RAREFAN: A webservice to identify REPINs and RAYTs in bacterial genomes

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Running title: REPIN/RAYT Finder and ANalyzer

Keywords: sequence analysis – mobile genetic elements – bacterial genomes – *Stenotrophomonas maltophilia*

Abstract

Compared to eukaryotes, repetitive sequences are rare in bacterial genomes and usually do not persist for long. Yet, there is at least one class of persistent prokaryotic mobile genetic elements: REPINs. REPINs are non-autonomous transposable elements replicated by single-copy transposases called RAYTs. REPIN-RAYT systems are mostly vertically inherited and have persisted in individual bacterial lineages for millions of years. Discovering and analyzing REPIN populations and their corresponding RAYT transposases in bacterial species can be rather laborious, hampering progress in understanding REPIN-RAYT biology and evolution. Here we present RAREFAN, a webservice that identifies REPIN populations and their corresponding RAYT transposase in a given set of bacterial genomes. We demonstrate RAREFAN's capabilities by analyzing a set of 49 *Stenotrophomonas maltophilia* genomes, containing nine different REPIN-RAYT systems. We guide the reader through the process of identifying and analyzing REPIN-RAYT systems across *S. maltophilia*, highlighting erroneous associations between REPIN and RAYTs, and providing solutions on how to find correct associations. RAREFAN enables rapid, large-scale detection of REPINs and RAYTs, and provides insight into the fascinating world of intragenomic sequence populations in bacterial genomes. RAREFAN is available at http://rarefan.evolbio.mpg.de.

35 **Introduction**

36 Repetitive sequences in bacteria are rare compared to most eukaryotic genomes. In eukaryotic
37 genomes, repetitive sequences are the result of the activities of persistent parasitic transposable
38 elements. In bacteria, in contrast, parasitic transposable elements cannot persist for long periods
39 of time (Park *et al.* 2021; van Dijk *et al.* 2022). To persist in the gene pool, transposable elements
40 have to constantly infect novel hosts (Sawyer *et al.* 1987; Lawrence *et al.* 1992; Bichsel *et al.*
41 2010; Rankin *et al.* 2010; Wu *et al.* 2015; Park *et al.* 2021). Yet, there is at least one exception: a
42 class of transposable elements called REPINs.

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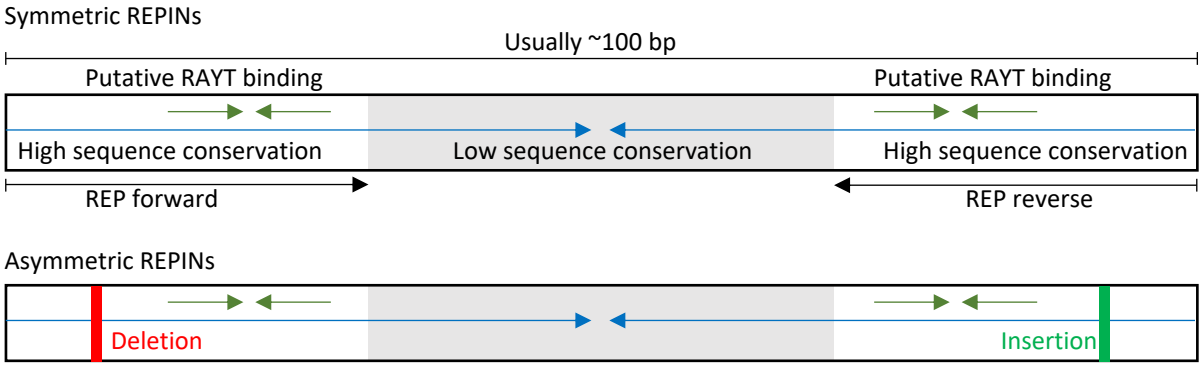

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Figure 1. The structure of symmetric and asymmetric REPINs. A typical REPIN consists of two highly conserved regions at the 5' and 3' end (white), separated by a spacer region of lower sequence conservation (grey). The entire REPIN is an imperfect palindrome (blue arrows), which means it can form hairpin structures in single stranded DNA or RNA. Each 5' and 3' region contains a nested imperfect palindrome, which is referred to as REP (repetitive extragenic palindromic) sequence and has first been described in *Escherichia coli* (Higgins *et al.* 1982). REPINs can be either symmetric or asymmetric. Asymmetric REPINs have a deletion and a corresponding insertion in the highly conserved 5' or 3' end, which leads to "bubbles" in the hairpin structure. REPINs in for example *Pseudomonas fluorescens* SBW25 are symmetric while REPINs in *E. coli* are asymmetric. Asymmetric REPINs make analyses with RAREFAN more challenging. Figure adapted from (Bertels, Rainey 2023), which is licensed under CC BY 4.0.

45 REPINs are short (~100 bp) nested palindromic sequences (**Figure 1**) that consist of two inverted
46 REP (repetitive extragenic palindromic (Higgins *et al.* 1982)) sequences that can be present
47 hundreds of times per genome (Bertels, Rainey 2011a). Most REPINs are symmetric where the 5'
48 REP sequences are identical to the 3' REP sequences, with the occasional substitution (Bertels,
49 Rainey 2011a; b). However, there are also asymmetric REPINs where the 5' REP sequence differs

50 from the 3' REP sequence by a point deletion or insertion (Bertels, Rainey 2011a, 2023), which
51 makes the analysis and detection of REPINs significantly more difficult (*e.g.*, *Escherichia coli*
52 REPINs). Isolated REP sequences, sometimes referred to as REP singlets, can also be found in the
53 genome. These sequences are decaying remnants of REPINs that are no longer mobile (Bertels,
54 Rainey 2011a). REPINs are non-autonomous mobile genetic elements, which means they require
55 a RAYT (**R**EP **A**ssociated t**Y**rosine **T**ransposase) transposase gene (also referred to as $tnpA_{REP}$) to
56 replicate inside the genome (Nunvar *et al.* 2010; Bertels, Rainey 2011a; Ton-Hoang *et al.* 2012).

57

58 Within a genome, each REPIN population is usually only associated with a single RAYT gene.
59 Hence, RAYT genes occur only in single copies per genome and do not copy themselves, unlike
60 for example insertion sequences where often multiple identical sequences are present inside the
61 genome. Unlike insertion sequences RAYT genes are almost exclusively inherited vertically,
62 meaning they are host-beneficial transposases that have been coopted by the host (Bertels,
63 Gallie, *et al.* 2017; Bertels, Rainey 2023). The fact that REPINs and their corresponding RAYT genes
64 are confined to a single bacterial lineage makes them unique, in comparison to all other parasitic
65 mobile genetic elements in bacterial genomes (Bertels, Rainey 2023).

66

67 Of a total of five different RAYT families, there are only two RAYT families that are associated
68 with repetitive sequences such as REPIN or REP sequences: Group 2 and Group 3 RAYTs (Bertels,
69 Gallie, *et al.* 2017). Group 2 RAYTs are present in most Enterobacteria and usually occur only once
70 per genome associated with a single REPIN population. In contrast, Group 3 RAYTs are found in
71 most *Pseudomonas* species and are usually present in multiple divergent copies per genome,
72 each copy associated with a specific REPIN population (Bertels, Gallie, *et al.* 2017).

73

74 REPINs and their corresponding RAYT genes occur exclusively in bacterial genomes and are
75 absent in eukaryotic or archaeal genomes (Bertels, Gallie, *et al.* 2017; Bertels, Rainey 2023).
76 Within bacterial genomes REPINs and RAYTs have been evolving in single bacterial lineages for
77 millions of years (Bertels, Gallie, *et al.* 2017). The long term persistence of REPINs in single

78 bacterial lineages can also be observed when analyzing REPIN populations (Bertels, Gokhale, *et*
79 *al.* 2017; Bertels, Rainey 2023).

80

81 Parasitic insertion sequences usually occur in identical copies in bacterial genomes, reflecting the
82 fact that insertion sequences persist only briefly before they are eradicated from the genome or
83 kill their host (Park *et al.* 2021). REPINs in contrast are only conserved at the ends of the sequence
84 (presumably due to selection for function), the rest of the sequence is highly variable and only
85 the hairpin structure is conserved (Bertels, Rainey 2011a). The sequence variability of REPINs
86 within the same genome reflects their long-term persistence in single bacterial lineages (Bertels,
87 Rainey 2023). REPINs cannot simply reinfect another bacterial lineage since they rely for mobility
88 on their corresponding RAYT, which itself is immobile.

