One More Decade of *Agrobacterium* Taxonomy

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Contents

Abstract This chapter presents a historical overview of the development and changes in scientific approaches to classifying members of the *Agrobacterium* genus. We also describe the changes in the inference of evolutionary relationships among *Agrobacterium* biovars and *Agrobacterium* strains from using the 16S rRNA

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marker to *recA* genes and to the use of multilocus sequence analysis (MLSA). Further, the impacts of the genomic era enabling low cost and rapid whole genome sequencing on *Agrobacterium* phylogeny are reviewed with a focus on the use of new and sophisticated bioinformatics approaches to refine phylogenetic inferences. An updated genome-based phylogeny of ninety-seven *Agrobacterium tumefaciens* complex isolates representing ten known genomic species is presented, providing additional support to the monophyly of the *Agrobacterium* clade. Additional taxon sampling within *Agrobacterium* genomovar G3 indicates potential exceptions to interpretation of the concept of bacterial genomics species as ecological species because the genomovar G3 genomic cluster, which initially includes clinical strains, now also includes plant-associated and cave isolates.

1 Introduction

Since the first uses of DNA sequences to classify relationship among bacterial strains became routine (Janda and Abbott 2007; Stackebrandt and Goebel 1994), new and increasing amounts of single-copy protein-coding DNA markers have been employed to revaluate and revise the taxonomy of *Agrobacterium*. Phylogenetic analyses based on increased taxon and gene sampling have led to the reclassification of the traditional *Agrobacterium* biovars 1 and 3 to two new genera (Costechareyre et al. 2010; Mousavi et al. 2014). With the now common practice of sequencing whole bacterial genomes, large data sets are increasingly available, and these sequences have become linked to more sophisticated approaches to analyse data using multiple and linear bioinformatical approaches. These approaches have provided new and improved insight into the evolutionary relationships among *Agrobacterium* species. In this review chapter, we first provide a historical overview of the molecular systematics of the genus *Agrobacterium* which led to an intense debate among the scientific community during the 16S rRNA era. We next review changes to the *Agrobacterium* taxonomy which is gradually embraced by the scientific community in the light of more recent and refined phylogenetic analyses using improved gene and taxon sampling. The unprecedented genetic information about *Agrobacterium* derived from the advent of next-generation sequencing and its impacts on the inference and delineation of *Agrobacterium* at the strain level is summarized. We also provide a genome-based phylogeny of ninety-seven *Agrobacterium tumefaciens* complex isolates, representing a significant increase in taxon sampling compared to a previous phylogenomic study (Ormeno-Orrillo et al. 2015). The validity of bacterial genome species being ecological species (Lassalle et al. 2011) is briefly assessed and discussed in the light of new phylogenomic inferences and observed ecological niche diversity among recently sequenced strains belonging to *Agrobacterium* genomovar 3.

2 Pre-2006 *Agrobacterium* Taxonomy

The use of 16S rRNA sequence as a genetic marker for microbial taxonomy brought about both chaos and order within the taxonomy of *Agrobacterium*. The availability of universal 16S rRNA primers and the inherent high copy number of 16S rRNA in most bacterial genomes are two of the main attributes promoting the inclusion of the 16S rRNA sequence as part of the developed polyphasic taxonomy for bacteria (Janda and Abbott 2007; Woo et al. 2008). Furthermore, the high sequence conservation of the 16S rRNA gene makes it a very powerful genetic marker when inferring deep relationships. However, at the species or genus level, the use of the 16 s rRNA gene to discriminate among species tends to be modest if not inferior to other universal genetic markers (Kisand and Wikner 2003; Stackebrandt and Goebel 1994).

