (Gtari et al. 2015). The seventh and last group is represented by *Candidatus* Frankia californiensis Dg2 (Nguyen et al. 2016). ANI calculations were made using EDGAR 3.0 platform (Dieckmann et al. 2021). See Fig. S2 for mean values.

Fig. S2 for mean values). Using the accepted 95–96% threshold to separate species (Goris et al. 2007, Richter and Rosselló-Móra 2009), we found that the samples from NZ and Patagonia, respectively, represent two novel *Frankia* species.

The *Frankia* MAGs of all NZ samples (Cas1_Cas_nod to Cas8_Cas_nod) share between 98.3% and 99.7% ANI, indicating they belong to the same species, different from the other *Frankia* cluster-2 MAGs included in the analysis. Cr1_Cr_nod, the MAG of the Patagonian soil sample which had been used to inoculate *C. ruscifolia* in the greenhouse, was found to belong to the same species as the NZ *Frankia* strains (95.8%-98.9% ANI, Fig. 3). Interestingly, the MAG of Cas1_Cas_nod additionally shared high similarity to *Ca.* F. meridionalis Cppng1 (97.9% ANI, Fig. 3). The ANI values of Cas1_Cas_nod compared to the other NZ MAGs showed a non-reciprocal pattern: while the other genomes shared between 98.8% and 99.2% ANI with Cas1_Cas_nod, Cas1_Cas_nod only shared between 95.2% and 96.6% ANI with the other genomes. This *Frankia* MAG has a much larger size than the others (Table 1b). It has been reported before that more than one closely related genome cannot be separated by bioinformatics means, leading to a mixed assembly (Nguyen et al. 2016, 2019). We thus conclude that Cas1_Cas_nod contains two genomes: one of which belongs to the new species from NZ, while the other is a representative of *Ca.* F. meridionalis. The position of this genome in the phylogenetic tree can be attributed to the fact that it represents a mix of two species (Fig. 2).

The *Frankia* MAGs of field samples from Patagonia (Cr2_Cr_nod to Cr5_Cr_nod) belong to a different, novel, *Frankia* species based on their shared high ANI values (99.8%-99.9%). Both the novel NZ

and the novel Patagonian *Frankia* species shared higher native to values with other *Frankia* MAGs from the island lineage, than with *Frankia* MAGs from the continental lineage.