89

90 RAYTs and REPINs are distinct from typical parasitic insertion sequences, yet we know very little
91 about their evolution or biology. Currently, it is completely unclear what kind of beneficial
92 function maintains REPINs and RAYTs as well as their association with each other. The reason for
93 our lack of knowledge is not because REPINs and RAYTs are rare. They are ubiquitously found in
94 many important and well-studied model bacteria such as Enterobacteria, Pseudomonads,
95 Neisseriads and Xanthomonads. Microbial molecular biologists presumably encounter REPINs
96 quite frequently. However, connecting the presence or absence of REPINs/RAYTs with
97 phenotypes is difficult if we do not know when it is a REPIN that is present close to a gene of
98 interest or a different type or repeat sequence. Even if the scientist knows about the presence of
99 a REPIN, it is also important to know whether a corresponding RAYT is present (Bertels, Rainey
100 2023).

101

102 The identification of REPIN populations and their corresponding RAYTs can be rather
103 cumbersome if done from scratch. This is particularly true if the microbial molecular biologist is
104 not aware of all the details of REPIN and RAYT biology. Identifying REPINs starts with an analysis
105 of short repetitive sequences in a genome. If there are excessively abundant short sequences

106 present in the genome, the distribution of these sequences is then analyzed. If these sequences
107 are exclusively identical tandem repeats without sequence variation, and present in only one or
108 two loci in the genome, then these sequences are probably part of a CRISPR array and not REPINs.
109 If the sequences are distributed across the genome, highly diverse and often present as inverted
110 repeats then it is likely that the repeats are indeed REPINs.

111

112 Here, we present RAREFAN (RAYT/REPIN Finder and Analyzer), a webservice that automates the
113 identification of REPINs and their corresponding RAYTs. RAREFAN is publicly accessible at
114 http://rarefan.evolbio.mpg.de and identifies REPIN populations and RAYTs inside a set of
115 bacterial genomes. RAREFAN also generates graphs to visualize the population dynamics of
116 REPINs, and assigns RAYT genes to their corresponding REPIN groups. Here we will demonstrate
117 RAREFAN's functionality by analyzing REPIN-RAYT systems in the bacterial species
118 *Stenotrophomonas maltophilia*.

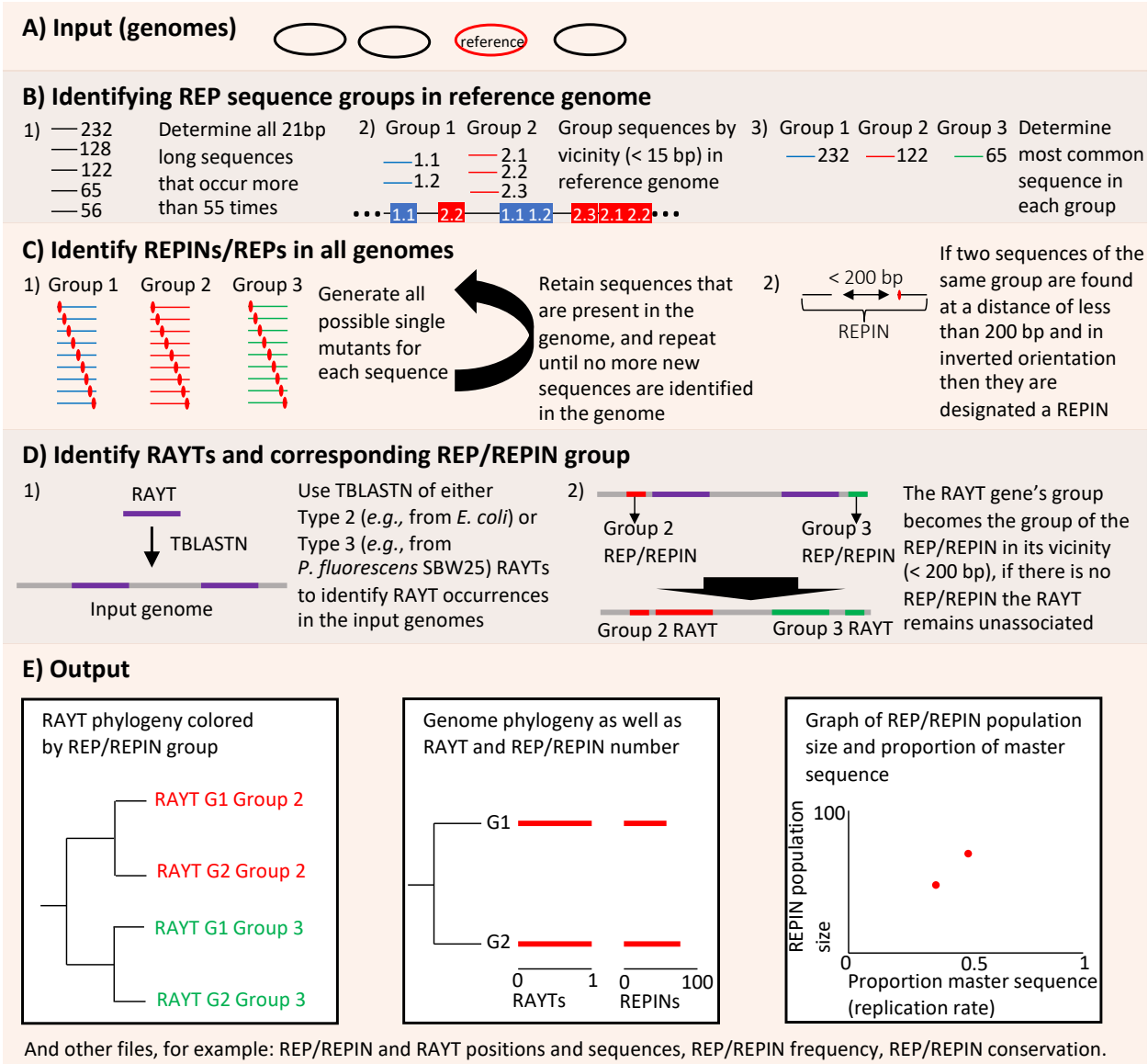

Figure 2. RAREFAN workflow. (**a**) By default RAREFAN requires the user to supply input sequences containing RAYTs and REPINs. These are fully sequenced and complete genomes. (**b**) RAREFAN then identifies seed sequence groups (potential REP sequences) in the reference genome by first isolating all 21 bp (adjustable parameter) long sequences that occur more than 55 times (adjustable parameter) in the reference genome. It is likely that a large number of these sequences belong to the same REPIN sequence type since the conserved part of REPINs is longer than 21 bp. Hence, we grouped all sequences together that occur within 15 bp (adjustable parameter) of each other anywhere in the genome. All further analyses are performed only with the most common sequence in each sequence group. This sequence will be called the seed sequence. (**c**) The occurrences of the seed and mutated seed sequences are identified in all submitted genomes. If a mutated seed sequence is identified in a genome, then all single mutants of that seed sequence are searched recursively in the same genome. All identified sequences that occur within 130 bp in inverted orientation of each other are designated REPINs. All other identified seed sequences and mutated seed sequences are REP singlets. (**d**) TBLASTN is used to

identify RAYT homologs (e-Value < 1e-30, adjustable parameter) of either *E. coli* (Group 2 RAYT) or from *P. fluorescens* SBW25 (Group 3 RAYT) across all submitted genomes. If a RAYT homolog is in the vicinity (default < 200 bp, adjustable parameter) of a previously identified REPIN or REP singlet, then this RAYT is designated as associated with this REPIN group. (**e**) The first graph contains a RAYT phylogeny computed from a nucleotide alignment of all identified RAYT genes. The RAYT phylogeny indicates what RAYTs are associated with what REPIN populations (largest sequence cluster calculated with MCL) via colour coding. In a second graph the abundance of each REPIN population and RAYT copy number are displayed on a genome phylogeny. In the last graph REPIN population sizes are plotted in relation to the proportion of master sequences. Master sequences are the most abundant REPIN in each population. RAREFAN also generates various files containing, for example, REP, REPIN, or RAYT sequences and their positions in the query genomes.

Methods

Identification of REPs and REPINs

The algorithm to determine REP sequence groups has been described in previous papers and is now slightly improved (Bertels, Rainey 2011a, 2023; Bertels, Gokhale, *et al.* 2017). The main difference between the implementations is that RAREFAN automatically links REPs/REPIN populations to RAYT genes, which was not possible previously.