It is important to note that 16S rRNA gene substitution rates appear to vary among different groups of bacteria (Ochman et al. 1999; Smit et al. 2007). In other words, if the 16S rRNA gene substitution rates are lower in the family Rhizobiaceae, this will translate into low 16S rRNA gene nucleotide divergence and/or phylogenetic signals among members of the Rhizobiaceae. This may negatively affect phylogenetic interpretation, raising doubts about the veracity of their inferred evolutionary relationships. An initial proposal by Young et al. (2001) to incorporate all species of *Agrobacterium* and *Allorhizobium* into the genus *Rhizobium* due to the lack of concordance between DNA hybridization, biochemical traits, and fatty acid profiles among members of the described genera sparked an intense response from the scientific community (Farrand et al. 2003; Young et al. 2001). Farrand et al. (2003) claimed that members of the genus *Agrobacterium* and *Rhizobium* can be distinguished based on chromosomal structure and phenotype (as an individual species but not genera). Young et al. (2001) replied to Farrand et al. (2003) defending the initial proposal in addition to highlighting that the proposal is in accordance with the rules/codes set out by the International Code of Nomenclature of Bacteria. Young et al. (2001) further cautioned that bending the codes to retain the genus *Agrobacterium* may trigger a potential return to unregulated and chaotic bacterial nomenclature. The initial classification of *Agrobacterium* species based on their pathogenicity has been problematic, as it is now well established that the virulence factors are usually encoded on plasmids and some of these can even be lost relatively easily through growth at elevated temperature (Genetello et al. 1977). For further reading on the change and development in *Agrobacterium* taxonomy until 2006, we direct reader to a comprehensive review by Young (2008).

3 Alternative Views of the *Agrobacterium* Phylogeny

3.1 *The* recA *Gene as an Alternative Genetic Marker to 16S rRNA for Inferring* Agrobacterium *Phylogeny*

The *recA* gene encodes a multifunctional and important enzyme involved in homologous recombination and DNA repair (Kowalczykowski et al. 1994). A *recA* mutant is therefore characterized by its high sensitivity to UV light in addition to being recombination-deficient, a desirable trait for genetic studies involving trans-complementation of mutations located on a chromosome or plasmid (Kanie et al. 2007; Kuzminov and Stahl 1997). The importance of a *recA* mutant is well recognized among *Agrobacterium* geneticists, leading to the construction of strains LBA4301 and UIA143, *recA* mutants of *Agrobacterium tumefaciens* Ach5, and *Agrobacterium tumefaciens* C58, respectively (Farrand et al. 1989). Beyond molecular genetics, the *recA* gene is also well known in molecular systematics (Lloyd and Sharp 1993) and has been incorporated as one of the main genes for multilocus typing (MLSA) (Bennasar et al. 2010; Delamuta et al. 2012; Huo et al. 2017; Martens et al. 2008; Menna et al. 2009; Sakamoto and Ohkuma 2011). Phylogenetic analysis based on the *recA* gene of 138 strains from 13 genomic species of *Agrobacterium* lends support to the use of this marker gene for speciation of the genus *Agrobacterium* (Costechareyre et al. 2010). Genomic species is a concept of bacterial species based on similarities among bacterial chromosomal DNAs as determined by DNA–DNA hybridization or alternatively by in silico calculation of pair-wise average nucleotide identity (ANI) using whole genome sequences (Konstantinidis et al. 2006; Stackebrandt and Goebel 1994). A genomic species is defined as a group of bacterial strains with DNA–DNA reassociation values of more than 70%, which corresponds closely to ~95% ANI (Konstantinidis et al. 2006). A *recA*-based phylogenetic analysis indicates that *Agrobacterium* biovar 2, typically represented by *Agrobacterium rhizogenes*, and biovar 3 represented by *Agrobacterium vitis* are distantly related to *Agrobacterium* biovar 1. In addition, inclusion of *recA* sequences from several *Rhizobium* type strains in the analysis showed a stronger affiliation of *Agrobacterium rhizogenes* and *Agrobacterium vitis* to the *Rhizobium* clade.