Dispersal of *Frankia* cluster-2 in the southern hemisphere

Combining the new genome data from *Frankia* with novel host plant biogeographic analysis, we can draw some conclusions on the dispersal of *Frankia* cluster-2 in the southern hemisphere (Fig. 4). Renner et al. (2020) concluded that *Coriaria* spp. in the southern hemisphere split between ca. 57 and 46 Mya from those in the northern hemisphere, and Coriariaceae arrived in America from Antarctica (Fig. 4A), rather than migrating over the Pacific Ocean, as proposed earlier (Yokoyama et al. 2000). The divergence of *C. ruscifolia* and *C. ruscifolia* subsp. *microphylla* from the other southern hemisphere *Coriaria* species, and the arrival to America, is estimated to have occurred before 36 Mya (Fig. 4A) (Renner et al. 2020). We found a *Frankia* species able to engage in symbiosis with both Patagonian *C. ruscifolia* (Cr1_Cr_nod) and NZ *Coriaria* spp. (Cas1_Cas_nod to Cas8_Cas_nod) (Fig. 2, illustrated in yellow). This species could have diverged from the other *Frankia* species and dispersed during the Eocene, post-Gondwana when South America and NZ were still loosely connected by Antarctica. It is currently not known how *Frankia* disperses, but several lines of evidence suggest through animals faeces (Burleigh and Dawson 1995, Chaia et al. 2012, Paschke and Dawson 1993). This implies the two novel *Frankia* species identified here, must have arrived in South America before the opening of the Drake passage, which is
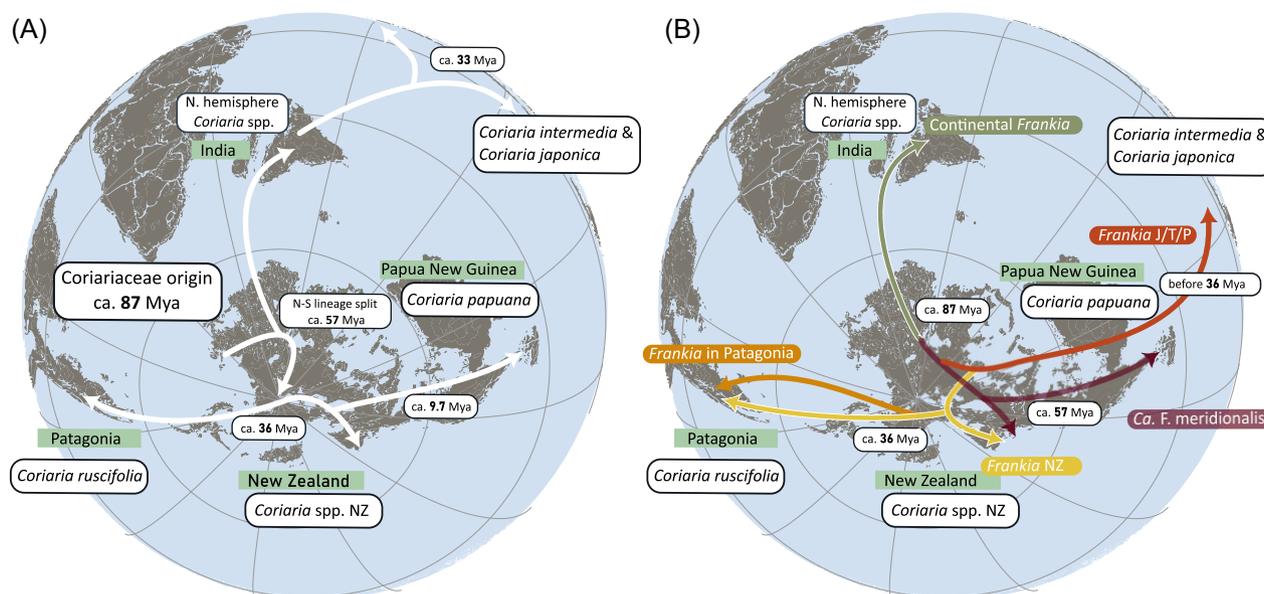
Figure 4. Paleogeographic reconstruction of (A) Coriariaceae and (B) *Frankia* cluster-2 dispersal patterns. Reconstruction of the continents is based on PALEOMAP available in GPlates 2.3 (Müller et al. 2018). (A) Coriariaceae dispersal is illustrated with white arrows. Dates are based on the most recent phylogeny (Renner et al. 2020). (B) Dispersal and speciation patterns of *Frankia* cluster-2. *Based* on the phylogeny of *Frankia* species and their associated host plants, we assume the continental *Frankia* species diverged from the island lineage already 87 Mya. The continental lineage of *Frankia* (green) would have dispersed together with the northern hemisphere Coriarie and Datiscaceae in India, and reached continental Eurasia *via* this route. In the island lineage, *Ca.* F. meridionalis is illustrated in purple; it is found in Papua New Guinea and New Zealand. *Frankia* found in Japan, Taiwan, and the Philippines (JTP) is illustrated in red. The New Zealand *Frankia* species is illustrated in yellow and can also be found in Patagonia. The Patagonian *Frankia* species is illustrated in orange.

in agreement with the arrival of *C. ruscifolia* in South America at 36 Mya (Reguero et al. 2014, Renner et al. 2020).

This would imply a scenario where the host plants and *Frankia* species evolved together. However, this scenario does not hold up based upon closer investigation of all *Frankia* cluster-2 species and *Coriaria* spp. In NZ, we were able to identify two different *Frankia* species: *Ca.* F. meridionalis, and a novel *Frankia* species (Fig. 2, yellow). *Ca.* F. meridionalis was first identified from samples originating in Papua New Guinea (Nguyen et al. 2019). *C. arborea* and *C. papuana,* the *Coriaria* species endemic to Papua New Guinea, are closely related species which diverged in the late Miocene (9 to 8 Mya). Therefore, one could assume that their associated *Frankia* evolved together with their host plant, i.e. *Ca.* F. meridionalis and the novel *Frankia* species are direct sister groups. However, this is not the case: *Ca.* F. meridionalis is the earliest divergent *Frankia* species within the island lineage, whereas the novel NZ *Frankia* species diverged from the Patagonian species relatively recently.

