Letter to the Editor: The Ralstonia research community rejects the proposal to classify phylotype I Ralstonia into the new species Ralstonia nicotianae

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Letter to the Editor: The *Ralstonia* research community rejects the proposal to classify phylotype I *Ralstonia* into the new species *Ralstonia nicotianae.*

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The *Ralstonia solanacearum* species complex (RSSC) is a group of globally important plant pathogens. Bacteria in this very large and genetically diverse group all colonize the xylem elements of angiosperm plants and cause high-impact wilting diseases of many crops. Because they threaten economic and food security, several RSSC subgroups are strictly regulated as quarantine pests (See “Regulation” section of References). Biologically meaningful and consistent nomenclature is essential for organisms that have major economic and regulatory importance like plant pathogenic *Ralstonia*. There are currently three species of *Ralstonia* wilt pathogens: *R. pseudosolanacearum* (corresponding to two phylogenetic groups that are described in the literature as phylotypes I and III), *R. solanacearum* (phylotypes IIA, IIB, and IIC), and *R. syzygii* (phylotype IV, containing three subspecies: subsp. *syzygii*, subsp. *celebensis*, and subsp. *indonesiensis*). A recent paper proposed re-classifying phylotype I as a new species named “*Ralstonia nicotianae”* (Liu et al. 2023). The purpose of this commentary is to register our objection to the taxon “*Ralstonia nicotianae*”.

Although changing bacterial taxonomy is sometimes necessary, the *Ralstonia nicotianae* proposal is not justified. Changing the taxonomy of any groups of organisms can be disruptive to both scientists and regulators, so it should not be proposed for trivial reasons, as explained by the code of the International Committee on Systematics of Prokaryotes (ICSP) (Oren et al. 2023). There are two main reasons to propose new bacterial species. First, the isolation and discovery of novel bacteria that do not belong to any named species justifies the naming of a new species. Second, better data from technological and analytical advances can change our understanding of the diversity and evolution of bacterial lineages. With sufficient evidence, these advances can justify taxonomic revisions so that the newly named species better reflect evolutionary relationships. However, the *R. nicotianiae* proposal is not based on discovery of a new lineage, nor does it reflect novel insight into the evolutionary relationships within the RSSC. As demonstrated below, the *R. nicotianiae* proposal ignores natural phylogenetic gaps among the existing three species. Moreover, it is based on inappropriately selective use of molecular analyses.

During their decades-long careers, Drs. Philippe Prior and Mark Fegan collected and studied the diversity of RSSC plant pathogens from around the world. Both research group leaders concurred that the RSSC is properly divided into three species (Fegan and Prior 2005; Remenant et al. 2010, 2011; Prior et al. 2016; Safni et al. 2014). Specifically, extensive genomic and biological analyses of phylotype I and III strains led these and other experts to conclude that phylotype I and III should not be divided into distinct

species (Prior et al. 2016; Truchon et al. 2023; Sharma et al. 2022). As a result, three RSSC species were validly published in 2014 as *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* (Safni et al. 2014). These names were subsequently validated by the list editors of the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM). Figure 1 shows the overall relationships among subgroups of the RSSC in a phylogenetic tree constructed using the core genome of the species complex. *R. pseudosolanacearum* is composed of two major subgroups (phylotype I and III). *R. solanacearum* is composed of three major subgroups (phylotype IIA, IIB, and IIC).

Natural gaps in genetic diversity separate the three RSSC species. Average nucleotide identity (ANI) is now an accepted way to use whole genome sequences to measure relationships between strains and propose species delineations (Oren et al. 2023). The ANI between any pair of genomes can be calculated based on different algorithms, such as BLAST comparisons ("ANIb") or the MUMMER index ("ANIm"). We used pyani (Pritchard et al. 2016), a Python-based ANIb software, to calculate pairwise ANIb values for 300 RSSC genomes, including genomes of 11 phylotype III strains and 148 phylotype I strains. When the resulting 90,000 ANIb values are hierarchically clustered and visualized as a heatmap, three obvious clusters correspond to the three accepted RSSC species (Fig. 2A).