3.2 *Four (or Six) Is Better Than One: Refining and Revising the* Agrobacterium *Genus Through Multilocus Sequence Analysis (MLSA)*

Phylogenetic tree construction based on six protein-coding housekeeping genes consisting of ATP synthase F1, beta subunit (*atpD*), glutamine synthetase type I (*glnA*), glutamine synthetase type II (*glnII*), recombinase A (*recA*), RNA polymerase beta subunit (*rpoB*), and threonine synthase (*thrC*) from 114 rhizo- and

agrobacteria reinforced the monophyly of the genus *Agrobacterium* which was previously reestablished based on the *recA* gene. In addition to resolving other pending taxonomic issues related to the family Rhizobiaceae, the substantial increase in gene and taxon sampling also lent support to the reclassification of *Agrobacterium vitis* to an existing genus *Allorhizobium* (Mousavi et al. 2014). Once belonging to three different biovars of the same genus, the phythopathogenic *Agrobacterium tumefaciens*, *Agrobacterium vitis* (now *Allorhizobium vitis*), and *Agrobacterium rhizogenes* (now *Rhizobium rhizogenes*) now belong to three different genera. Furthermore, with the creation of the genus *Neorhizobium* which is a sister group to *Agrobacterium*, *Agrobacterium* can now remain a suitable genus name for a monophyletic clade within the Rhizobiaceae family. A follow-up study based on three housekeeping genes and the 16S rRNA gene again supported the monophyly of the revised *Agrobacterium* clade in addition to expanding the membership of the genus *Allorhizobium* to include *R. taibanshenase*, *R. paknamense*, *R. oryzae*, *R.psuedoryzae*, *R. qilianshanense*, and *R. borbori*. However, in contrast to a previous study based on six housekeeping genes, a sister grouping of *Agrobacterium–Neorhizobium* was not observed. The *Agrobacterium* clade instead shared a sister grouping with the *R. aggregatum* complex (Mousavi et al. 2015). Mousavi et al., however, did not suggest the reclassification of members from the *R. aggregatum* complex to the genus *Agrobacterium* as members of this sister clade, citing the lack of *Agrobacterium*-specific genome architecture (linear chromosome and the presence of the protelomerase-coding gene, *telA* (Ramirez-Bahena et al. 2014).

4 *Agrobacterium* and the Genomic Era

4.1 Pre-next-Generation Sequencing Period

Whole genome sequencing provides an unprecedented view into the evolutionary relationships of microorganisms. With a repertoire of single-copy and near-universal genes, usually in the range of hundreds, that can be used for phylogenetic inference, there is no longer a limitation to gene sampling, one of the main requirements for accurate phylogenetic analysis (Hedges 2002; Rosenberg and Kumar 2003). *Agrobacterium tumefaciens* C58 (now *Agrobacterium fabrum* C58) is the first *Agrobacterium* strain to have its complete genome sequenced by two separate research groups using conventional Sanger sequencing (Goodner et al. 2001; Wood et al. 2001) and subsequently revised with improved annotation (Slater et al. 2013). Approximately nine years later, the complete genome for members from the remaining two biovars, e.g. *Agrobacterium vitis* (biovar 3, now *Allorhizobium vitis*) and *Agrobacterium radiobacter* (biovar 2, now *Rhizobium* sp.; Slater et al. 2009), was reported. In addition, for the first time a high-resolution phylogeny of *Agrobacterium* was constructed based on the concatenated protein

alignment of 507 single-copy orthologous gene families encoded on the primary chromosomes. Phylogenetic clustering patterns indicated that biovar 2 should be grouped to the genus *Rhizobium*, whereas biovar 3 and biovar 1 are still members of the *Agrobacterium* genus. The limited taxon sampling resulting from the high cost of whole genome sequencing at the time unfortunately prevented Slater et al. (2009) from inferring the delineation of biovar 3 and biovar 1 into two separate genera.

