Taxogenomic status of phylogenetically distant *Frankia* clusters warrants their elevation to the rank of genus: A description of *Protofrankia* gen. nov., *Parafrankia* gen. nov., and *Pseudofrankia* gen. nov. as three novel genera within the family *Frankiaceae*

Maher Gtari*

USCR Bactériologie Moléculaire & Génomique, Institut National des Sciences Appliquées et de Technologie, Université de Carthage, Tunis, Tunisia

The genus *Frankia* is at present the sole genus in the family *Frankiaceae* and encompasses filamentous, sporangia-forming actinomycetes principally isolated from root nodules of taxonomically disparate dicotyledonous hosts named actinorhizal plants. Multiple independent phylogenetic analyses agree with the division of the genus *Frankia* into four well-supported clusters. Within these clusters, *Frankia* strains are well defined based on host infectivity range, mode of infection, morphology, and their behaviour in culture. In this study, phylogenomics, overall genome related indices (OGRI), together with available data sets for phenotypic and host-plant ranges available for the type strains of *Frankia* species, were considered. The robustness and the deep radiation observed in *Frankia* at the subgeneric level, fulfilling the primary principle of phylogenetic systematics, were strengthened by establishing genome criteria for new genus demarcation boundaries. Therefore, the taxonomic elevation of the *Frankia* clusters to the rank of the genus is proposed. The genus *Frankia* should be revised to encompass cluster 1 species only and three novel genera, *Protofrankia* gen. nov., *Parafrankia* gen. nov., and *Pseudofrankia* gen. nov., are proposed to accommodate clusters 2, 3, and 4 species, respectively. New combinations for validly named species are also provided.

**KEYWORDS**

*Frankiaceae*, bacterial genus boundaries, phylogenomics, pangenome, AAI, POCP, ANI, 16S rRNA gene
Introduction

The genus *Frankia* Brunchorst, 1886 (Approved Lists 1980) (Brunchorst, 1886; Becking, 1970; Skerman et al., 1980; Lechevalier and Lechevalier, 1989) is currently the solitary genus within the family *Frankiaceae* (Becking, 1970; Hahn et al., 1989; Normand et al., 1996; Stackebrandt et al., 1997) of the order *Frankiales* (Sen et al., 2014; Normand and Benson, 2015). The genus encompasses soil-inhabiting mesophilic actinomycetes mostly are able to fix dinitrogen and to establish symbiosis with pioneer and economically important plants, collectively named actinorhizal plants (Normand and Fernandez, 2019). Both in culture and the host root nodules, *Frankia* strains produce branched septate hyphae which, for most analysed strains, can carry multicellular sporangia, while the diazotrophic strains produce vesicles, where nitrogen fixation occurs (Normand et al., 2014; Gtari et al., 2019). Based on the 16S rRNA gene phylogeny (Normand et al., 1996), which was further substantiated by internal transcribed spacer (ITS) 16S-23S rRNA genes (Ghodhbane-Gtari et al., 2011; Persson et al., 2011; Sen et al., 2014; Gtari et al., 2015; Pozzi et al., 2018), amplified fragment length polymorphism (AFLP) (Bautista et al., 2011) and whole-genome analyses (Gtari et al., 2019; Nouioui et al., 2019b), four phylogenetic clusters are consistently delineated within the genus, grouping strains with similar cultural behaviour, morphology, host range, and mode of infection (Benson and Silvester, 1993). Cluster 1 members colonise species of *Alnus* (Betulaceae), *Allocasuarina* and *Casuarina* (Casuarinaceae), and *Comptonia* and *Myrica* (Myricaceae), while cluster 2 contains strains that infect *Corigiaceae*, *Datiscaceae*, *Dryadoideae* (*Rosaceae*), and *Ceanothus* (Rhamnaceae). Cluster 3 contains *Frankia* associated with *Elaeagnaceae*, *Colletettia* (*Rhamnaceae*), *Morella* (Myricaceae), and *Gymnostoma* (Casuarinaceae). Members of cluster 3 have been also occasionally isolated from *Alnus*, *Allocasuarina*, *Casuarina*, *Ceanothus*, and *Dryadoideae* root nodules (Benson and Dawson, 2007). Finally, cluster 4 includes asymbiotic *Frankia* strains, which are unable to fix nitrogen and/or to re-infect their host plants.

Prior to the genomic era, bacterial taxonomy relied on the polyphasic approach (Colwell, 1970) which integrates morphological, metabolic, and chemotaxonomic markers, 16S rRNA phylogeny, and pairwise dissimilarity. For ambiguous situations, the gold standard DNA–DNA hybridization (DDH) was used for drawing conclusions on species delineation (Wayne et al., 1987). For the genus *Frankia*, problems of applying traditional bacteriological techniques persisted due primarily to the high proportion of uncultivable strains and, secondly, to the very slow growing rate of most cultivated strains. Metabolic behaviours and wet-lab experimental DNA relatedness were, thus, inconsistent when the polyphasic taxonomic approach was applied in the case of the genus *Frankia* (Normand and Fernandez, 2008; Gtari et al., 2013).