An ANI threshold of 96% is not the appropriate cut-off for delineating species in the RSSC. Depending on the taxon, bacterial species borders can be drawn using ANI threshold values of 95-96% (Oren et al. 2023; Chun et al. 2018). However, ANI <95% is the most widely used cut-off for dividing species. This threshold has been applied across the bacterial domain by the Genome Taxonomy Database (Parks et al. 2020). We investigated the distributions of 90,000 ANIb comparisons among 300 RSSC genomes to determine if there is a biologically relevant cut-off that separates RSSC species.

The *R. nicotianae* proposal applied an ANI = 96% species threshold value. However, our analysis of 300 RSSC genomes suggests that 95% is the appropriate threshold for delineating species within the RSSC (Fig. 2). Visualizing the distribution of ANI values reveals an obvious natural gap in ANIb values: no pairwise comparison yields an ANI value between 92.57% and 95.06% (Fig. 2B). Applying an ANI cut-off of 96% (indicated by the red lines in Figure 2 graphs) would interrupt a continuous distribution of genetic distances within the RSSC as a whole (Fig. 2B), within *R. solanacearum* (Fig. 2C) and within *R. pseudosolanacearum* (Fig. 2D). In contrast, an ANI = 95% cut-off (indicated by the blue lines) separates the RSSC into three species with clear gaps that suggest that these groups have distinct evolutionary

histories (Fig. 2E) and the existing 3-species nomenclature may thus represent their natural phylogenetic order.

The data presented in the *R. nicotianae* proposal do not support a division of phylotype I into a new species. This section provides a detailed dissection of ANI data to highlight the methodological problems in the *R. nicotianae* proposal.

The *R. nicotianae* proposal was based on limited analyses that compared genomes of a single phylotype I and a single phylotype III genome against other *R. pseudosolanacearum* genomes. This approach significantly biased the statistics and phylogenetic analyses, as it does not reflect the diversity of a representative population of isolates. The focal strains were the established type strain of *R. pseudosolanacearum* (phylotype III strain LMG9673^{T}) and a phylotype I strain (RS) that was proposed as a type strain for the novel species. Hereafter, we refer to this strain as RS^{proposed_T}.

The *R. nicotianae* proposal calculated ANI with three methods: FastANI using the Genome Taxonomy Database website interface, ANIb using the JSpeciesWS website interface, and MUMMER-based ANI (ANIm) using the JSpeciesWS website interface. The authors then carried out 434 FastANI comparisons (LMG9673^{T} and RS^{proposed_T} vs. 204 phylotype I and 11 phylotype III strains), 24 ANIb comparisons (LMG9673^{T} and RS^{proposed_T} vs. 1 phylotype I and 11 phylotype III strains), and 24 ANIm comparisons (LMG9673^{T} and RS^{proposed_T} vs. 1 phylotype I and 11 phylotype III strains).

Comparing RS^{proposed_T} to the 11 phylotype III genomes yielded FastANI values from 95.85 to 96.06%, ANIm values from 96.12 to 96.26%, and ANIb values from 94.95 to 95.33% as described in the *R. nicotianae* proposal. We also computed ANIb values, but we used the Python-based pyani tool over a larger sample size of phylotype I and III genomes (Fig. 2). In the subset of comparisons that overlap between our analysis and that of the *R. nicotianae* proposal, pyani yielded ANIb values from 95.77-96.02%. An overview of these data are presented in Fig. 3A, which compares the ANI values obtained for each of the comparisons and methods.

For taxonomic classification, the most important ANI comparisons are between type strains. In the *R. nicotianae* proposal, comparisons between RS^{proposed_T} and the *R. pseudosolanacearum* type strain

LMG9673^{T} yielded values of 95.97 to 96.02% (FastANI), 96.14 to 96.15% (ANIm), and 95.23 to 95.30% (ANIb). Our pyani calculation of ANIb yielded a narrow range of values from 95.81 to 95.82%.