4.2 Next-Generation Sequencing and Agrobacterium

The advent of next-generation sequencing brought about a revolution in microbial genomics by enabling the whole genome sequence of a pure culture to be obtained at a small fraction of the cost and time initially required by Sanger sequencing (MacLean et al. 2009; Metzker 2010). Coupled with advances in algorithms for quick and accurate microbial genome assembly and annotation (Bankevich et al. 2012; Seemann 2014), the scientific community is now blessed with an explosion of publicly available microbial genomic resources which naturally invite a new investigation of the phylogeny of *Agrobacterium*. Ormeno-Orrillo and workers used a sophisticated and reproducible bioinformatics pipeline (Segata et al. 2013) to reconstruct the *Agrobacterium* phylogeny based on the concatenated alignment of 384 universal proteins identified from 113 sequenced strains from the family Rhizobiaceae (Ormeno-Orrillo et al. 2015). In contrast to the previously inferred whole genome phylogeny, *Agrobacterium vitis* S4 no longer formed a tight cluster with *Agrobacterium tumefaciens* C58. Instead, the increased taxon sampling supported previous *recA* and MLSA-based analyses indicating the monophyletic clustering of *Agrobacterium vitis* S4 with members of the genus *Allorhizobium* such as *Allorhizobium undicola* (de Lajudie et al. 1998), lending further support to the revival of *Allorhizobium* as a genus within the Rhizobiaceae (Mousavi et al. 2014). By reclassifying *Agrobacterium* biovars 2 and 3 into separate genera (Mousavi et al. 2014, 2015; Velázquez et al. 2010), a monophyletic cluster consisting solely of members from the genus *Agrobacterium* can be obtained with maximal support, indicating that at the genomic level, *Agrobacterium* is a definable genus of the family Rhizobiaceae (Ormeno-Orrillo et al. 2015). The author noted, however, the exclusion of an important Agrobacterium genome, e.g. *Agrobacterium radiobacter* NCPPB 3001 = DSM30147^T (accession number ASXY01, Bioproject PRJNA212112; Zhang et al. 2014) from their analysis, citing unusual genomic anomalies such as low sequence homology (<97%) to some of its published gene sequences. Leveraging the recent availability of key *Agrobacterium* species genomes, Kim and Gan (2017) performed a smaller scale phylogenomic analysis of the genus *Agrobacterium* showing the monophyletic clustering of *A. tumefaciens* B6 and *A. radiobacter* NCPPB 3001^T = DSM30147^T with high pair-wise ANI value (>95%), providing conclusive genomic evidence that both strains are identical species (Kim and Gan 2017).

4.3 *Updating the* Agrobacterium *Phylogeny in the Light of More Publicly Available Genomic Resources*

In this chapter, we present an updated phylogeny of *Agrobacterium* and more generally the Rhizobiaceae using a similar PhyloPhlAn approach implemented by Ormeno-Orrillo et al. (2015). PhyloPhlAn is a bioinformatics pipeline which takes the predicted proteomes from multiple microbial strains in fasta format as input and uses an ultra-fast protein similarity search (Edgar 2010) to identify more than 400 single-copy and conserved proteins within each predicted proteome. The identified proteins are aligned individually using MUSCLE (Edgar 2004), concatenated, and used for maximum likelihood tree reconstruction with FastTree2 (Price et al. 2010). Consistent with previous reports, a cluster consisting of mainly *Agrobacterium* strains could be recovered with maximal support, with *Agrobacterium rubi* and *Agrobacterium larrymoorei* being basal to the rest of *Agrobacterium* (Figs. 1 and 2). The presence of a substantial number of *Rhizobium* strains in the *Agrobacterium* clade (Fig. 2) is an aftermath of Young et al.'s initial proposal (2001) for merging *Agrobacterium* with the genus *Rhizobium*. In addition, the phylogenetic placement of *A. radiobacter* DSM 30147^T basal to the rest of agrobacteria genomovar 4, which now includes a more recent and improved genome of *A. radiobacter* DSM 30147^T