Taking advantage of incorporating Taxogenomic and omniLog® phenoarray into the polyphasic approach (Gtari et al., 2019), 13 species with validly published names have been described based on accepted thresholds for bacterial species delineation, i.e., 99.0% (with a maximum probability of error of 1.0%) for 16S rRNA similarities (Meier-Kolthoff et al., 2013), 70% for digital DDH (Auch et al., 2010), and 95% for average nucleotide identity (ANI) (Konstantinidis et al., 2006). Ten of these species are facultative symbiotic species from clusters 1, 2, and 3 and include the type species *Frankia alni* (Nouioui et al., 2016), *Frankia canadensis* (Normand et al., 2018), *Frankia casuarinae* (Nouioui et al., 2016), and *Frankia torreyi* (Nouioui et al., 2019a) of cluster 1; *Frankia coriariae* (Nouioui et al., 2017b) of cluster 2; and *Frankia elaeagni* (Nouioui et al., 2016), *Frankia discariae* (Nouioui et al., 2017d), *Frankia irregularis* (Nouioui et al., 2018b), *Frankia soli* (Gtari et al., 2020), and *Frankia colletiae* (Nouioui et al., 2022) of cluster 3. Cluster 4 includes *Frankia asymbiotica* (Nouioui et al., 2017c), *Frankia ineflissax* (Nouioui et al., 2017a), and *Frankia saprophytica* (Nouioui et al., 2018a). Additionally, four candidate species are also defined to accommodate uncultured taxa: *Candidatus Frankia datisiae* (Persson et al., 2011) and *Candidatus Frankia californiensis* (Normand et al., 2017) from cluster 2, as well as *Candidatus Frankia alpina* (Pozzi et al., 2020) and *Candidatus Frankia nodulisporulans* (Herrera-Belaroussi et al., 2020) from cluster 1.

Bacterial classification at higher taxonomic ranks relies primarily on phylogenetic systematics (Ludwig and Klenk, 2005; Oren and Garrity, 2014) which has been greatly improved through phylogenomics (Dagan, 2011; Oren and Garrity, 2014; Hugenholtz et al., 2021). Genomic criteria for demarcating genus boundaries include conserved indels and proteins signatures (Naushad et al., 2014), comparative pangenome (Caputo et al., 2019), average amino acid identity (AAI) (Konstantinidis and Tiedje, 2007), percentage of conserved proteins (POCP) (Qi et al., 2014), and ANI (Barco et al., 2020). The gold standard overall genome related indices (OGRI) for genus demarcation remains pending inquiry (Sant’Anna et al., 2019).

The commonplace availability of genomic tools and algorithms for phylogenetic purposes and OGRI has motivated our interest to review the taxonomic structure of the genus *Frankia* in relation to the evolutionary history of its well-known phylogenetic clusters. The results obtained in the present study support the taxonomic elevation of each of the four clusters to the rank of the genus. Hence, three novel genera, *Protofrankia*...
gen. nov., Parafrankia gen. nov., and Pseudofrankia gen. nov., to accommodate clusters 2, 3, and 4, respectively, with 9 related new combinations, are proposed.

Materials and methods

Complete and draft genomes for type strains and candidate species together with other selected published genomes covering current Frankia diversity were used in this study and are listed in Table 1.

Phylogenetic analysis

Phylogenetic analyses based on 16S rRNA gene sequences were carried out using the GGDC web server adapted to single genes (Meier-Kolthoff et al., 2013). Maximum-likelihood (ML) and maximum-parsimony (MP) trees were inferred with RAxML (Stamatakis, 2014) and tree analysis new technology (TNT) (Goloboff et al., 2008), respectively. For MP, rapid bootstrapping was used in conjunction with the autoMRE bootstrapping criterion (Pattengale et al., 2010), followed by a search for the best tree. For MP, 1,000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and 10 random addition-sequence replicates. The sequences were checked for compositional bias using the χ² test as implemented in phylogenetic analysis using parsimony (PAUP)* (Swofford, 2002).

The whole-genome-based taxonomic analysis was performed through the Type Strain Genome Server® (Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022). Pairwise genomic comparisons were calculated and intergenomic distances were inferred under the algorithm “trimming” and distance formula dS using 100 distance replicates in FastME 2.0 (Lefort et al., 2015).

The pangenome analysis was performed using Roary (Page et al., 2015) implemented in the "Pan" module of the Prokaryotic Genomics and Comparative Genomics Analysis Pipeline (PGCGAP v1.0.21) (Liu et al., 2020). Single-copy analysis of core proteins, alignment of sequences, sequence concatenation, best model, and phylogenetic tree reconstruction based on 303 single copies of core proteins were performed with the “CoreTree” module of PGCGAP v1.0.21.