Before genome sequences were readily available, the gold standard for classifying bacterial strains into species was a wet-lab technique called DNA-DNA Hybridization (DDH). A 70% DDH threshold was used to delineate bacterial species. The *R. nicotianae* proposal used three digital DDH calculations (dDDH) to estimate DDH between RS^{proposed_T} and LMG9673^{T}. Two dDDH calculations yielded values above the standard 70% threshold (74.9% and 75.8%) while a third dDDH calculation yielded a value of 66.2%. If averaged, the three calculations yield 72.3%, above the 70% species cut-off. Fig. 3B shows the full distribution of dDDH scores from the *R. nicotianae* proposal. However, the text of the *R. nicotianae* proposal emphasized only the lowest of these three DDH values.

The careful assessment above reveals that the conclusions in the *R. nicotianae* proposal were based on the sole DDH analysis and the sole ANI analysis where comparisons of type strains yielded a value less than the 70% DDH threshold and an ANI value in the gray zone of 95-96% ANI. This ignored the molecular phylogenomic analysis results that suggested that phylotype I should remain within the *R. pseudosolanacearum* species. Selecting among obtained results to present only the subset of results that support a preferred narrative is not consistent with good scientific practice (Casadevall and Fang 2016).

Even if there was genomic and biological justification for the separation of phylotype I into a novel species, *nicotianae* would be a misleading species epithet for phylotype I. The epithet *nicotianae* was suggested because pathogenic bacteria are sometimes named for their host of isolation, usually the primary host, and the proposed Type strain RS^{proposed_T} was isolated from an experimental tobacco plot. However, this name would be misleading because infecting tobacco is not a distinguishing trait of phylotype I. RSSC strains from each of the four phylotypes have been isolated from tobacco (Lowe-Power et al. 2020). Furthermore, phylotype I strains have the broadest host range within the RSSC; phylotype I strains have been isolated from 95 plant species in 79 genera in 46 families (Lowe-Power et al. 2020). In comparison, the other three phylotypes combined have been isolated from only 69 plant species in 40 genera in 28 families (Lowe-Power et al. 2020).

Proposing new names without careful consideration can create confusion in the research community and potentially in the published literature. For example, the widely-used NCBI genome database transiently

adopted the *R. nicotianae* proposal. Within two weeks of the publishing of the *R. nicotianae* proposal in *Frontiers in Microbiology*, we noticed that NCBI had renamed the genome of the much studied model *R. pseudosolanacearum* strain GMI1000 to "*Ralstonia nicotianae*". This occurred before the IJSEM list editors had the opportunity to consider this proposal and issue a decision about publishing the new name. Although GMI1000 is a phylotype I *R. pseudosolanacearum* strain, the GMI1000 genome was still labeled in NCBI as "*Ralstonia solanacearum*" for historical reasons: the genome was sequenced and deposited 14 years before the RSSC was formally divided into three species (Salanoubat et al. 2002). Importantly, this error was promptly corrected when it was brought to the attention of NCBI.

Summary. Adopting "*R. nicotianae*" as a newly named species corresponding to phylotype I and reducing the validly published species *R. pseudosolanacearum* to include only phylotype III is not justified based on either genomic similarity or evolutionary relationships. On the contrary, the comparative genomics analyses presented in the *R. nicotianae* proposal are consistent with the conclusion that phylotype I and phylotype III are two subgroups of the same species, *R. pseudosolanacearum*. Furthermore, accepting a division of phylotype I and III into separate species would complicate and disrupt scientific and regulatory communication about strains and genomes of plant pathogenic *Ralstonia*. Changing the name of a taxon that has been established and validated through multiple rigorous studies would create unnecessary confusion. This proposal violates three of the four essential elements of Principle 1 of the International Code of Nomenclature of Prokaryotes, which states that nomenclature should: "1) Aim at stability of names; 2) Avoid or reject names that create error or confusions; and 3) Avoid the useless creation of names" (Oren et al. 2023). Finally, the chosen species name would be misleading regarding the host range of the strains that belong to it and to the related strains in other species within the RSSC, and is thus in conflict with International Code of Nomenclature of Prokaryotes Recommendation 12(c) 2: "Avoid [epithets] that express a character common to all, or nearly all, the species of a genus" (Oren et al. 2023). These reasons, together with the analyses presented in this letter, establish that "*Ralstonia nicotianae"* Liu et al. 2023 is at most a junior heterotypic synonym of *Ralstonia pseudosolanacearum* Safni et al. 2014.