However, as argued above, both *Frankia* and its host plants arrived in Patagonia ca. 36 Mya. The divergence of the two *Frankia* species from the other species must have occurred before this arrival. As a consequence, the *Frankia* species occurring in Japan/Taiwan/the Phillipines as well as *Ca.* F. meridionalis have to have separated more than 36 Mya. This is in agreement with our previous work, where we showed that the species in Japan/Taiwan/the Philippines dispersed from the south to the north by an unknown host plant (Fig. 4B), which has either gone extinct or lost its symbiotic capability (Berckx et al. 2022a). Upon the arrival of *C. japonica* and *C. intermedia* on these islands, this *Frankia* species was already present. *C. japonica* and *C. intermedia* have been dated to have separated from the other northern hemisphere *Coriaria* spp. at least 33 Mya.

C. terminalis has also been shown to be nodulated by *Ca.* F. meridionalis (Berckx et al. 2022a). *C. terminalis* is endemic to Nepal,

and belongs to the northern hemisphere *Coriaria* clade, diverging from the southern clade 57–46 Mya. The most parsimonious conclusion therefore would be that *Ca.* F. meridionalis speciated before this time. To our knowledge, members of the island lineage are unable to nodulate Datiscaceae, whereas the members of the continental lineage can. We were unable to induce nodules on Datiscaceae by *Ca.* F. meridionalis (Nguyen et al. 2019), or the *Frankia* species from the Philippines (Berckx et al. 2022a). We were also unable to induce nodules on Datiscaceae using the soil collected in New Zealand. However, the plants died six to eight weeks post-infection, and thus we cannot exclude other factors impacting nodulation. This implies the divergence of the island lineage from the continental lineage *Frankia* species around the same time as the divergence of Coriariaceae from the Corynocarpaceae, ca. 86–82 Mya.

In summary, the different *Frankia* cluster-2 species of the island diverged between 82 and 36 Mya. *Ca.* F. meridionalis then split off from the other island lineage *Frankia* between 87 and 57 mya. We cannot determine when the next divergence of species occurred, but the most recent divergence, i.e. the Patagonian and NZ species from the Japanese/Taiwanese/Philippine species occurred more than 36 Mya. The NZ and Patagonian *Frankia* species are more closely related to each other, and belong to the island lineage, instead of the continental lineage. This contradicts previously published data based on the use of single phylogenetic marker genes (Nouioui et al. 2014).

Our data did not allow us to test our second hypothesis, whether NZ is a hotspot of species diversity for *Frankia* as well. Further investigation is needed to sequence more root nodules collected from other *Coriaria* spp. in other locations in New Zealand. Similarly, more sampling of soil *C. ruscifolia* in Patagonia and other parts of South and Central America is needed to assess the occurrence of the different *Frankia* species. As mentioned above,

the monophyletic position of *C. ruscifolia* is unclear, and therefore *Frankia* associating with *C. ruscifolia* and *C. ruscifolia* subsp. *microphylla* might belong to a species different from the *Frankia* we identified in this study.

Frankia strains compete for host inoculation and can persist under unfavourable conditions

The genome of Cr1_Cr_nod belongs to the same species as the NZ *Frankia* strains (Fig. 3). This species was not identified in any of the four field nodules sequenced directly from Patagonia. It was only found in the nodules induced in the greenhouse by soil collected under *C. ruscifolia* in Patagonia. While the directly sequenced nodules and the soil originate from two different sampling trips, cross contamination in our greenhouse can be excluded. It can be expected that in the soil, both species are present. Our data indicates that some competition between the different *Frankia* species takes place, and our greenhouse conditions promoted nodulation by the NZ *Frankia* species on *C. ruscifolia*. Under field conditions, which could include many different environmental factors such as climate or pH of the soil, the Patagonian *Frankia* species seemed to induce nodules on *C. ruscifolia*. In our nodule samples, the NZ species was either present on the nodule and below the detection limit, or it was not present in or on these particular nodules altogether.