The algorithm starts by extracting all N bp (21 bp by default) long seed sequences that occur more than M times (55 by default) from the reference genome (**Figure 2B**). All sequences occurring within the reference genome at least once within 15 bp of each other are then grouped together into n REP sequence groups (numbered 0-[n-1]). The most common sequence in each group, named REP seed sequence, is used for further analyses in each input genome.

In the next step all possible point mutants of the seed sequences are generated and searched for in the genome (**Figure 2C**). If a sequence is found in the genome, then all possible point mutants are generated for this sequence as well and searched against the genome and so on until no more sequences can be identified. Once all sequences related to the seed sequence are found, RAREFAN determines whether the sequences form REPINs. Two sequences form a REPIN when they are located within 130 bp of each other in inverted orientation.

REP and REPIN sequences of the same type form REPIN populations. A REPIN population is defined as the largest coherent sequence cluster. To identify the largest sequence cluster MCL is applied to a network of REP/REPIN sequences where all sequences that differ by exactly one

139 nucleotide are connected using an inflation parameter of 1.2 (Van Dongen 2000). The clustering
140 results are stored in a file ending in `.mcl`. The sequences of the largest REPIN population
141 (excluding REP singlets) are stored in a file ending in `largestCluster.nodes`. The largest
142 REPIN populations are shown in the REPIN population plot and the master sequence correlation
143 plot (**Figure 4**).

144 *Identification of RAYTs*

145 RAYTs are identified using TBLASTN (Camacho *et al.* 2009) with either a protein sequence
146 provided by the user, a Group 2 RAYT from *E. coli* (yafM, Uniprot accession Q47152) or a Group
147 3 RAYT from *P. fluorescens* SBW25 (yafM, Uniprot accession C3JZZ6). The presence of RAYTs in
148 the vicinity (default maximum of 200 bp) of a particular REPIN can be used to establish the
149 association between the RAYT gene and a REPIN group (**Figure 2D**). If a REP sequence or a REPIN
150 of a particular group occur within 200 bp (by default) of a RAYT gene then the RAYT gene is linked
151 to the REP/REPIN group. REP/REPIN associations are stored in the file
152 `repin_rayt_association.txt`. REPIN or REP sequences are almost always present in the
153 extragenic spaces of the RAYT and this linkage is consistent across the RAYT phylogeny (as shown
154 in **Figure 5**). However, what causes the REP/REPIN group to be linked with the RAYT gene is
155 unclear.

156 *Implementation*

157 RAREFAN is a modular webservice. It consists of a web frontend written in the Python
158 programming language (Van Rossum, Drake Jr 1995) using the Flask framework (Grinberg 2018),
159 a Java (Arnold *et al.* 2005) backend for genomic sequence analysis and an R (R Core Team 2016)
160 Shiny app (RStudio, Inc 2013) for data visualization. The software is developed and tested on the
161 Debian GNU/Linux operating system (Kleinmann *et al.* 2021). All components are released under
162 the MIT opensource license (Initiative 2021) and can be obtained from our public GitHub
163 repository at https://github.com/mpievolbio-scicomp/rarefan.

164 The public RAREFAN instance at http://rarefan.evolbio.mpg.de runs on a virtual cloud server with
165 four single-threaded CPUs and 16GB of shared memory provided and maintained by the

166 Gesellschaft für Wissenschaftliche Datenverarbeitung Göttingen (GWDG) and running the Debian
167 GNU/Linux Operating System (Kleinmann *et al*. 2021).

168 The Java backend drives the sequence analysis. It makes system calls to TBLASTN (Altschul *et al.*
169 1990) to identify RAYT homologs and to MCL (Van Dongen 2000) for clustering REPIN sequences
170 in order to determine REPIN populations.

171 Jobs submitted through the web server are queued and executed as soon as the required
172 resources become available. Users are informed about the status of their jobs. After job
173 completion, the user can trigger the R Shiny app to visualize the results.

174 The Java backend can also be run locally *via* the command line interface (available for download
175 at https://github.com/mpievolbio-scicomp/rarefan/releases).

176 *Usage of the webservice*

177 The front page of our webservice allows users to upload their bacterial genomes in FASTA (.fas)
178 format (**Figure 2A**). Optionally, users may also provide RAYT protein FASTA sequences (.faa) or
179 phylogenies in NEWICK (.nwk) format. After successful completion of the upload process, the
180 user fills out a web form to specify the parameters of the algorithm:

181 - Reference sequence: Which of the uploaded genome sequences will be designated as
182 reference genome (see below for explanations). Defaults to the first uploaded filename
183 in alphabetical order.

184 - Query RAYT: The RAYT gene that is used to identify homologous RAYTs in the query
185 genomes. If the user does not provide a protein sequence file then the user can choose
186 one of two RAYT sequences (one from Group 2 and one from Group 3 RAYTs (Bertels,
187 Gallie, *et al.* 2017)) as RAYT query.

188 - Tree file: A phylogenetic tree of the reference genomes that can be provided by the user,
189 otherwise the tree will be calculated using andi (Haubold *et al.* 2015).

190 - Minimum seed sequence frequency: Lower limit on seed sequence frequency in the
191 reference genome to be considered as a REP candidate. Default is 55.

192 • Seed sequence length: The seed sequence length (in base pairs) is used to identify REPIN
193 candidates from the input genomes. Default is 21 bp.

194 • Distance group seeds: The maximum distance between a single occurrence of short
195 repetitive sequences to still be sorted into the same sequence group.

196 • REPIN-RAYT association distance: The maximum distance at which a REP sequence can be
197 located from a RAYT gene to be linked to that RAYT gene. Default is 200 bp.

198 • e-value cut-off: Alignment e-value cut-off for identifying RAYT homologs with TBLASTN.
199 Default is 1e-30.

200 • Analyse REPINs: Ticked REPINs will be analysed (two inverted REP sequences found at a
201 distance of less than 130 bp), if not ticked only short repetitive 21 bp long sequence will
202 be analysed.

203 • User email (optional): If provided, then the user will be notified by email upon run
204 completion.

205 The job is then ready for submission to the job queue. Upon job completion, links to browse and
206 to download the results, as well as a link to a visualization dashboard are provided. If a job runs
207 for a long time then users may also come back to RAREFAN at a later time, query their job status
208 and eventually retrieve their results by entering the run ID into the search field at
209 http://rarefan.evolbio.mpg.de/results. Relevant links and the run ID are communicated either on
210 the status site or by email if the user provided their email address during run configuration. Runs
211 are automatically deleted from the server after six months.

212 *Visualizations*

213 For each REPIN-RAYT group summary plots are generated. These include plots showing the RAYT
214 phylogeny (calculated from a nucleotide alignment using MUSCLE (Edgar 2004) and PHYML
215 (Guindon *et al.* 2010) to generate a phylogeny), REPIN population sizes in relation to the genome
216 phylogeny (provided by the user or if not provided calculated by andi (Haubold *et al.* 2015)) as
217 well as the proportion of master sequences (most common REPIN in a REPIN population) in
218 relation to REPIN population size (**Figure 2E**).

219 *Other outputs*

220 Identified REPINs, REP singlets as well as RAYTs are written to FASTA formatted sequence files
221 and to tab formatted annotation files that can be read with the Artemis genome browser
222 (Rutherford *et al.* 2000). The REPIN-RAYT associations as well as the number of RAYT copies per
223 genome are written to tabular data files. A detailed description of all output files is provided in
224 the manual (http://rarefan.evolbio.mpg.de/manual) and in the file "readme.md" in the output
225 directory.

226 ***Sequence analysis and annotation***

227 For verification of RAREFAN results, REPIN-RAYT-systems were analysed in their corresponding
228 genomes using Geneious Prime version 2022.2.2 (Kearse *et al.* 2012). Nucleotide sequences and
229 positions of REP singlets, REPINs, and RAYTs were extracted from output files generated by
230 RAREFAN and mapped in the relevant *S. maltophilia* genome. Complete RAREFAN data used for
231 analysis can be accessed by using the run IDs listed in **Table 1.**

232

233 **Table 1. RAREFAN IDs linking to the raw data of the presented analyses.**

Run ID	Reference genome
_1a8l7wu	*S. maltophilia* Sm53
mknhxpv8	*S. maltophilia* AA1
pgfmaxx5	*S. maltophilia* FDAARGO_649
yy72i755	*S. maltophilia* AB550
78eu9zl0	*S. maltophilia* ISMMS3

234 Associated data can accessed by entering the run ID at http://rarefan.evolbio.mpg.de/results.

235

236 **Results**

237 RAREFAN can identify REPINs and their corresponding RAYTs in a set of fully sequenced bacterial
238 genomes. The RAREFAN algorithm has been used in previous analyses to identify and
239 characterize REPINs and RAYTs in Pseudomonads (Bertels, Rainey 2011a, 2023), Neisseriads
240 (Bertels, Rainey 2023), and Enterobacteria (Bertels, Gallie, *et al.* 2017; Park *et al.* 2021). To
241 demonstrate RAREFAN's capabilities, we are presenting an analysis of 49 strains belonging to the
242 opportunistic pathogen *S. maltophilia*.