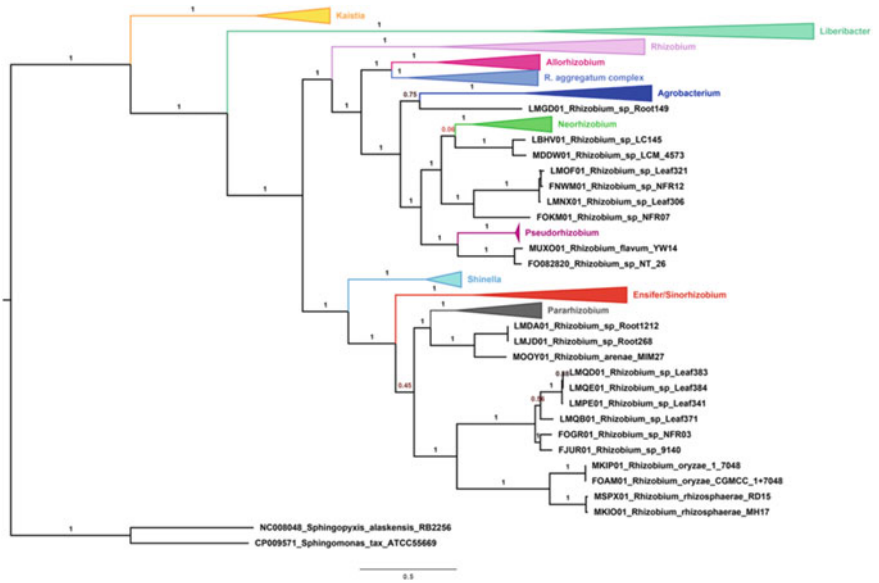
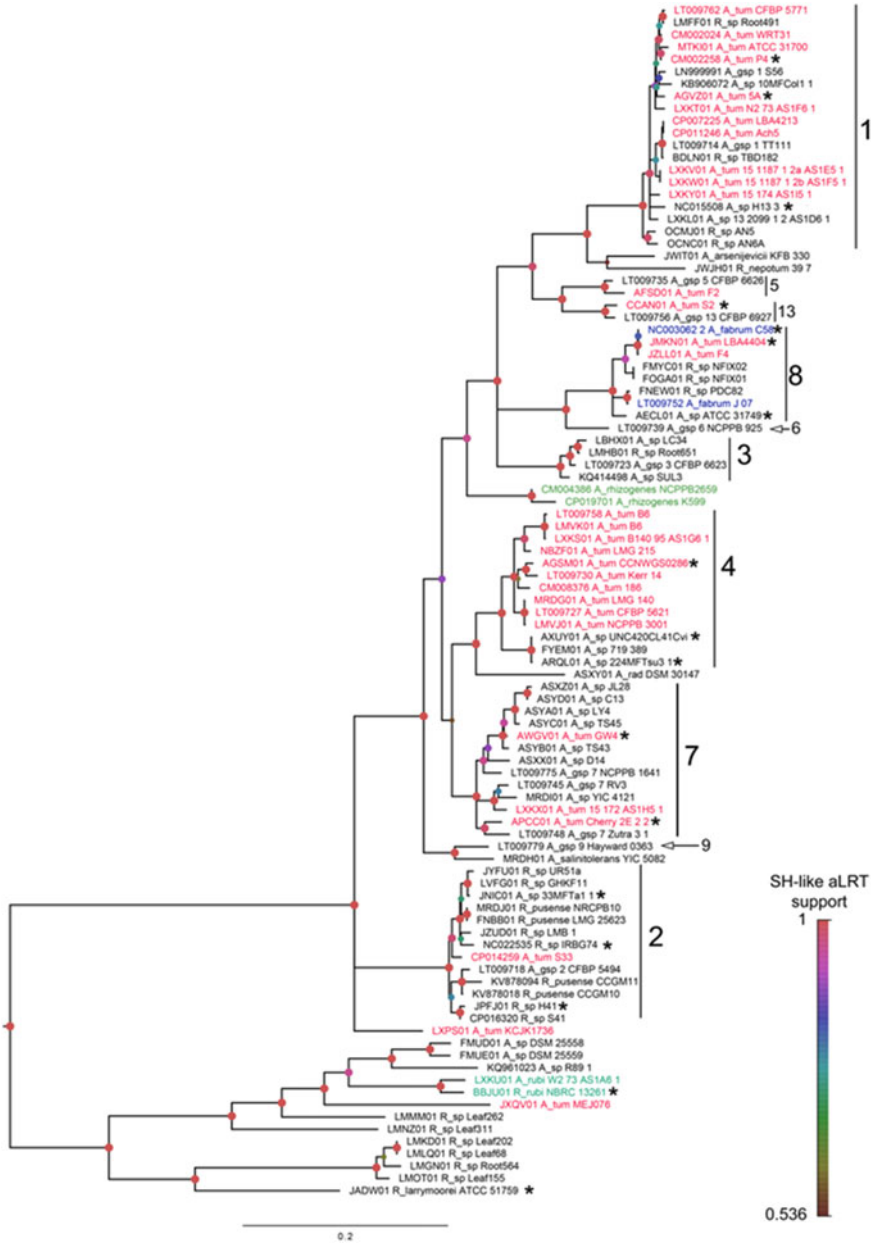