Overall genome related indices calculations

Overall genome related indices, including pairwise ANI, were calculated through the IMG/M data management and analysis system (Varghese et al., 2015). Pairwise AAI was calculated with the EzAAI tool (v1.1) (Kim et al., 2021) with default settings, which use MMSeqs2 for protein comparisons and consider a minimum query coverage of 50% and a minimum identity of 40% for AAI calculations. Pairwise POCP was calculated according to Qin et al.’s (2014) findings, following the steps described by Adamek et al. (2018) and Margos et al. (2018). Reciprocal BlastP (Altschul, 1997) for each pair of genomes used an E-value of < 1 × 10⁻⁵, > 40% sequence identity and > 50% of the query sequence. The pairwise POCP value was then deduced as [(C1 + C2)/(T1 + T2)] × 100, where C1 and C2 represent the conserved proteins numbers between the genome pair, while T1 and T2 are the total numbers of compared proteins in each genome (Qin et al., 2014).

Results and discussion

Phylogenetics and phylogenomics

An update of the 16S rRNA phylogeny was, here made available using the largest possible dataset of sequences (n = 72) from all type strains and candidate species, and those available in public databases or extractable from genome sequences providing a size of ≥ 900 nt (Figure 1). A more robust phylogenetic history was obtained using a single copy of core proteins (Figure 2) and whole-genome sequences (Figure 3), as shown by higher bootstrap and posterior probability values supporting the branching for the four clusters. Otherwise, in congruence with Salam et al. (2020), the most closely related Actinomycetia to Frankiaceae are Acidothermus (Acidothermaceae and Acidothermales) and Jatrophihabitans (Jatrophihabitantaecae and Jatrophihabitantes). The trees obtained, in this study, showed broadly similar patterns and topology with those previously reported for the 16S rRNA gene (Normand et al., 1996), ITS 16S-23S rRNA (Ghodhbane-Gtari et al., 2010), AFLP (Bautista et al., 2011), or combined data sets; 16S rRNA and glnA (Clawson et al., 2004), GyrB, glnII, and nifH (Nouioui et al., 2011), atpD, dnaA, ftsZ, pgk, and rpoB (Sen et al., 2014; Gtari et al., 2015; Nouioui et al., 2017ab, 2018a,b, 2019a; Pozzi et al., 2018), as well as 50 (Sen et al., 2014; Persson et al., 2015) or up to 200 gene sequences (Gtari et al., 2019; Nouioui et al., 2019b). Alongside the trees’ robustness and topology congruencies for phylogenetic splitting, no ambiguous or shifting affiliations between clusters were observed for any of the studied strains regardless of the algorithms or the extent of the genomic region used for inferring Frankia evolutionary history.