243 *S. maltophilia* strains contain Group 3 RAYTs, which are also commonly found in plant-associated
244 *Pseudomonas* species such as *P. fluorescens* or *P. syringae* (Bertels, Rainey 2011a, 2023). Similar
245 to Group 3 RAYTs in other species, *S. maltophilia* contains multiple REPIN-RAYT systems per
246 genome. Group 2 RAYTs, in contrast, tend to contain only one REPIN-RAYT system per genome
247 (Bertels, Rainey 2023).

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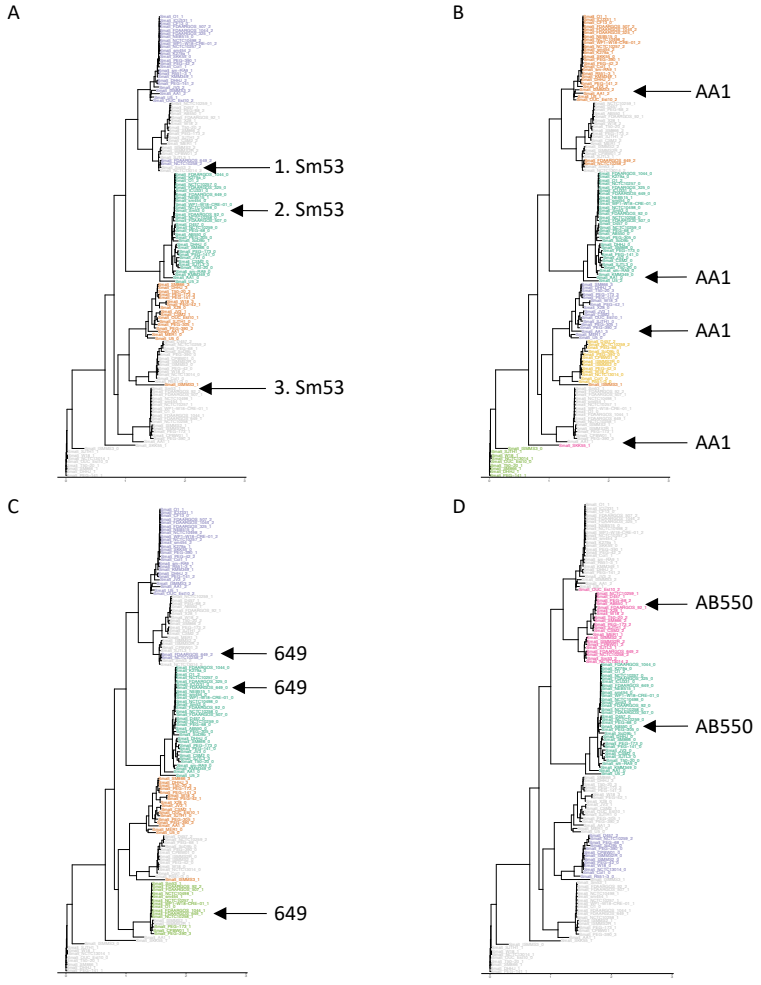

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Figure 3. Phylogenetic trees built from RAYT genes extracted from *S. maltophilia* genomes. RAYT genes are coloured by RAREFAN according to their association with REPIN populations in the reference genome. If a REPIN population of a query genome is not present in the reference genome, then the REPIN population cannot be identified in the query genome and the corresponding RAYT gene cannot be linked and is coloured in grey. The four panels **A-D** show phylogenies for four different reference strains. *S. maltophilia* strains Sm53, AA1, 649 and AB550 were used in panels **A** to **D**, respectively. Locations of a reference strain's RAYT genes in the tree are indicated by arrows. An association between almost all RAYTs and REPIN populations could

be made by using four different reference genomes. Most of the RAYT genes are coloured (associated with a REPIN group) in at least one of the trees. The three numbered RAYT genes from the Sm53 RAREFAN run are referenced in the text.

250 *Nine different REPIN-RAYT systems in* S. maltophilia

251 REPIN-RAYT systems in *S. maltophilia* are surprisingly diverse compared to other species. For
252 example, *Pseudomonas chlororaphis* contains three separate REPIN populations that are present
253 in all *P. chlororaphis* strains, each associated with its cognate RAYT gene (Bertels, Rainey 2023).
254 *S. maltophilia,* in contrast*,* contains only one REPIN-RAYT system that is present across almost
255 the entire species (green clade in **Figure 3**), and at least eight REPIN-RAYT systems that are
256 present in subsets of strains (nine clades in **Figure 5**).

257 The patchy presence-absence pattern of REPIN-RAYT systems in *S. maltophilia,* makes the dataset
258 quite challenging to analyse. If a REPIN population is not present in the reference strain then
259 RAREFAN will not be able to detect it in any other strain. Yet, it is possible to detect RAYT genes
260 in all strains of a species independent of the reference strain selection. RAYT genes that are not
261 associated with a REPIN population are displayed in grey (**Figure 3A**). While these RAYT genes are
262 not associated with REPIN populations detected in the reference strain, they might still be
263 associated with a yet unidentified REPIN type present in the genome the unassociated RAYT gene
264 is located in.

265 In order to identify all REPIN populations across a species, multiple RAREFAN runs with different
266 reference strains should be performed. The RAREFAN web interface supports re-launching a
267 given job with modified parameters. To identify as many different REPIN-RAYT systems as
268 possible in each subsequent run the reference should be set to a genome that contains RAYTs
269 that were not associated with a REPIN population previously (*i.e.*, genomes containing grey RAYTs
270 in **Figure 3**). However, this strategy may also fail when the REPIN population size falls below the
271 RAREFAN seed sequence frequency threshold. In that case reducing the frequency threshold will
272 be more productive.

273 For example, *S. maltophilia* Sm53 contains three RAYTs only one of which is associated with a
274 REPIN population (RAYT genes indicated in **Figure 3A**). However, the remaining two RAYTs are
275 indeed associated with a REPIN population, but these REPIN populations are too small to be

detected in *S. maltophilia* Sm53 (the seed sequence frequency threshold is set to 55 by default). In other *S. maltophilia* strains the REPIN populations are large enough to exceed the threshold. For example, if *S. maltophilia* AB550 is set as reference, RAYT number 1 from Sm53 (**Figure 3A**) is associated with the pink REPIN population (**Figure 3D**). If *S. maltophilia* 649 is set as reference RAYT number 3 from Sm53 (**Figure 3A**) is associated with the light green REPIN population (**Figure 3C**). RAYTs from the bottom clade are only associated with REPIN populations when *S. maltophilia* AA1 is chosen as reference (**Figure 3B**). While lower thresholds can guarantee that all REPINs will be identified in the genome, the number of sequence groups that are not REPINs quickly explodes. This is especially true for genomes that contain large numbers of mobile genetic elements or CRISPRs (Bertels, Rainey 2023).