Similar phenomena have been reported before: after sequencing field nodules of *C. intermedia* from the Philippines, we assembled island lineage *Frankia* MAGs. Using the same nodules in the greenhouse on *C. myrtifolia,* we could only obtain continental *Frankia* MAGs, namely CiP1_Cm_nod1 and CiP1_Cm_nod2 (Berckx et al. 2022a). In the same study, we could assemble *Frankia* MAGs from the island lineage from field nodules of *C. japonica* (Berckx et al. 2022a). It had previously been assumed that only *Frankia* species of the continental lineage were present in Japan, as inoculation of *Datisca glomerata* (Nguyen et al. 2019) or of *C. myrtifolia* followed by isolation (Gtari et al. 2015) with nodules from Japan, led to nodules containing *Frankia* from the continental lineage. Another notable example is cluster-3 *Frankia irregularis*. This species was first isolated from *Casuarina* spp. nodules (Casuarinaceae, Fagales), which are cluster-1 host plants (Mansour et al. 2017, Nouioui et al. 2018, Pujic et al. 2015). However, upon investigation, *F. irregularis* was unable to re-infect *Casuarina* spp. but inducing nodules on Rhamnaceae (Rosales), and on *Gymnostoma* spp., which are cluster-3 host plants. A later study showed that *F. irregularis* was present on the surface of nodules of *Casuarina* spp. (Vemulapally et al. 2019). Lastly, non-symbiotic cluster-4 strains have been isolated from nodules of actinorhizal Cucurbitales but could not nodulate them (Hafeez 1983, Hameed et al. 1994, Mirza et al. 1994). In conclusion, *Frankia* strains, including those of cluster-2, can persist under non-symbiotic conditions in the soil or on the surface of actinorhizal nodules, and the symbiotic strains among them can maintain their ability to nodulate once optimal conditions occur.

Nod genes are not required for the establishment of symbiosis in Coriariaceae

Unlike rhizobial symbiosis, actinorhizal symbiosis is not established through lipochito-oligosaccharide (LCO) Nod factor signalling as most *Frankia* genomes do not contain the canonical *nodABC* genes, encoding the proteins responsible for the Nod factor backbone production. *Candidatus* Frankia datiscae Dg1 was the first *Frankia* in which the canonical *nodABC* genes could be identified (Persson et al. 2011). These genes have been reported in

most *Frankia* cluster-2 genomes of the continental lineage, and the North American strains additionally contain the sulfotransferase gene *nodH* (Nguyen et al. 2016, 2019). However, they have not been identified in strains infecting only Coriariaceae such as *F. coriariae* (Gtari et al. 2015, Gueddou et al. 2019), the new species of the continental lineage that was part of the *Coriaria intermedia* inoculum (CiP1_Cm_nod1, Berckx et al. 2022a), or members of the island lineage. Instead, *Ca.* F. meridionalis Cppng1 had been reported to contain a *nodB'C* operon, as well as a carbamoyl transferase gene *nodU*, but not a *nodBA* operon (Nguyen et al. 2019). In most other members of the island lineage, no *nod* genes could be identified (Berckx et al. 2022a). We, therefore, wanted to investigate the potential presence of *nod* genes in the new genomes (Table S4C). While the *Frankia* MAGs from NZ all contained the *nodC-nltIJ-nodU* operon, identical to the one found in *Ca.* F. meridionalis, we only able to identify *nodC* in the Patagonian MAGs (Fig. 5, Table S4C, Fig. S3).

While the separation between the island and the continental lineage is clear, the *nodC* (Fig. S3) and the *nodC-nltIJ* operon phylogenies (Fig. 5) do not follow the core gene phylogeny. At some point, a gene duplication event of *nodC* took place in the island lineage, before the split of the Patagonian and the NZ species. With the limited data available, it is unclear whether this duplication event happened earlier and the Patagonian *Frankia* species contained two *nodC* copies at some point. It also cannot be determined if only the *nodC* gene was duplicated, or the entire operon, followed by sequential loss of the other genes. In the Patagonian MAGs, only one *nodC* copy can be found and this copy is not part of an operon including *nltIJ*. Both copies are present in the MAGs of Cas3_Cas_nod, Cas5_Cas_nod and Cas8_Cas_nod. However, while in Cas3_Cas_nod and in Cas8_Cas_nod the single *nodC* groups together with the *nodC* of the Patagonian species, the single *nodC* of Cas5_Cas_nod groups together with the *nodC* of other NZ MAGs in which *nodC* is present as part of an operon. The Cas5_Cas_nod *nodC-nltIJ* operon groups together with the operons of the other NZ MAGs.

As illustrated above (Fig. 2), *Ca.* F. meridionalis diverged first within the island lineage. The *nodC-nltIJ* operon of the other *Frankia* MAGs of the island lineage, do not follow the core phylogeny. The *Frankia* cluster-2 *nod* genes are surrounded by many transposons (Nguyen et al. 2019), and the neighbourhood of the *nod* genes in the MAGs of this study also contain many transposons. These transposons allow a different selection pressure, evolution, and horizontal gene transfer, compared to the rest of the genome. In addition, they enable the loss of many genes within the *nod* operon, i.e. *nodA, nodB, nodU, nltI, nltJ,* in the different species. Based on the limited data on genes which were maintained, it is unclear if the *nod* genes were acquired by the ancestor of symbiotic *Frankia*, or if multiple horizontal gene transfer events took place later.