The relative positioning of each of the four Frankia clusters, and the timeline assumed for their separation, have been for a long a source of debate. While 16S rRNA (Normand et al., 1996), ITS 16S-23S rRNA (Ghodhbane-Gtari et al., 2010), AFLP (Bautista et al., 2011), and MLSA (Nouioui et al., 2011) based
| Strains | Scaffold | Genome size bp | Gene | G + C% | Protein coding gene | RNA | rRNA | tRNA | CRISPR | COG | Enzyme | KEGG | HTG |
|----------|----------|----------------|-------|--------|---------------------|-----|------|------|--------|-----|---------|------|-----|
| Cluster 1 |          |                |       |        |                      |     |      |      |        |     |         |      |     |
| Frankia alni ACN14aT | 1 | 7,497,934 | 6,795 | 72.83 | 6,723 | 72 | 6 | 46 | 11 | 3,434 | 1,465 | 1,437 | 429 |
| Frankia torreyi Cpl1T | 153 | 7,624,758 | 6,448 | 72.43 | 6,373 | 75 | 5 | 47 | 4 | 3,634 | 1,362 | 1,347 | 41  |
| Frankia canadensis ARgP5T | 568 | 7,673,585 | 6,894 | 72.39 | 6,799 | 57 | 3 | 52 | 7 | 4,642 | 1,393 | 1,343 | 0   |
| Candidatus Frankia alpina | 669 | 5,504,816 | 5,659 | 71.57 | 5,574 | 57 | 8 | 47 | 2 | 3,435 | 1,029 | 1,011 | 234 |
| Candidatus Frankia nodulisporulans | 612 | 4,882,652 | 4,602 | 71.61 | 4,528 | 55 | 6 | 46 | 1 | 2,973 | 988  | 972  | 223  |
| Frankia sp. QA3 | 90 | 7,521,104 | 6,312 | 72.59 | 6,493 | 53 | 4 | 46 | 8 | 3,434 | 1,029 | 1,011 | 234 |
| Frankia sp. ACN1Ag | 77 | 7,741,902 | 6,530 | 72.74 | 6,470 | 60 | 5 | 46 | 5 | 3,672 | 1,412 | 1,396 | 45   |
| Frankia sp. AvcI.1 | 77 | 7,741,902 | 6,530 | 72.74 | 6,470 | 60 | 5 | 46 | 5 | 3,672 | 1,412 | 1,396 | 45   |
| Frankia casuarinae Ccl3T | 7 | 5,433,628 | 4,621 | 70.08 | 4,548 | 73 | 6 | 46 | 7 | 2,438 | 1,211 | 1,160 | 151  |
| Frankia sp. CeD | 120 | 5,004,595 | 4,466 | 70.2 | 4,403 | 63 | 7 | 45 | 1 | 2,372 | 1,092 | 1,062 | 3    |
| Frankia sp. KBS | 420 | 5,455,564 | 4,675 | 70.11 | 4,622 | 53 | 6 | 45 | 2 | 2,416 | 1,107 | 1,093 | 243  |
| Cluster 2 |          |                |       |        |                      |     |      |      |        |     |         |      |     |
| Frankia coriariae BMG5.1T | 102 | 5,789,716 | 5,333 | 70.24 | 5,277 | 56 | 3 | 45 | 7 | 2,491 | 1,044 | 1,073 | 593  |
| Frankia sp. BMG5.30 | 94 | 5,818,019 | 5,034 | 70.21 | 4,976 | 58 | 5 | 45 | 5 | 2,662 | 1,132 | 1,153 | 15   |
| Candidatus Frankia datiscae Dg1 | 1 | 5,323,186 | 4,254 | 70.04 | 4,202 | 52 | 6 | 44 | 4 | 2,452 | 1,090 | 1,078 | 1    |
| Candidatus Frankia californiensis Dg2 | 2,738 | 5,896,456 | 7,108 | 68 | 7,022 | 65 | 4 | 39 | 8 | 4,102 | 988  | 978  | 893  |
| Cluster 3 |          |                |       |        |                      |     |      |      |        |     |         |      |     |
| Frankia elaeagni BMG5.12T | 135 | 7,589,313 | 6,342 | 71.67 | 6,253 | 89 | 5 | 51 | 1 | 3,516 | 1,390 | 1,356 | 564  |
| Frankia discariae BCU110501 | 194 | 7,891,711 | 6,839 | 72.39 | 6,742 | 97 | 8 | 47 | 7 | 3,671 | 1,399 | 1,350 | 954  |
| Frankia irregularis G2T | 83 | 9,573,992 | 7,873 | 70.95 | 7,789 | 84 | 9 | 47 | 3 | 4,538 | 1,635 | 1,605 | 7,525 |
| Frankia soli C1T | 289 | 8,022,739 | 6,296 | 71.73 | 6,244 | 52 | 5 | 45 | 3 | 3,609 | 1,390 | 1,378 | 531  |
| Frankia colletiae Ccl.17T | 195 | 8,361,025 | 6,392 | 71.44 | 6,343 | 49 | 0 | 47 | 0 | 3,870 | 1,440 | 1,424 | 335  |
| Frankia sp. Eal.12 | 749 | 8,022,419 | 7,429 | 71.67 | 7,308 | 63 | 4 | 55 | 1 | 4,995 | 1,412 | 1,371 | 200  |
| Frankia sp. BMG5.11 | 219 | 11,255,272 | 10,281 | 69.87 | 10,106 | 122 | 7 | 109 | 4 | 7,207 | 2,369 | 2,326 | 2,876 |
| Frankia sp. EUN1f | 31 | 10,442,526 | 8,596 | 70.91 | 8,523 | 73 | 9 | 45 | 4 | 4,797 | 1,775 | 1,721 | 1,400 |
| Frankia sp. BMG5.11 | 155 | 6,617,243 | 5,515 | 72.19 | 5,452 | 63 | 4 | 46 | 2 | 3,260 | 1,318 | 1,302 | 215  |
| Cluster 4 |          |                |       |        |                      |     |      |      |        |     |         |      |     |
| Frankia inefficax EuI1cT | 1 | 8,815,781 | 7,262 | 72.31 | 7,205 | 57 | 9 | 46 | 1 | 4,499 | 1,764 | 1,738 | 3    |
| Frankia asymbiotica M16386T | 174 | 9,435,764 | 7,904 | 71.97 | 7,821 | 83 | 3 | 70 | 5 | 4,483 | 1,586 | 1,562 | 651  |
| Frankia saprophytica CN3 | 2 | 9,978,592 | 8,411 | 71.81 | 8,332 | 79 | 5 | 68 | 6 | 4,930 | 1,662 | 1,633 | 211  |
| Frankia sp. EUN1h | 129 | 9,910,952 | 7,477 | 71.86 | 7,405 | 72 | 2 | 68 | 4 | 4,617 | 1,586 | 1,562 | 14   |
| Frankia sp. BMG5.36 | 280 | 11,203,906 | 8,330 | 71.26 | 8,250 | 80 | 11 | 67 | 3 | 4,966 | 1,722 | 1,701 | 1,287 |
| Frankia sp. DC12 | 1 | 6,884,336 | 5,933 | 71.93 | 5,858 | 75 | 9 | 46 | 2 | 3,162 | 1,313 | 1,254 | 89   |
phylogenies placed cluster 4 at the base of the tree, followed by clusters 3 and then clusters 1 and 2 which form sister groups, and other MLSA studies (Pozzi et al., 2018) or concatenation of proteins (Sen et al., 2014; Gtari et al., 2015; Persson et al., 2015) showed cluster 2 as basal, followed by cluster 4 and then the symbiotic cluster 3, and finally, cluster 1 as the most derived. Both situations were here obtained based on the whole genome (Figure 3) and on core proteins (Figure 2), respectively, which could imply a different evolution pressure of the whole genome versus the core genome or a bias in codon usage.