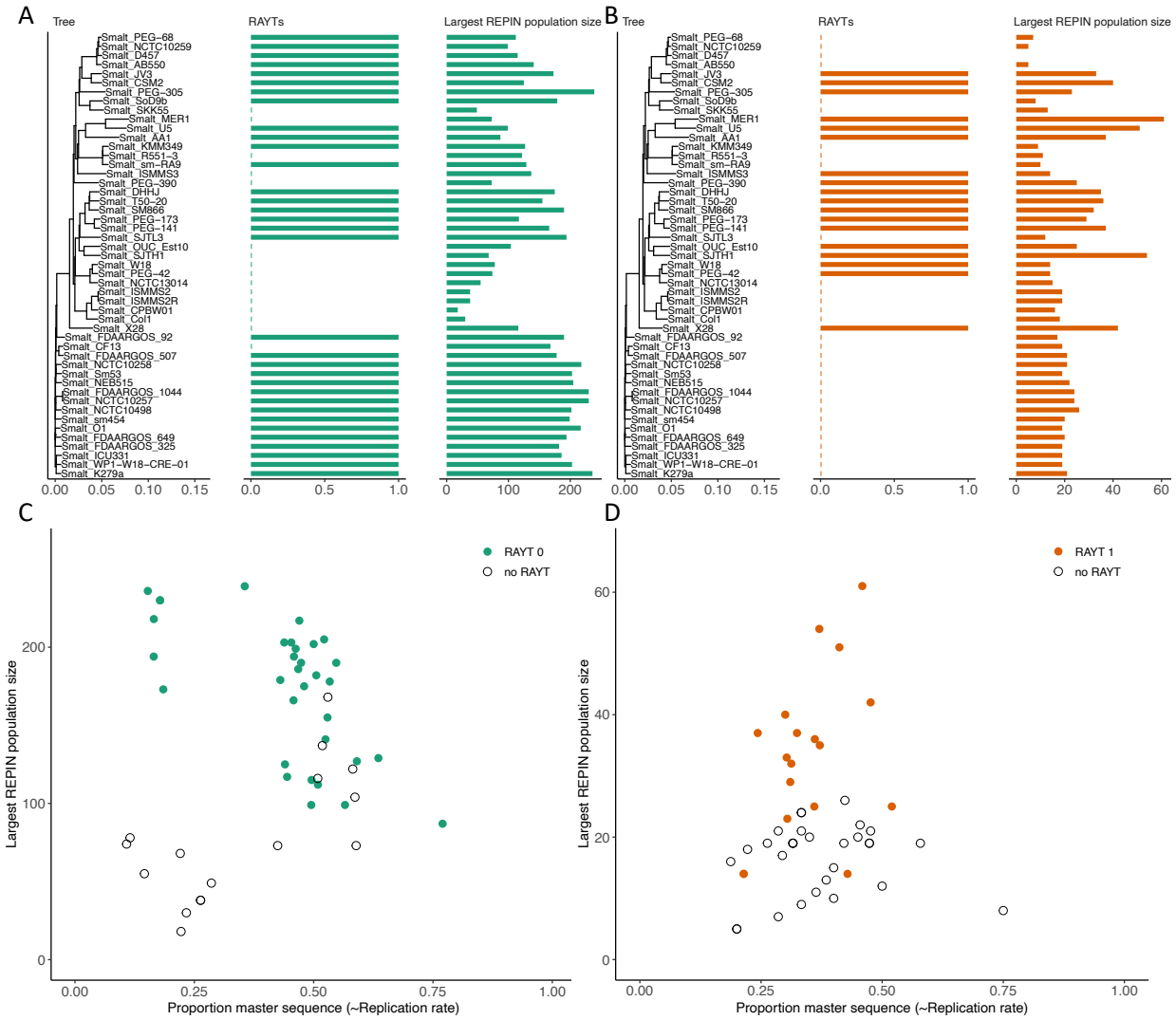

Figure 4. REPIN population sizes and conservation. The plots show two REPIN populations and their associated RAYTs that were identified in *S. maltophilia* using *S. maltophilia* Sm53 as reference. (**A**) The phylogenetic tree on the left side is a whole genome phylogeny generated by andi (Haubold *et al.* 2015). Shown on the right are REPIN population sizes (which is the largest REPIN cluster calculated by MCL) and the number of associated RAYTs sorted by the genome phylogeny. The green REPIN populations and associated RAYTs are present in most strains in high abundance (maximum of 239 occurrences in *S. maltophilia* K279a, left panel). (**B**) The orange population in contrast is present in much lower numbers (maximum of 61 occurrences in *S. maltophilia* MER1, right panel). Note, REPIN populations are assigned consistent colours based on their abundance in the reference genome. For example, the most abundant REPIN population in the reference is always coloured in green, and the second most abundant population is always coloured in orange. (**C and D**) Proportion of master sequence in *S. maltophilia* REPIN populations. The master sequence in a REPIN population is the most common REPIN sequence. At equilibrium the higher the proportion of the master sequence in the population the higher the replication rate (Bertels, Gokhale, *et al.* 2017). The presence and absence of an associated RAYT is also indicated by the colours of the dots. Empty circles indicate that the REPIN population is not associated with a RAYT gene in that genome.

287 *RAREFAN visualizes REPIN population size and potential replication rate*

288 The RAREFAN webserver visualizes REPIN population size and RAYT numbers in barplots. Barplots
289 are ordered by the phylogenetic relationship of the submitted bacterial strains using ggtree (Yu
290 *et al.* 2018). RAREFAN detects three populations when *S. maltophilia* Sm53 is selected as
291 reference strain (**Figure 3A**). The largest REPIN population (calculated by MCL from all REPINs of
292 that type) has a corresponding RAYT gene in almost all strains (first barplot in **Figure 4A**) and
293 most REPIN populations contain more than 100 REPINs (second barplot in **Figure 4A**). The second
294 largest REPIN population in Sm53 (orange population in **Figure 4B**) is significantly smaller and
295 contains no more than 61 REPINs in any strain and most strains do not contain a corresponding
296 RAYT for this population.

297 RAREFAN also provides information on REPIN replication rate (**Figure 4C** and **D**). REPIN replication
298 rate can be estimated by dividing the number of the most common REPIN sequence (master
299 sequence) by the REPIN population size if the population is in mutation selection balance (Bertels,
300 Gokhale, *et al.* 2017). If a REPIN replicates very fast most of the population will consist of identical
301 sequences because mutations do not have enough time to accumulate between replication
302 events. Hence, the proportion of master sequences will be high in populations that have a high
303 replication rate. Transposable elements such as insertion sequences consist almost exclusively of

304 identical master sequences because the time between replication events is not sufficient to
305 accumulate mutations and because quick extinction of the element usually prevents the
306 accumulation of mutations after replication (Park *et al.* 2021; Bertels, Rainey 2023). Sequence
307 diversity of REPIN populations in contrast is much higher suggesting that REPINs replicate slowly
308 and persist for long periods of time.

309 In *S. maltophilia* the proportion of master sequences in the population does not seem to correlate
310 well with REPIN population size, both in the green and the orange population (**Figures 4C and D**).
311 Similar observations have been made in *P. chlororaphis* (Bertels, Rainey 2023), and may suggest
312 that an increase in population size is not caused by an increase in replication rate. Population size
313 is likely to be more strongly affected by other factors such as the loss of the corresponding RAYT
314 gene, which leads to the decay of the REPIN population. One could even speculate that high
315 REPIN replication rates are more likely to lead to the eventual demise of the population due to
316 the negative fitness effect of high replication rates on the host (Park *et al.* 2021; Bertels, Rainey
317 2023).

318 The presence of RAYTs and the size of the corresponding REPIN population do correlate
319 surprisingly well (**Figure 4A** and **B**, p-value = 0.008 of a linear model of independent contrasts
320 (Felsenstein 1985) of green RAYT and REPIN number, p-value = 0.003 for orange REPIN
321 populations). Green RAYTs are absent from an entire *S. maltophilia* clade (middle of **Figure 4A**).
322 This clade has also lost a significant amount of green REPINs, and the proportion of the master
323 sequences in these populations is low (**Figure 4C**). Similarly, genomes without orange RAYTs have
324 smaller REPIN populations in the orange population than genomes with the corresponding RAYT
325 (**Figure 4D**). A similar observation has been made previously in *E. coli, P. chlororaphis, N.*
326 *meningitidis* and *N. gonorrhoeae* where the loss of the RAYT gene is followed by a decay of the
327 associated REPIN population (Park *et al.* 2021; Bertels, Rainey 2023).