Fig. 1 Reconstruction of the Rhizobiaceae phylogeny using maximum likelihood inference based on the concatenated amino acid alignment of universal single-copy genes as implemented in the PhyloPhlAn pipeline (Segata et al. 2013). Members of the family Sphingomondaceae were rooted as the outgroup. Values along branch indicate SH-like aLRT support values (Shimodaira and Hasegawa 1999) calculated using FastTree2 (Price et al. 2010)



(=NCPPB3001^{T}; WGS Accession: LMVJ01; Lee et al., unpublished), is unusual, suggesting a genome assembly anomaly as previously noted (Ormeno-Orrillo et al. 2015). Another notable anomaly revealed by increased taxon sampling is the unexpected clustering of strain LBA4404, a disarmed derivative of the wild-type

◂**Fig. 2** Expanded *Agrobacterium* clade from Fig. 1 depicting the evolutionary relationships among *Agrobacterium* strains. First text strings are the WGS accession numbers, and the first letters after the strings represent the submitted genus name (R = *Rhizobium*; A = *Agrobacterium*). Taxon name is as per species name deposited into the NCBI whole genome shotgun database. Taxa coloured green: *Agrobacterium rubi*; taxa coloured red: *Agrobacterium tumefaciens*; taxa coloured blue: *Agrobacterium fabrum*. Nodes were coloured according to their SH-like local support values, and genomic species clusters were indicated by the vertical lines or arrows next to the tree. Asterisk signs indicate taxa that were included in a previous large-scale phylogenomic analysis by Ormeno-Orrillo et al. (2015). The tree was constructed using a whole genome-based (400 universal single-copy genes) approach

Ach5 Tn*904* mutant (strain LBA4213), with members from the genovar 8 containing *Agrobacterium fabrum* C58 (Ooms et al. 1982). Recently, both strains Ach5 and LBA4213 have been sequenced by two independent groups (Henkel et al. 2014; Huang et al. 2015) and in contrast to strain LBA4404, both strains resided in the genomovar 1 clade, forming a monophyletic group. Given the known divergence between strain Ach5 and strain C58, this strongly indicates that the currently deposited whole genome sequence of strain LBA4404 is incorrect and warrants future investigation. The abnormal phylogenetic placement of strain LBA4404 was similarly observed but not explicitly mentioned in a study by Ormeno-Orrillo et al. (2015). Clustering based on genospecies is apparent; albeit the relationships among some of the genospecies are not strongly supported, suggesting the limitation of amino acid-based phylogenomic analysis for fully resolving strain, subspecies, and/or species-level relationships similarly observed in a recent genome-based phylogeny of *Pseudomonas* (Tran et al. 2017). To infer accurately the phylogeny of the currently well-supported *Agrobacterium* clade, future work utilizing the newly published phylogenetic-aware pan-genome analysis tool (Ding et al. 2017) to improve the recovery of core *Agrobacterium* single-copy genes, coupled with complementary analysis based on pair-wise average nucleotide identity (ANI) (Richter et al. 2016), will be instructive.