Whole-genome-based criteria

Inter-cluster pairwise AAI (Figure 4 and Supplementary Table 1), POCP (Figure 4 and Supplementary Table 2), and ANI (Supplementary Table 3) values ranged between 66.5 and 72.2, 33.5 and 61.3, and 78 and 81.5%, respectively. A cut-off AAI value at 72.2 ± 0.03%, which is within the 60–80% threshold recommended for the delineation of genera (Konstantinidis and Tiedje, 2005; Rodriguez-R and Konstantinidis, 2014), permitted a congruent regrouping with phylogenetic clusters. The POCP threshold of 50% originally proposed for genus delineation by Qin et al. (2014) has been shown to be inappropriate for multiple taxa (Aliyu et al., 2016; Pannekoek et al., 2016; Li et al., 2017; Lopes-Santos et al., 2017; Orata et al., 2018; Wirth and Whitman, 2018; Park et al., 2022). Sangal et al. (2022) considered this POCP cutoff as overly stringent and proposed its reappraisal to 58–66%. The inter-cluster pairwise POCP values obtained in this study (33.5–61.3%) are within this proposed new range.
When ANI values are compared between more divergent taxa than species, they are prone to saturation and loss of information and, hence, considered inappropriate standards for genus delineation (Konstantinidis and Tiedje, 2007; Kim et al., 2014; Qin et al., 2014; Rodriguez-R and Konstantinidis, 2014; Gosselin et al., 2022; Park et al., 2022). The ANI values in the present study (78–81.5%) were consistently lower than the ANI species thresholds of 95–96.5%. Values closer to the species threshold (89–90%) were, however, seen with the quartic function of 750 genomes analysed (Barco et al., 2020).

Other genomic criteria

Some genes and operon organisation show some distinctiveness for each of the four clusters (Supplementary Figure 1). There are two ribosomal operons for clusters 1 and 2 strains and three for clusters 3 and 4 (Gtari et al., 2007). Nitrogenase complexes encoding (nif) genes are totally absent in the asymbiotic cluster 4 (not retrieved in the draft genome sequence, except for F. asymbiotica strain M16386T). The nif genes are, however, organised in different ways with respect to each cluster (Tisa et al., 2016; Nouioui et al., 2019b). The Ni-Fe hydrogenase or uptake hydrogenase (hup) genes are clustered into two operons for clusters 1 and 3, while only one operon is present for clusters 2 and 4 (Tisa et al., 2016). Another important distinctive gene is murC, related to peptidoglycan biosynthesis. Different copies in the genomes of the four different clusters were found (Berckx et al., 2020). Two copies, murC1 and murC2, are present in clusters 1 and 2, 3, which differ in their orientation with the presence of an open reading frame (ORF) encoding a nitroreductase family deazaflavin-dependent oxidoreductase in cluster 3. Clusters 1 and 4 contain only one copy of murC2 with the ORF encoding a nitroreductase family deazaflavin-dependent oxidoreductase only present in cluster 1.

Overall differentially encoded proteins between genomes of the four clusters were provided in Supplementary Table 4. The presence/absence profiles of the protein clusters for the four phylogenetic clusters were illustrated in Figure 5 and Supplementary Figure 2.

Ecological and phenotypic features

The phylogenetic segregation of Frankia clusters found additional support in the ecological lifestyle interaction with host plants and cultural behaviours among the Frankia strains of each cluster. Frankia strains from cluster 1 (except those nodulating Allocasuarina and Casuarina) and cluster 3 are...
globally distributed in soils irrespective of the presence or absence of compatible host plants (Smolander and Sundman, 1987; Põlme et al., 2014). The distribution of cluster 1 of Casuarinaceae infective and cluster 2 strains is restricted to the native range of their respective host plants (Simonet et al., 1999). Cluster 4 ineffective strains (nodulating but non-nitrogen fixing) were shown to represent the most prominent Frankia population, exhibiting a higher diversity in prairie soils in the absence of actinorhizal host plants (Ben Tekaya et al., 2017) and wet soils under Alnus glutinosa (Hahn et al., 1988; van Dijk and Sluimer, 1994; Wolters et al., 1997a,b).