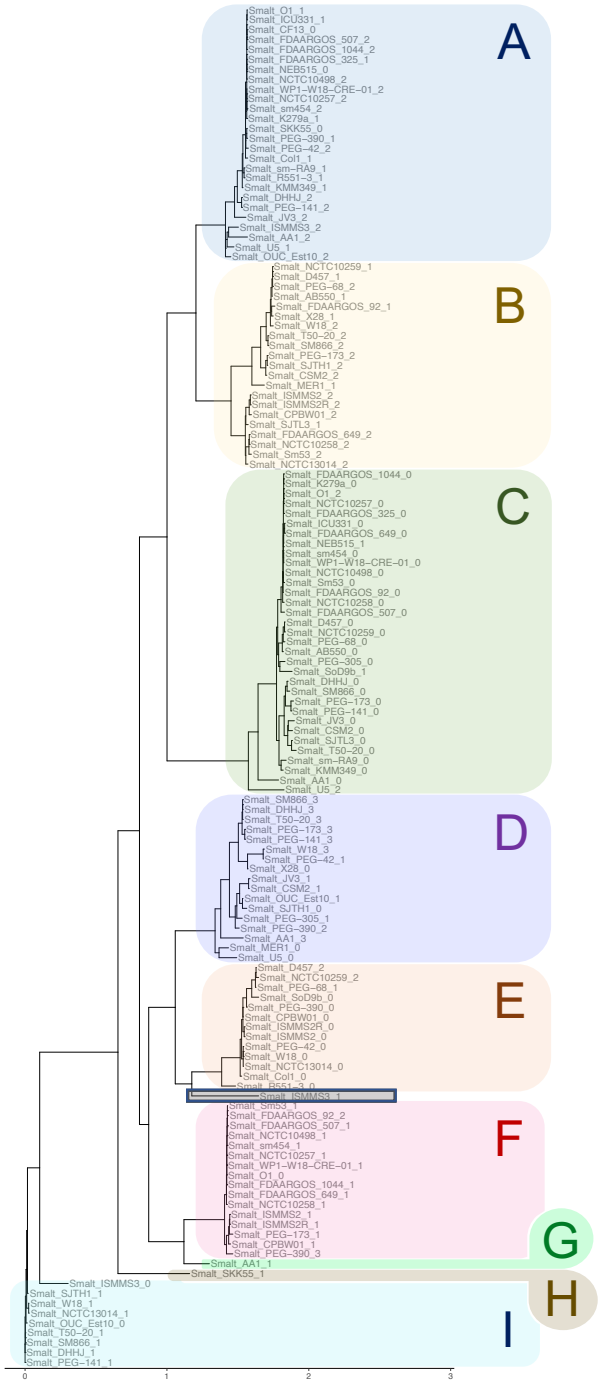

Figure 5. Phylogeny of RAYT genes and their associated REPINs. The tree shows RAYT genes from 49 *S. maltophilia* strains. Colours of clades A-I are assigned according to their association with a REPIN found within 200 bp of the RAYT gene (see **Table 2**). Except for a single RAYT gene ISMMS3_1 (grey box), which could not be linked to a REPIN population.

Table 2. REPIN palindromes associated with each RAYT clade.

RAYT population	REPIN palindromes

A	CCGACC**AAC**GGTCGG
B	CCAACC**AA**GGTTGGC
C	CCGGCC**AGC**GGCCGG
D	TCCACGC**ATG**GCGTGGA
E	CCGAGC**CCAT**GCTCGG
F	TCGACT**AAC**AGTCGA
G	TCGACC**AAC**GGTCGA
H	GCCGGGC**ATG**GCCCGGC
I	AGTCGAGC**TT**GCTCGACT

Each RAYT clade from **Figure 5** is associated with a unique imperfect palindrome that is present at the 5' and/or 3' end of the RAYT gene.

Linking REPIN populations with RAYT genes can be challenging

Unfortunately, RAREFAN is not always able to link the correct REPIN population with the correct RAYT gene. In some RAREFAN runs, associations between RAYTs and REPINs are not monophyletic, as for example the red RAYT clade in **Figure 3A**. However, the same clade of RAYTs is uniformly coloured in yellow in **Figure 3D**, suggesting that the entire RAYT clade is associated with the same REPIN group.

An analysis of all REPIN groups that were identified by RAREFAN across four different RAREFAN runs (**Table 1**, one additional analysis was performed with ISMMS3) showed that there are a total of nine different REPIN groups, each defined by an individual central palindrome (**Table 2**). Each REPIN group is associated with a monophyletic RAYT group (**Figure 5**). Only a single RAYT is not associated with a REPIN population (ISMMS3_1).

RAREFAN could not link a REPIN to the RAYT gene ISMMS3_1 (**Figure 5**, grey box). While there is a sequence that resembles the A palindrome as well as variants of the C palindrome flanking both sides of the RAYT gene (**Supplementary Figure 2**), none of the sequences formed REPIN populations large enough to be identified by RAREFAN. Presumably the RAYT ISMMS3_1, which

348 is only present in a single *S. maltophilia* strain, is at the early stages of establishing a REPIN
349 population, and the REPIN population has not spread to a considerable size yet.

350 If the maximum REPIN-RAYT distance parameter is too small then RAREFAN will also fail to
351 correctly link REPINs and RAYTs. For example, when the maximum REPIN-RAYT distance
352 parameter is set to 130bp there are two cases where RAREFAN fails to link RAYT genes with
353 REPINs (ISSMS2_ and ISSMS2R_1**, Supplementary Figure 1 D** and **E**). When the parameter is set
354 to a distance of 200 bp (default RAREFAN setting), RAREFAN correctly links these REPINs to the
355 RAYT gene.

356 In three cases the wrong REPIN population was linked to a RAYT gene. In our dataset this can
357 happen when RAYTs are flanked by seed sequences from two different REPIN populations
358 **(Supplementary Figure 1 A-C)**. A single REP sequence from the "wrong" (non-monophyletic
359 RAYT) clade occurs together with multiple REP or REPIN sequences from the "right"
360 (monophyletic in a different RAREFAN run) clade. REPINs are linked to the "wrong" RAYT when
361 the correct REPIN population is absent in the chosen reference genome. This problem can be
362 alleviated by performing analyses with multiple reference genomes and comparing the results.

363 *REPIN groups may be lost when the seed distance is too large*

364 The seed distance parameter determines whether two highly abundant sequences are sorted
365 into the same or different REPIN groups (**Figure 2B**). If two REPINs from two different groups
366 occur next to each other, at a distance of less than the seed distance parameter, then the two
367 seeds are erroneously sorted into the same group. If two different REPIN groups are sorted into
368 the same group then one of the groups will be ignored by RAREFAN, because only the most
369 abundant seed in each group will be used to identify REPINs.

370 A manual analysis (*e.g.*, multiple sequence alignment) of sequences in the groupSeedSequences
371 folder of the RAREFAN output can identify erroneously merged REPIN groups. In *S. maltophilia*,
372 groups are separated well when the distance parameter is set to 15 bp and Sm53 is used as a
373 reference. When the parameter is set to 30 bp instead, one of the REPIN groups will be missed
374 by RAREFAN.

A small seed distance parameter will separate seed sequences belonging to the same REPIN group into different groups. Hence, RAREFAN will analyse the same REPIN group multiple times. While this will lead to increased RAREFAN runtimes, these errors, are easy to spot, because (1) the same RAYT gene will be associated to multiple REPIN groups, (2) the central palindrome between the group is identical and (3) the master sequence between the groups will be very similar.