5 Genomic Species Within *Agrobacterium*

Traditionally, genome–genome hybridization has been used to establish genomic relatedness among strains, and a hybridization ratio of approximately 70% between two strains usually indicates a species-level relationship (Wayne et al. 1987; Stackebrandt et al. 2002). Average nucleotide calculation (ANI) is becoming increasingly popular for in silico species delineation in the light of genomic data availability. An initial genomic comparison indicated 95% pair-wise ANI as correlated with 70% DNA–DNA hybridization (DDH), and this correlation was consistently observed in various subsequent studies (Auch et al. 2010; Colston et al. 2014). Using the established 70% DDH criterion in addition to a follow-up validation based on mathematical models and amplified fragment length polymorphism (AFLP) data, members within the *Agrobacterium tumefaciens* complex were

classified into ten distinct genomic species with a non-continuous genomovar numbering, e.g. G1–G9 followed by G13, as a consequence of the reclassification of some initially established genomovars to a different genus, e.g. *Agrobacterium rhizogenes* (genomovar 10) to *Rhizobium rhizogenes* or to a greater extent *Agrobacterium* clade, e.g. *Agrobacterium rubi* (genomovar 11; clade 2 in Fig. 2). To date, most of the genomovars have not received official Latin binomials due to the lack of differentiating biochemical features that are traditionally used to describe new bacterial species. Lassalle et al. (2011) took one of the first initiatives to differentiate the *Agrobacterium tumefaciens* species complex by identifying the gene repertoire specific to *Agrobacterium* genospecies 8 which includes strain C58, a widely used strain among *Agrobacterium* geneticists that has had its genome sequenced and annotated. By comparing the C58 genome against 25 strains from different *Agrobacterium* genospecies based on hybridization to DNA microarrays spanning the whole genome of strain C58, genes relevant to the speciation and ecological isolation of genomovar G8 were identified. Phenotypic traits specific to genomovar G8 initially inferred from microarray data, such as ferulic acid degradation and curdlan production, were subsequently validated using HPLC and Congo red assays, respectively. As a result, the species name *Agrobacterium fabrum* was suggested for strains of *Agrobacterium* genomovar G8, from the Latin plural genitive of *smith*, in reference to the pioneer isolator of an *Agrobacterium* strain (Smith and Townsend 1907).

Based on identification of a gene repertoire unique to genomovar G8 that is associated with commensal interactions with plants, and by citing several similar studies linking ecological niche and genomic species beyond the genus *Agrobacterium* (Cai et al. 2009; Johnson et al. 2006; Lefébure et al. 2010; Porwollik et al. 2002), Lassalle et al. (2011) suggested the generalization of the concept of bacterial genomic species as ecological species. A potential exception to this generalization is currently emerging within *Agrobacterium* genomovar G3. The *Agrobacterium* genomovar G3 initially consisted of strains isolated from clinical environments, e.g. human host and antiseptic flask (Popoff et al. 1984). However, based on the newly constructed phylogenomic tree, in addition to the classical *Agrobacterium* sp. CFBP 6623, the agrobacteria G3 clade now consists of strains LC34, SUL3, and Root651 which were isolated from a diverse and non-clinical environment. Notably, *Agrobacterium* sp. LC34 originated from the rock surface of the Lechuguilla Cave which has been isolated from humans for over four million years (Bhullar et al. 2012), an environment that substantially differs from that of strain CFBP 6623. On the contrary, *Agrobacterium* sp. Root651 may share a similar ecological niche with that of G8 agrobacteria given that it is a member of the *Arabidopsis* plant root microbiota (Bai et al. 2015). *Agrobacterium* sp. SUL3 was isolated from a laboratory culture of the hydrocarbon-producing *Botryococcus braunii*, a non-plant photosynthetic organism (green microalga; (Jones et al. 2016). Taken together, it will be hard to convince microbial ecologists that members of the *Agrobacterium* genomovar G3 are a single ecological species despite their high genomic relatedness.

6 Concluding Remarks

The progress of using whole genome sequence data for establishing relatedness among members of the Rhizobiaceae family is presented. As additional whole genome sequences of these members are elucidated, further insight into the complex phylogeny of *Agrobacterium* will become available. Further and rigorous analysis of large data sets will validate or further contest the concept of bacterial genomic species as ecological species.

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