Most Frankia strains from clusters 1 and 3 have been cultured in axenic conditions with relative ease. Strains from cluster 4, which have been isolated as a “by-product” in studies aimed at the cultivation of the “true” beneficial microsymbionts in actinorhizal root nodules, show very similar cultural behaviours to other soil actinomycetes (Normand and Chapelon, 1997; Nouioui et al., 2017a,b, 2018a). Cluster 1 contains as-yet-uncultured Frankia microsymbionts, which are characterised by profuse sporulation within nodules (Sp + types; Schwinzter, 1990), and a very higher proportion of cluster 2 strains are as-yet-uncultured. The Candidatus status has been used to accommodate some of these uncultured Frankia which were defined based on genome sequences (Persson et al., 2011, 2015; Nguyen et al., 2016, 2019; Normand et al., 2017; Herrera-Belaroussi et al., 2020; Pozzi et al., 2020; Berckx et al., 2022). Two strains from cluster 2 have been successfully cultivated following a dual approach consisting of comparative genomics and direct physiological assay on nodule tissues (Gtari et al., 2015; Gueddou et al., 2019).

While filamentous hyphae are the primary vegetative state for all growing Frankia strains, the extent of sporangia and vesicle formation varies from cluster to cluster. The sporulation of strains from clusters 1, 3, and 4 may be readily detected in vitro or may depend on the composition of media and cultural conditions (Tisa et al., 1983; Krumholz et al., 2003). The sporulation of the two cultivated strains of cluster 2 seems to be completely suppressed (Gtari et al., 2015; Nouioui et al., 2017b). In general, vesicles containing nitrogenase are formed in
response to the limited availability of nitrogen (Fontaine et al., 1984; Murry et al., 1984). Some *Frankia* strains, belonging to cluster 3, continue to form vesicles even in the presence of a nitrogen source, but the numbers are reduced compared with growth in nitrogen-depleted media (Gauthier, 1983; Meesters et al., 1985). Strains of cluster 4 are unable to fix dinitrogen and thus to form vesicles, with the exception of *F. asymbiotica*.
### TABLE 2 Phenotypic and host-plant-related features.

| Cluster 1 species | Cluster 2 species | Cluster 3 species | Cluster 4 species |
|-------------------|-------------------|-------------------|-------------------|
|                  |                   |                   |                   |
| Colony colour     | White             | White             | Brown             | White             |
| Vesicles/N2 fixation | +                 | +                 | +                 | +                 |
| Sporangia         | +                 | +                 | +                 | +                 |
| Major fatty acids (> 15%) | iso-C16:0, C17:1 08c | iso-C16:0, C17:1 08c | iso-C16:0, C17:1 08c | iso-C16:0, C17:1 08c |
| Predominant menaquinones (> 20%) | MK-9(H4) | MK-9(H4) | MK-9(H4) | MK-9(H4) |
| Phospholipid 1 | PI, DPG, GPL1–3, PG (L) | PI, DPG, GPL1–3, PG (L) | PI, DPG, GPL1–3, PG (L) | PI, DPG, GPL1–3, PG (L) |
| Cell wall sugars | Galactose, glucose, mannose, rhamnose, ribose, and xylose | Galactose, glucose, mannose, rhamnose, ribose, and xylose | Galactose, glucose, mannose, rhamnose, ribose, and xylose | Galactose, glucose, mannose, rhamnose, ribose, and xylose |
| Host specificity group | HSG1 | HGS2 | HSG3/HGS4 | HSG3 |
| Mode of infection | RHI | CE/IC | IC/CE | – |

1DPG, diphosphatidylglycerol; GL, glycolipid; GPL, unknown glycosphospholipid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids; UL, unidentified lipids. Phospholipid between brackets is yet uncharacterized. 2HSG1 infecting Alnus (Betulaceae), Comptonia, Morella, and Myrica (Myricaceae) species; HSG2 infecting Casuarina and Allocasuarina (Casuarinaceae) and Morella species; HSG3 infecting Elaeagnaceae, Colletieae (Rhamnaceae), Gymnostoma (Casuarinaceae), and Myrica (Myricaceae) species; HSG4 strains nodulate members of the Elaeagnaceae but not the promiscuous hosts in the Myricaceae or Gymnostoma (Casuarinaceae) and HSG5, strains nodulate members of Coriariaceae, Datiscaeae, Dryodoidae (Rosaceae), and Ceanothus (Rhamnaceae) species. 3RHI, root-hair infection; CE, crack entry; IC, intercellular (Nguyen and Pawlowski, 2017).
Strains from clusters 1 and 3 grow well in nitrogen-depleted media and metabolise short-chain fatty acids, TCA cycle intermediates, and carbohydrates. Strains in cluster 4 are similar to other saprophytic actinomycetes, more active physiologically, grow more rapidly and utilise a variety of monosaccharides and disaccharides, and produce hydrolytic enzymes, such as pectinases, cellulases, amylases, and proteases (Lechevalier, 1994). The two cultivated strains from cluster 2 are more slowly growing and have an unusual physiological requirement for alkalophilic growth media (Giari et al., 2015; Nouioui et al., 2017b). Other phenotypic markers, including chemotaxonomy, are provided in Table 2.