Therefore, we strongly encourage our fellow scientists in the RSSC community not to adopt *R. nicotianae* in publications and scientific communication in general. We further respectfully request that the IJSEM list editors review the evidence presented here when considering whether *R. nicotianae* should be validly published.

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European Union: Regulation (EU) 2016/2031 of the European Parliament of the Council of 26 October 2016 on protective measures against pests of plants, amending Regulations (EU) No 228/2013, (EU) No 652/2014 and (EU) No 1143/2014 of the European Parliament and of the Council and repealing Council Directives 69/464/EEC, 74/647/EEC, 93/85/EEC, 98/57/EC, 2000/29/EC, 2006/91/EC and 2007/33/EC https://eur-lex.europa.eu/eli/reg/2016/2031/oj

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Fig. 1. Core genome phylogenetic tree demarcating the three RSSC species and their major subdivisions: *R. pseudosolanacearum* (phylotype I and III subdivisions), *R. solanacearum* (phylotype IIA, IIB, and IIC subdivisions (Sharma et al. 2022)), and *R. syzygii*. Tree was built using IQtree (Minh et al. 2020) using the core-genome alignments obtained with PIRATE (Bayliss et al. 2019) as input.

Fig. 2. The biologically relevant ANI threshold for delineating RSSC species is 95%. (A) Robust ANI analysis of 300 RSSC genomes reveals three species clusters corresponding to *R. pseudosolanacearum, R. solanacearum,* and *R. syzygii*. Pairwise comparisons are shown in an ANI heatmap calculated with the BLAST-based ANIb method using pyani (Pritchard et al. 2016). (B) The distribution of pairwise ANIb values between 300 RSSC strains reveals a natural gap between pairs sharing 92.57% and 95.06% ANIb. ANIb was calculated with pyani (Pritchard et al. 2016). (C) Comparison of ANI values within the *R. pseudosolanacearum* species and its two major subdivisions. (D) Comparison of ANI values within the *R. solanacearum* species and its three major subdivisions. (E) Comparison of ANI values between the three validated RSSC species. Blue lines show the biologically relevant ANI threshold of 95% and red lines show the biologically inappropriate threshold of 96%.

Fig 3. The *R. nicotianae* proposal focused on outlier ANI and dDDH calculations that supported a new species. (A) Comparison of ANI values from the 12 pairs of genomes that were shared between the *R. nicotianae* proposal and our larger-scale analysis (Figs 1 and 2). The *R. nicotionae* proposal analyzed ANI between six phylotype III genomes to two strains: the *R. pseudosolanacearum* type strain (LMG9673^{T}) and the phyl. I strain proposed as a new type strain (RS^{proposed_T}). (B) Comparison of dDDH calculations from the *R. nicotianae* proposal. Lines connect the same strain pairings that were analyzed using three different dDDH tools. ANI and DDH comparisons of *R. pseudosolanacearum* type strain LMG9673^{T} and RS^{proposed_T} are shown in red. Arrows indicate the outlier results favored in the *R. nicotianae* proposal.