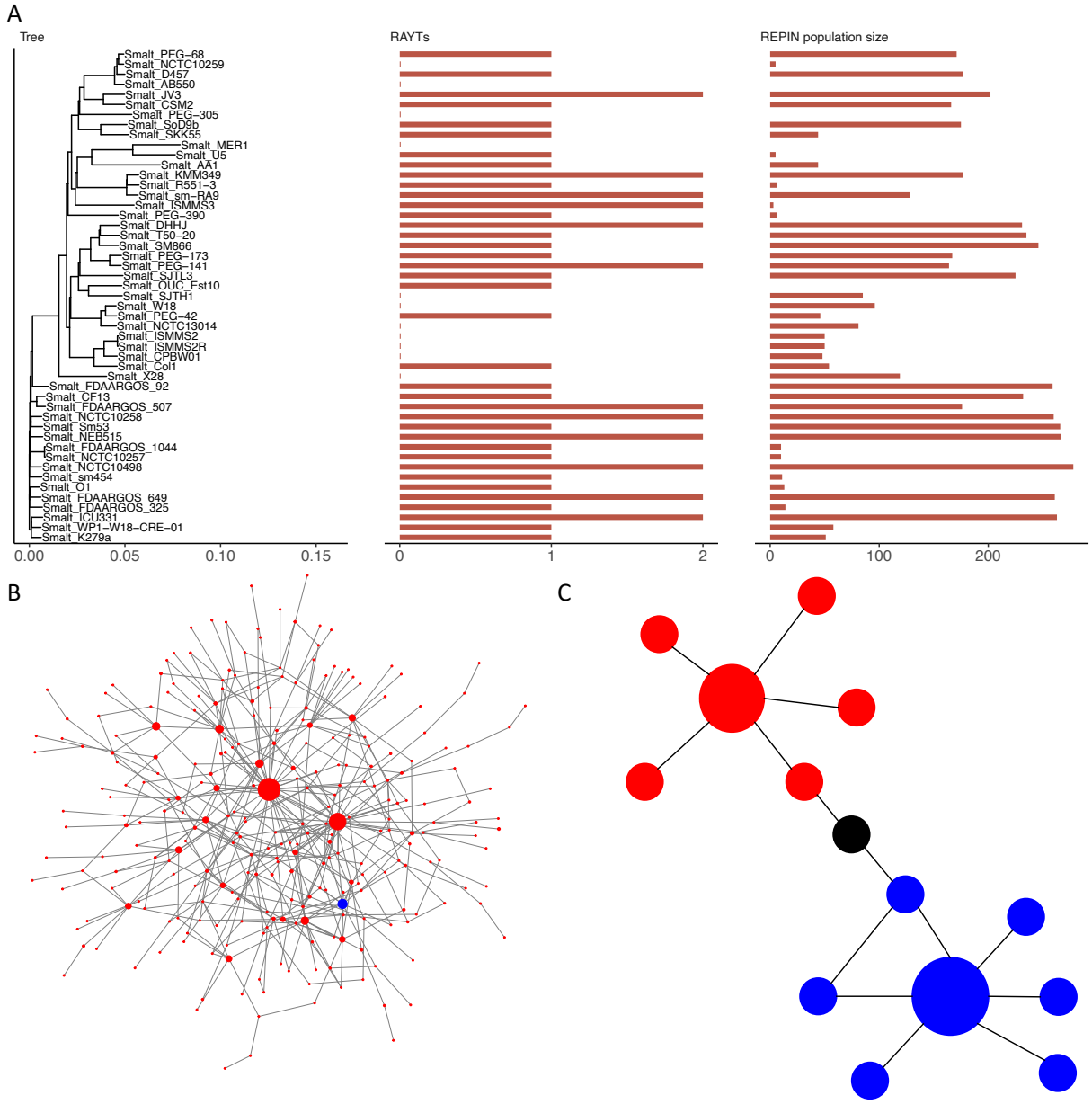

Figure 6. Closely related REPIN populations may be merged by RAREFAN. (A) REPIN group 2 identified in a *S. maltophilia* Sm53 RAREFAN run. The RAREFAN result suggests that REPIN group 2 is sometimes associated with two RAYTs. (**B**) A closer inspection of the data shows that group 2 is a combination of two different REPIN groups, the "real" group 2 and group 0. The network shown, visualizes all REP sequences identified as group 2. Nodes in the network represent 21 bp long REP sequences. Two nodes are connected if the sequences they represent differ by exactly one nucleotide. The node size indicates the abundance of the sequence in the genome. The blue node represents the most common group 2 sequence, occurring 65 times in the genome. The largest red node occurs 407 times in the genome and resembles a group 0 REP sequence. (**C**) Illustration of how small changes to a single sequence can connect two sequence clusters. The most common 21 bp long sequence in group 0 differs in only four positions from the most common 21 bp long sequence in group 2. There is a set of sequences that connects these two groups that only differ in exactly one position each (nodes connected by an edge), which passes through the black node. If there is such an unbroken path between REP sequences, then REPIN groups will be merged.

383

384 *Closely related REPIN groups may be merged into a single group by RAREFAN*

385 Incorrect merging of REPIN groups can occur when two REPIN groups are closely related. We
386 identified merged REPIN groups in *S. maltophilia* because RAREFAN linked some REPIN groups
387 with two RAYT genes in the same genome (**Figure 6A**). While REPIN groups linked to two RAYTs
388 have been observed before in *Neisseria meningitidis* (Bertels, Rainey 2023), it is particularly
389 unusual in *S. maltophilia* due to some key differences between REPIN-RAYT in the two bacterial
390 species. First, *N. meningitidis* contains Group 2 RAYTs and *S. maltophilia* only contains Group 3
391 RAYTs (Bertels, Gallie, *et al.* 2017), two very divergent RAYT gene families. Second, RAYTs that
392 are associated with the same REPIN group in *N. meningitidis* are almost identical, since they are
393 copied by an insertion sequence *in trans* (Bertels, Rainey 2023), something that is not the case
394 for *S. maltophilia*, where the two RAYTs are very distinct and quite distantly related from each
395 other (green and red clade in **Figure 3A**, or clade A and C in **Figure 5**).

396 A closer inspection of all sequences identified in REPIN group 2 shows that it also contains
397 sequences belonging to REPIN group 0 (palindromes linked to clade A and C in **Table 2**). The
398 relationship between the sequences shows that there is a chain of sequences that all differ by at
399 most a single nucleotide between the most abundant sequence in group 2 to the most abundant
400 sequence in group 0 (**Figure 6B and C**). Hence, the reason group 0 and group 2 are merged is that

401 they are too closely related to each other and hybrids of the two REPIN groups exist. Because
402 sequence groups are built by identifying all related sequences in the genome recursively, closely
403 related groups (the REPIN group 0 seed only differs in four nucleotides from the REPIN group 2
404 seed sequence) can be merged into a single REPIN group. REPIN population size and RAYT number
405 are the sum of REPIN group 0 and 2. There are various possibilities to resolve this issue: (1)
406 subtract sequences from group 0 (which does not contain group 2) from REPIN group 2; (2) use
407 a different sequence seed from the group 2 seed collection in the seed sequence file
408 (`groupSeedSequences/Group_Smalt_Sm53_2.out`); (3) sometimes it may be possible to
409 rerun RAREFAN with a different reference strain where the issue does not occur; or (4) increase
410 the length of the seed sequence.

411 *Performance*

412 We measured the elapsed time for a complete RAREFAN run for three different species and for
413 5, 10, 20, and 40 genomes with randomly selected reference strains and the two query RAYTs
414 (yafM_Ecoli and yafM_SBW25). For a given number N of submitted genomes of average
415 sequence length L (in megabases), a RAREFAN run completes in approximately T = (8-10 seconds)
416 * N * L on our moderate server hardware (4 CPU cores, 16 GB shared RAM) (**Supplementary**
417 **Figure 3** and **4)**.

418 **Discussion**

419 RAREFAN allows users to quickly detect REPIN populations and RAYT transposases inside
420 bacterial genomes. It also links the RAYT transposase genes to the REPIN population it duplicates.
421 These data help the user to study REPIN-RAYT dynamics in their strains of interest without a
422 dedicated bioinformatician, and hence will render REPIN-RAYT systems widely accessible.

423 One limitation of RAREFAN is that REPINs can only be identified in genomes when they are
424 symmetric (**Figure 1**). Symmetric REPINs have seed sequences that can morph into each other by
425 a series of single substitutions (intermediate sequences need to be present in the genome). A
426 REPIN consists of a 5' and a 3' REP sequence. If one of these REP sequences contains an insertion
427 or deletion, which the other REP sequence does not contain then RAREFAN will not recognize the
428 second repeat of the seed sequence. In this case, RAREFAN will not be able to identify REPINs but

429 can still be used to analyze REP singlet populations. To date, the only asymmetric REPIN
430 populations known to us are found in *E. coli.* However, it is likely that asymmetric REPINs also
431 exist in other microbial species.

432 RAREFAN sometimes cannot correctly divide REPINs into REPIN groups. Either because REPINs
433 from different groups occur in close proximity in the genome, an issue that can easily be solved
434 by adjusting a RAREFAN parameter, or because two REPIN groups are very closely related (**Figure**
435 **6**). Unfortunately, RAREFAN is not able to automatically detect and resolve the assignment of
436 closely related REPINs into groups yet. Hence it is advisable to manually check associations
437 between REPIN groups and RAYT genes by analyzing the composition of REPIN groups.

438 In the future we aim to make RAREFAN even more versatile and easier to use by, for example,
439 automatically integrating data from public databases such as GenBank, and creating a RAREFAN
440 Galaxy workflow (Afgan *et al.* 2018).

441 RAREFAN makes the study of REPIN-RAYT systems more accessible to any biologist or
442 bioinformatician interested in studying intragenomic sequence populations. Our tool will help
443 understand the purpose and evolution of REPIN-RAYT systems in bacterial genomes.

444 **Acknowledgements**

445 We would like to thank Prajwal Bharadwaj for assisting us with the sequence analysis and Jenna
446 Gallie for valuable feedback on the manuscript.