We hypothesize that the Nod factors, i.e. the LCOs produced by the Nod proteins, play a potential role in suppressing plant immunity during nodulation, a role which has been attributed to LCOs in general (Feng et al. 2019). We previously reported that *Frankia* cluster-2 strains lacking in *nod* genes are unable to nodulate Datiscaceae (Berckx et al. 2022a), which do not occur in the southern hemisphere. If the infection of Coriariaceae does not require LCO suppression of the plant immune system, there would be no need for *Frankia* to retain the *nod* genes. Interestingly, the MAG of the continental Cm1_Cm_nod, originating from *Coriaria myrtifolia* (Nguyen et al. 2019), retained a *nodB1A* and a *nodB2CnltIJ* operon and was able to infect both Datiscaceae and *C. myrtifolia,* similar to *Ca.* F. datiscae Dg1 which contains both operons and was able to infect both Datiscaceae and *C. nepalensis* (Nguyen et al. 2019). It does not seem that the absence of *nod* genes is a require-

Figure 5. Phylogenetic tree of *nodC-nltIJ* operon. Sequences were obtained using searches for the operon and BLAST using GenDB. Concatenation of *nodC*, *nltI*, and *nltJ*, alignment and maximum likelihood phylogenetic tree construction based on 100 bootstrap replicates, were performed in SeaView. The branches were colored as followed: the new *Frankia* species from Patagonia is given in orange, the new species from NZ is given in yellow, the species from Japan/Taiwan/Philippines is given in red, *Candidatus* Frankia meridionalis is given in purple, and the continental lineage is given in green.

ment for nodulating Coriariaceae, but rather that the presence of *nodA*, *nodB*, and *nodC* is required for nodulating Datiscaceae. Further analysis, including cross-inoculation studies with other cluster-2 host plants such as *Ceanothus* spp., and with the novel *Frankia* species described here are needed to understand the role of *nod* genes, nodulation, and host-specificity in *Frankia* cluster-2.

Coriaria root nodules encompass more than *Frankia*

As outlined above, *Frankia* strains can be part of the inocula while not inducing nodules. Actinorhizal nodules are known to harbour a more diverse microbiome than only *Frankia* strains. *Micromonospora* spp. have been suggested to commonly occupy actinorhizal, as well as leguminous, nodules (Trujillo et al. 2006, 2010, Carro et al. 2013). We previously isolated and identified a novel *Streptomyces* spp. from *C. intermedia*, originating in the Philippines (Berckx et al. 2022b). Therefore, we set out to investigate the microbiome composition of the *Coriaria* field nodules sequenced in this study. In addition, field samples from *Coriaria japonica* were also included. This species is native to Japan, and its nodules were previously sampled and analysed (Berckx et al. 2022a). Due to the limitation in DNA extracts available, no other samples were included.

Analysing 16S rRNA gene profiles, we found that Actinobacteria, Cyanobacteria, and Proteobacteria were the three most dominant phyla in the samples (Fig. S2A). A large proportion of the Cyanobacteria reads are assumed to originate from the host plant plastids, while *Frankia* would account for the majority of Actinobacteria reads. Excluding Cyanobacteria, we indeed found that *Frankia* dominates the 16S profiles (Fig. S2B). To identify which other bacteria were present, a threshold of 2% was set to identify the nodule bacterial community, excluding *Frankia* and Cyanobacteria. The top 10 most abundant genera are presented in Fig. 6 (Fig. S3C for top 30).

Our data indicate that the most abundant bacteria, overall, were rhizobia (*Bradyrhizobium* and Rhizobium complex, consist-

ing of *Allorhizobium, Neorhizobium, Pararhizobium,* and *Rhizobium* i), *Corynebacterium, Mycobacterium,* and *Streptomyces* (Fig. 6A). Some differences could be seen between the samples originating from the same geographical region and plant species (Fig. 6B). *Streptomyces* was predominantly present in samples from New Zealand (yellow) or Patagonia (orange), but almost lacking in samples from Japan (red). Similarly, *Finegoldia* and *Acidothermus* were found to be much less abundant to lacking in Patagonian samples, compared to Japanese or New Zealand ones. Such differences could be explained by many variables, such as the host plant, soil and climate conditions, or adjacent plants. From the sequencing data alone, it also cannot be determined whether the bacteria are present inside the nodules or attached to the surface and if they are metabolically active. Further research into the roles of different nodule-occupying bacteria is needed, as well as determining which factors play an important role in determining the composition. However, our results do show that some genera, such as *Mycobacterium, Haliangium, Corynebacterium,* rhizobia, and *Actinoplanes,* are commonly present on actinorhizal nodules of Coriariaceae, regardless of the specific conditions.