Conclusion and description of the new taxa

Evidence of the splitting of Frankia into novel genera is here provided based on phylogenomics and OGRI recommended for bacterial genus boundary demarcation. The taxonomic elevation of phylogenetically distant Frankia clusters is clearly supported through the consistent sequence divergence in phylogenetic trees, OGRI analysis, and other genome-related criteria. The genus Frankia should be revised to accommodate cluster 1 species only, while clusters 2, 3, and 4 are taxonomically elevated to the rank of the type species. Therefore, new genera as Protofrankia and Parafrankia are recommended for bacterial genus boundary demarcation.

The taxonomic revision provided, in this study, will help clarify the confusing past classification of the actinorhizal cycle intermediates, and carbohydrates. Strains in cluster 4 are similar to other saprophytic actinomycetes, more active physiologically, grow more rapidly and utilise a variety of monosaccharides and disaccharides, and produce hydrolytic enzymes, such as pectinases, cellulases, amylases, and proteases (Lechevalier, 1994). The two cultivated strains from cluster 2 are more slowly growing and have an unusual physiological requirement for alkalophilic growth media (Giari et al., 2015; Nouioui et al., 2017b). Other phenotypic markers, including chemotaxonomy, are provided in Table 2.

Description of Protofrankia gen. nov.

Protofrankia (Pro.to.fr.an’ki.a. Gr. masc. adj. protos, earlier than, prior to; N.L. fem. n. Frankia a bacterial genus name; N.L. fem. n. Protofrankia, a genus considered here as phylogenetically basal to Frankia).

The genus is defined by the taxonomic elevation of the taxon previously defined as Frankia phylogenetic cluster 2. Host plants include Coriariaceae, Datiscaceae, Dryadoideae (Rosaceae), and Ceanothus (Rhamnaceae) genera. Genome sizes in the range of 5.3–5.8 Mb with G + C mol% content of 68.0–70.2%. The type species is Protofrankia coriaria.

Protofrankia coriaria comb. nov.

Protofrankia coriaria (co.ri.a’ri.ae. N.L. gen. fem. n. coriariae, of Coriaria, referring to the origin of isolation of the type strain).

Basonym: Frankia coriaria (Nouioui et al., 2017b,c).

The description of Protofrankia coriaria comb. nov. is identical to that given by Nouioui et al. (2017b) for F. coriaria. The type strain is BMG5.1T (= DSM 100624T = CECT 9032T).

Description of Parafrankia gen. nov.

Parafrankia (Pa.ra fran’ki.a. Gr. prep. para, beside; N.L. fem. n. Frankia, a bacterial genus name; N.L. fem. n. Parafrankia, beside Frankia).

The genus Parafrankia is defined by the taxonomic elevation of the taxon previously defined as Frankia phylogenetic cluster 3. Host plants include members of Elaeagnaceae, Colletieae, Morella, and Gymnnothesta. Genome sizes range from 6.6 to 11.2 Mb with G + C mol% of 69.7–72.3. The type species is Parafrankia elaeagni. In addition, four species can be reclassified as members of the genus. Frankia discariae (Nouioui et al., 2018b), F. irregularis (Nouioui et al., 2018a), F. soli (Giari et al., 2020), and F. colletiae (Nouioui et al., 2022) should be named Parafrankia discariae, Parafrankia irregularis, Parafrankia soli, and Parafrankia colletiae, respectively.

Parafrankia elaeagni comb. nov.

Parafrankia elaeagni (e.lae.ag’ni. N.L. gen. masc. n. elaeagni, of Elaeagnus, referring to the source of the isolate).

Basonym: Frankia elaeagni (Nouioui et al., 2016).

The description of Parafrankia elaeagni is the same as that given by Nouioui et al. (2016) for F. elaeagni. The type strain is BMG5.12T (= DSM 46783T = CECT 9031T).

Parafrankia discariae comb. nov.

Parafrankia discariae (dis.ca.ri.ae. N.L. gen. fem. n. discariae, of Discaria, the host plant origin of isolation of the type strain).

Basonym: Frankia discariae (Nouioui et al., 2017d).