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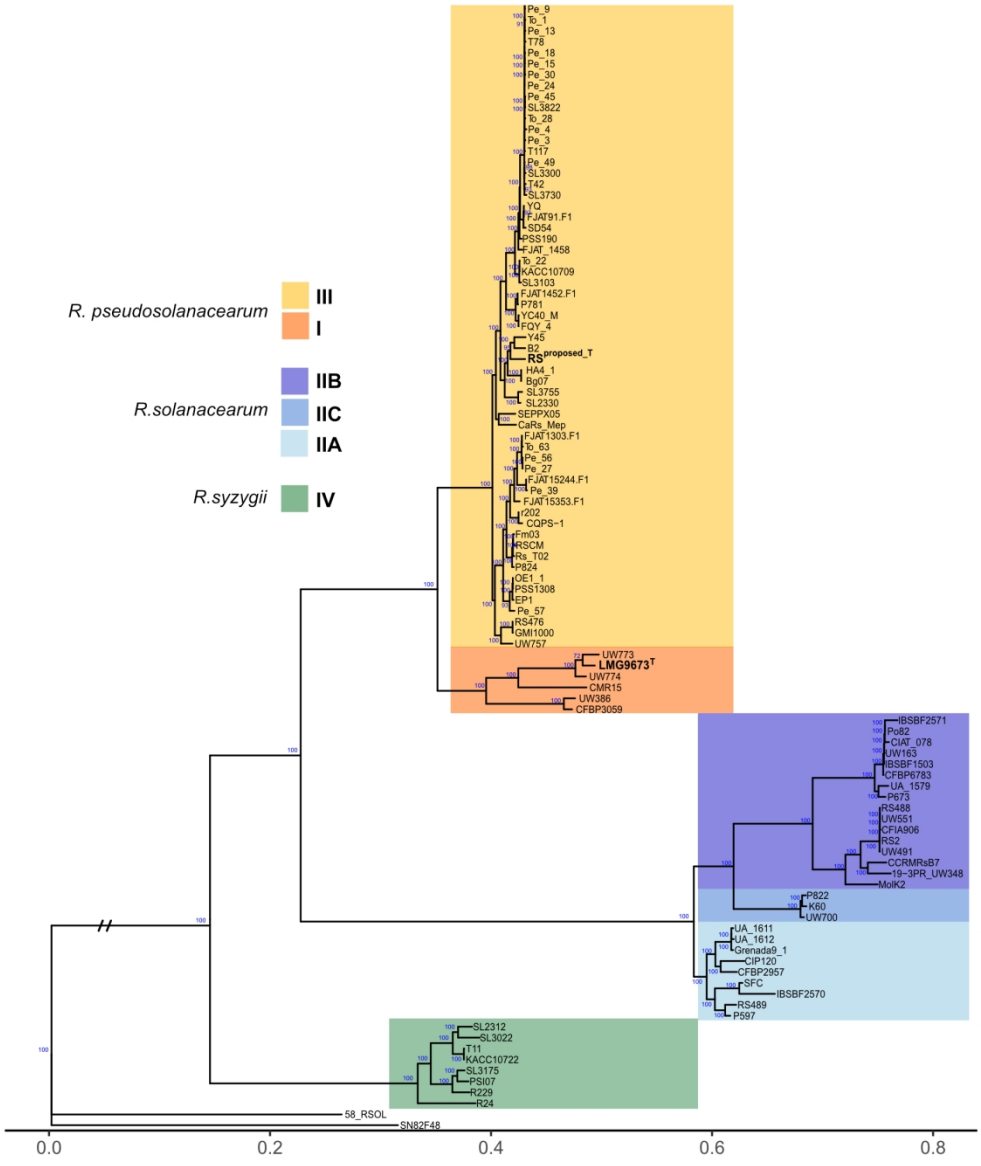