Conclusion

Our study has led to the identification of two novel *Frankia* cluster-2 species, associated with Coriariaceae in the southern hemisphere. Phylogenetic analysis of endosymbiont and host plant, together with the geographic distribution over a geological time scale from 87 Mya, indicate that the different *Frankia* species diverged early in evolution and independently of host plant speciation. Although speciation of the two novel species occurred at the latest 36 Mya, little genetic changes took place. During the dispersal towards Patagonia, strains of the two species lost *nod-CnltIJnodU* operon independently. Microbial communities associated with *Coriaria* spp. nodules are dominated by *Mycobacterium, Haliangium, Corynebacterium,* rhizobia, and *Actinoplanes,* regardless of specific climate or soil conditions. Our study was only able to analyse *Frankia* strains associated with a small number of the New
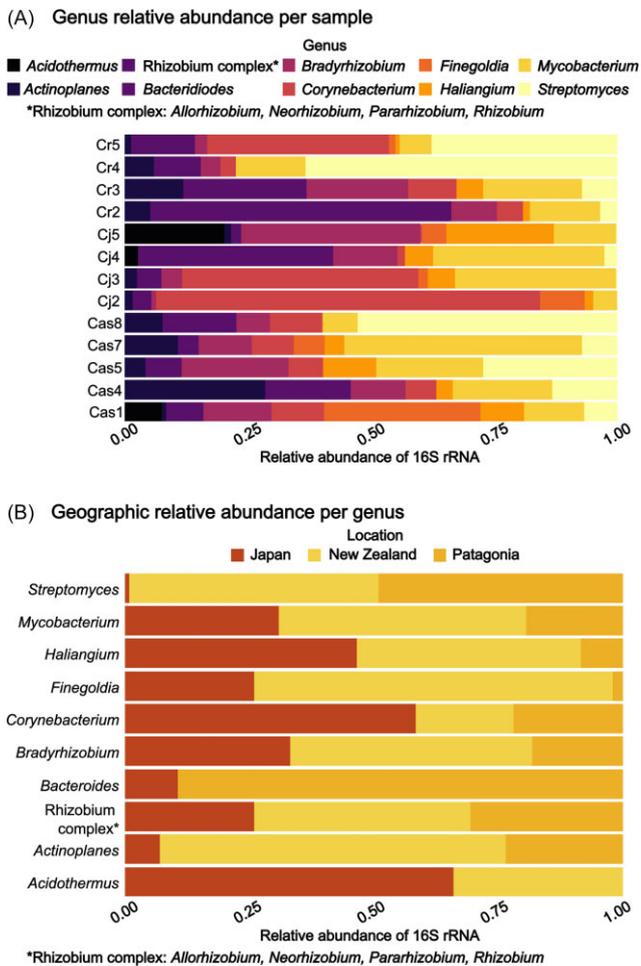
Figure 6. Top 10 most abundant bacterial genera identified in Coriariaceae field samples based on V3-V4 region of the 16S rRNA. The sample names refer to from which the corresponding *Frankia* MAG was assembled. Cr5, Cr4, Cr3, Cr2: nodules from *Coriaria ruscifolia* growing in Patagonia, Cj5, Cj4, Cj3, Cj2: nodules from *Coriaria japonica* growing in Japan, Cas8, Cas7, Cas5, Cas4, Cas1: *Coriaria* sp. growing in New Zealand. A) The relative abundance of each genus in each sample. Rhizobium complex refers to *Allorhizobium, Neorhizobium, Pararhizobium,* and *Rhizobium,* which were assigned to the same taxonomic group. B) The relative geographic distribution of each genus is illustrated for samples coming from Japan (red), New Zealand (yellow) and Patagonia (orange).

Zealand *Coriaria* species. Further research is needed to identify if more *Frankia* spp. evolved together with *Coriaria.* in New Zealand, as well as identifying the exact factors which determine the recruitment and role of the nodule microbiome.

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Supplementary data

Supplementary data is available at *FEMSEC* Journal online.

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