The description of Parafrankia discariae is the same as that given by Nouioui et al. (2017d) for F. discariae. The type strain is BCU110501T (= DSM 46785T = CECT 9042T).

Parafrankia irregularis comb. nov.

Parafrankia irregularis (ir.re.gu.la’ris. L. fem. adj. irregularis, of irregular, referring to the inability of the species to
infect its original host plant and to infect taxonomically disparate host plants).

Basonym: Frankia irregularis (Nouioui et al., 2018b).

The description of Parafrankia irregularis is the same as that given by Nouioui et al. (2018b) for F. irregularis. The type strain is G2\(^T\) (= DSM 45899\(^T\) = CECT 9038\(^T\)).

Parafrankia soli comb. nov.

Parafrankia soli (so’li. L. gen. neut. n. soli, of soil, referring to the isolation source of the type strain).

Basonym: Frankia soli (Gtari et al., 2020).

The description of Parafrankia soli comb. nov. is the same as that given by Gtari et al. (2020) for F. soli. The type strain is Cj\(^T\) (= DSM 100623\(^T\) = CECT 9041\(^T\) = NRRL B-16219\(^T\)).

Parafrankia colletiae comb. nov.

Parafrankia colletiae (col.let’i.ae. N.L. gen. n. colletiae of Colletia, referring to the host plant, Colletia, origin of isolation of the strain).

Basonym: Frankia colletiae (Nouioui et al., 2022).

The description of Parafrankia colletiae comb. nov. is the same as that given by Nouioui et al. (2022) for F. colletiae. The type strain is Cc1.17\(^T\) (= DSM 43829\(^T\) = CECT 9313\(^T\)).

Description of Pseudofrankia gen. nov.

Pseudofrankia (Pseu.do.franks’i.a. Gr. masc. adj. pseudes, false; N.L. fem. n. Frankia, a bacterial genus name; N.L. fem. n. Pseudofrankia, a false Frankia).

Pseudofrankia gen. nov. is defined by the taxonomic elevation of the taxon previously defined as Frankia phylogenetic cluster 4. Members of the genus have been isolated from actinorhizal root nodules and are non-infective and/or non-nitrogen-fixing taxa. The size range of the genomes is 6.6–9.9 Mb with 71.2–72.3 of G + C mol%. The type species is Pseudofrankia inefficax. In addition, two other species can be reclassified as members of the genus. Frankia asymbiotica (Nouioui et al., 2018a) and F. saprophytica (Nouioui et al., 2018) should be named Pseudofrankia asymbiotica and Pseudofrankia saprophytica, respectively.

Pseudofrankia inefficax comb. nov.

Pseudofrankia inefficax (in.ef ‘fi.cax. L. fem. adj. inefficax, inefficient in reference to the inability of the bacterium to form the effective nitrogen-fixing symbiosis with its plant host).

Basonym: Frankia inefficax (Nouioui et al., 2017a).

The description of Pseudofrankia inefficax comb. nov. is the same as that given by Nouioui et al. (2017a) for F. inefficax. The type strain is EuI1c\(^T\) (=DSM 45817\(^T\) = CECT 9037\(^T\)).

Pseudofrankia asymbiotica comb. nov.

Pseudofrankia asymbiotica (a.sym.bi.o’ti.ca. Gr. pref. a-, not; N.L. fem. adj. symbiotica, living together; N.L. fem. adj. asymbiotica, not symbiotic).

Basonym: Frankia asymbiotica (Nouioui et al., 2017c).

The description of Pseudofrankia asymbiotica comb. nov. is the same as that given by Nouioui et al. (2017c) for F. asymbiotica. The type strain is M16386\(^T\) (= DSM 100626\(^T\) = CECT 9040\(^T\) = NRRL B-16386\(^T\)).

Pseudofrankia saprophytica comb. nov.

Pseudofrankia saprophytica (sa.pro.phy’ti.ca. Gr. masc. adj. sapros, rotten; Gr. masc. adj. phytikos, belonging to plants; N.L. fem. adj. saprophytica, growing on rotten material, referring to the asymbiotic lifestyle of the type strain).

Basonym: Frankia saprophytica (Nouioui et al., 2018a).

The description of Pseudofrankia saprophytica comb. nov. is the same as that given by Nouioui et al. (2018a) for F. saprophytica. The type strain is CN3\(^T\) (=DSM 105290\(^T\) = CECT 9314\(^T\)).

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MG conceived the study, performed the analyses, and wrote the manuscript.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1041425/full#supplementary-material

Supplementary Figure 1
Comparative representations of the nif operons in strains representing Frankia clusters 1, 2, and 3 (A) and hup gene cluster (B) and biosynthetic peptidoglycan genes murC (C) in clusters 1, 2, 3, and 4.

Supplementary Figure 2
Clusters of Orthologous Genes (COG) categories distribution for all studied strains.

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