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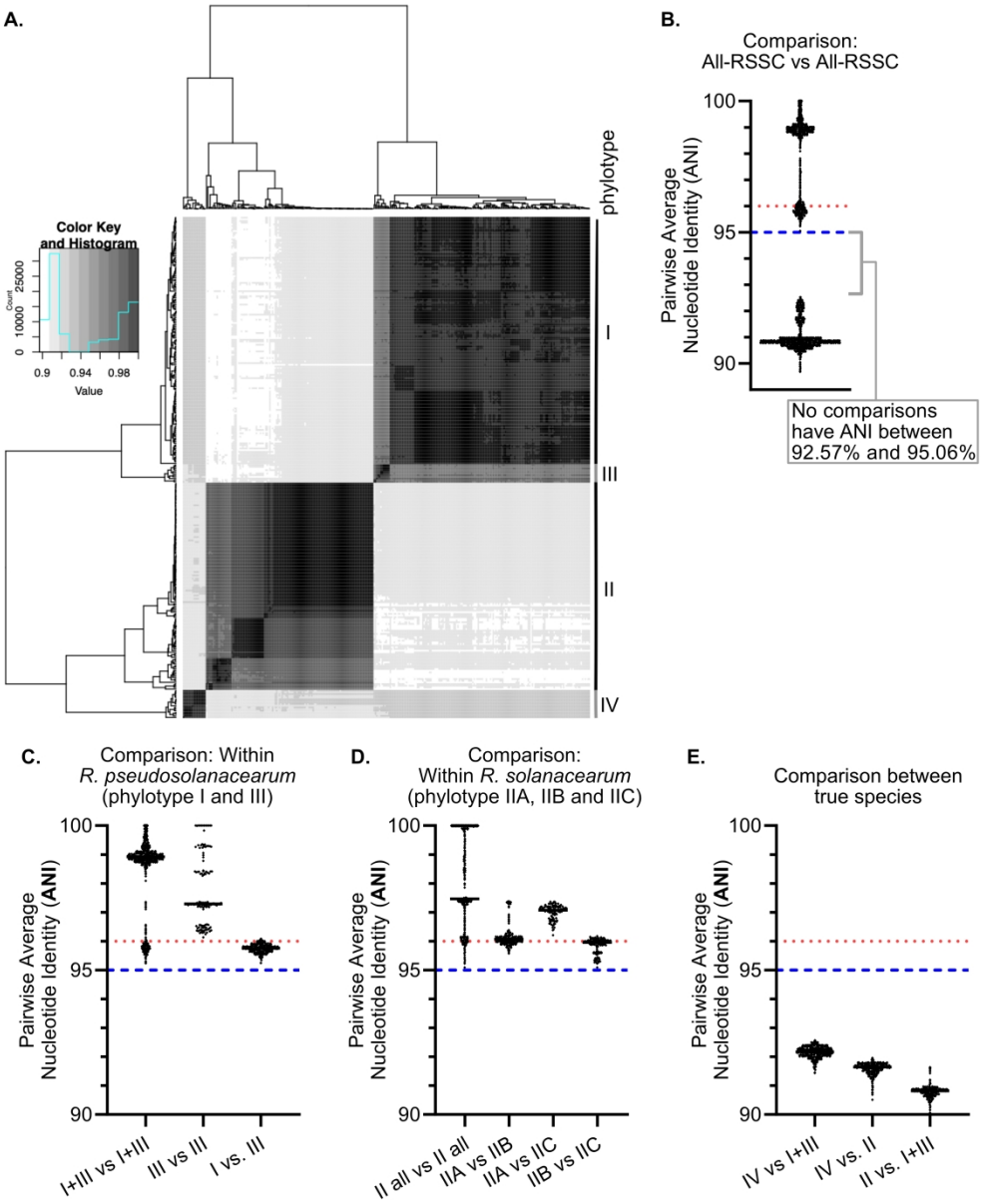

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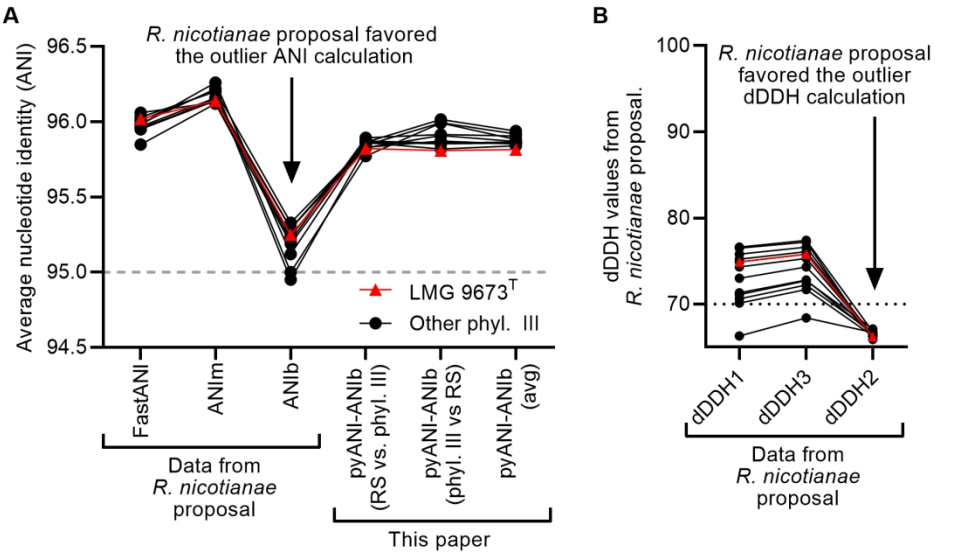

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