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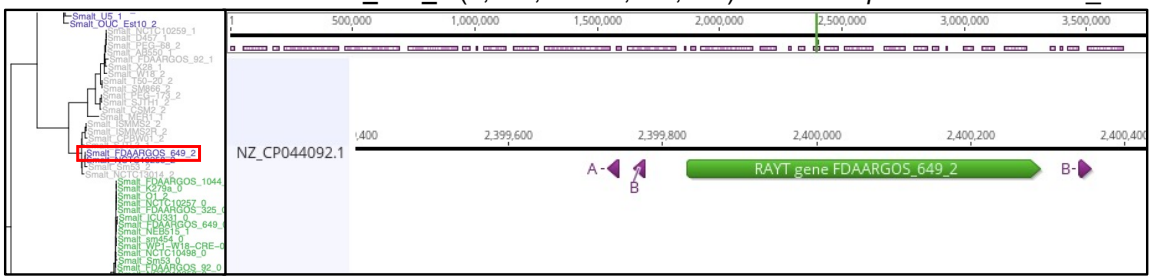
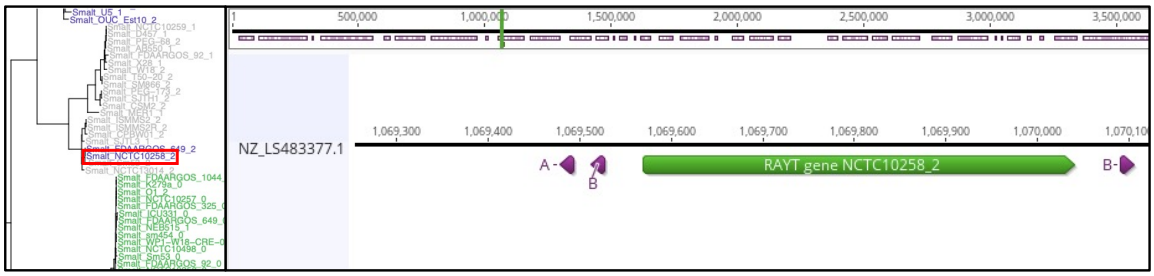
529 **Supplementary Figures**

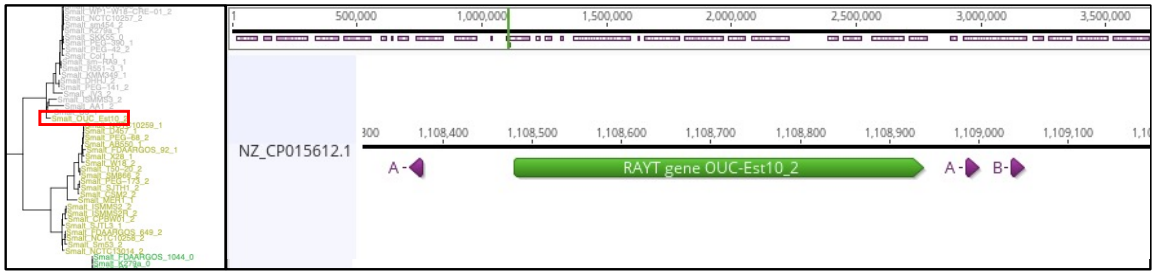
A **FDAARGOS_649_2** (2,399,832 - 2,400,290) in *S. maltophilia* FDAARGOS_649



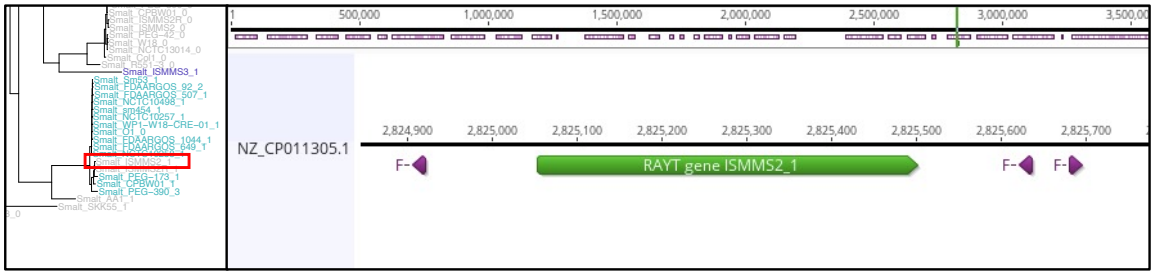
B **NCTC10258_2** (1,069,570 – 1,070,040) in *S. maltophilia* NCTC10258



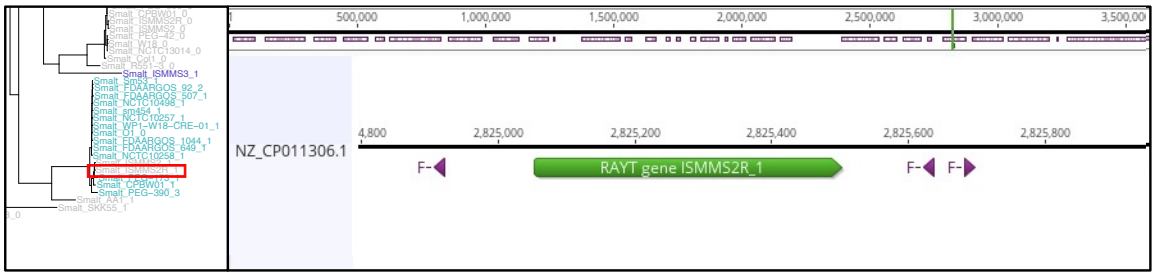
C **OUC_Est10_2** (1,108,480 – 1,108,938) in *S. maltophilia* OUC_Est10



D **ISMMS2_1** (2,825,502 – 2,825,053) in *S. maltophilia* ISMMS2

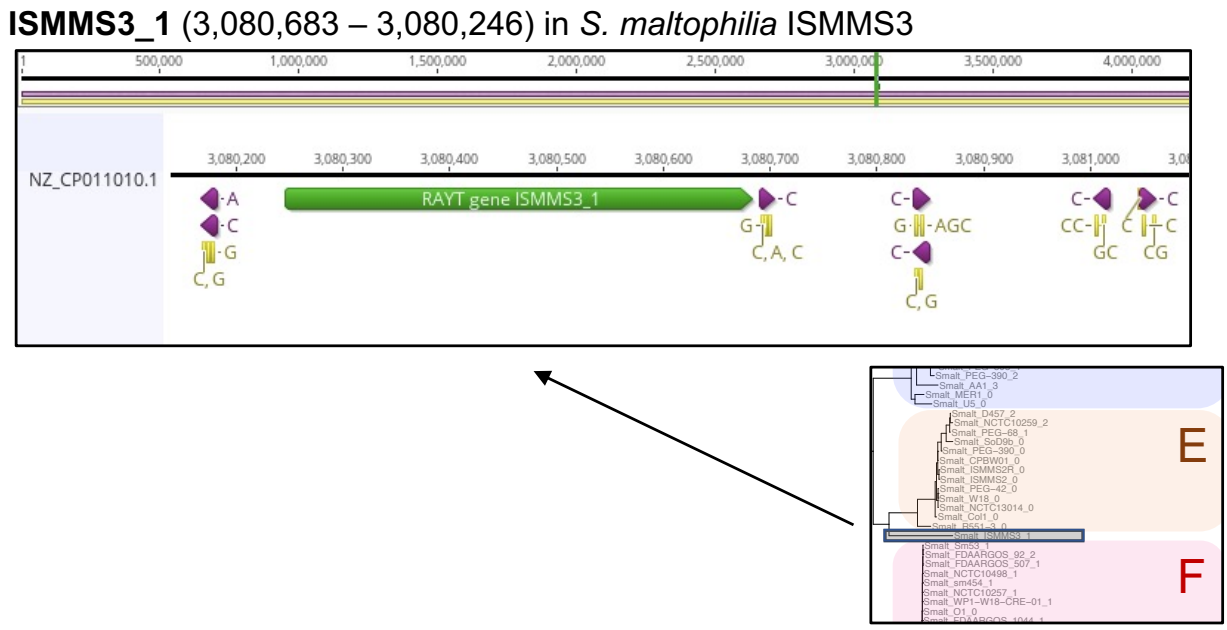


E **ISMMS2R_1** (2,825,502 – 2,825,053) in *S. maltophilia* ISMMS2R



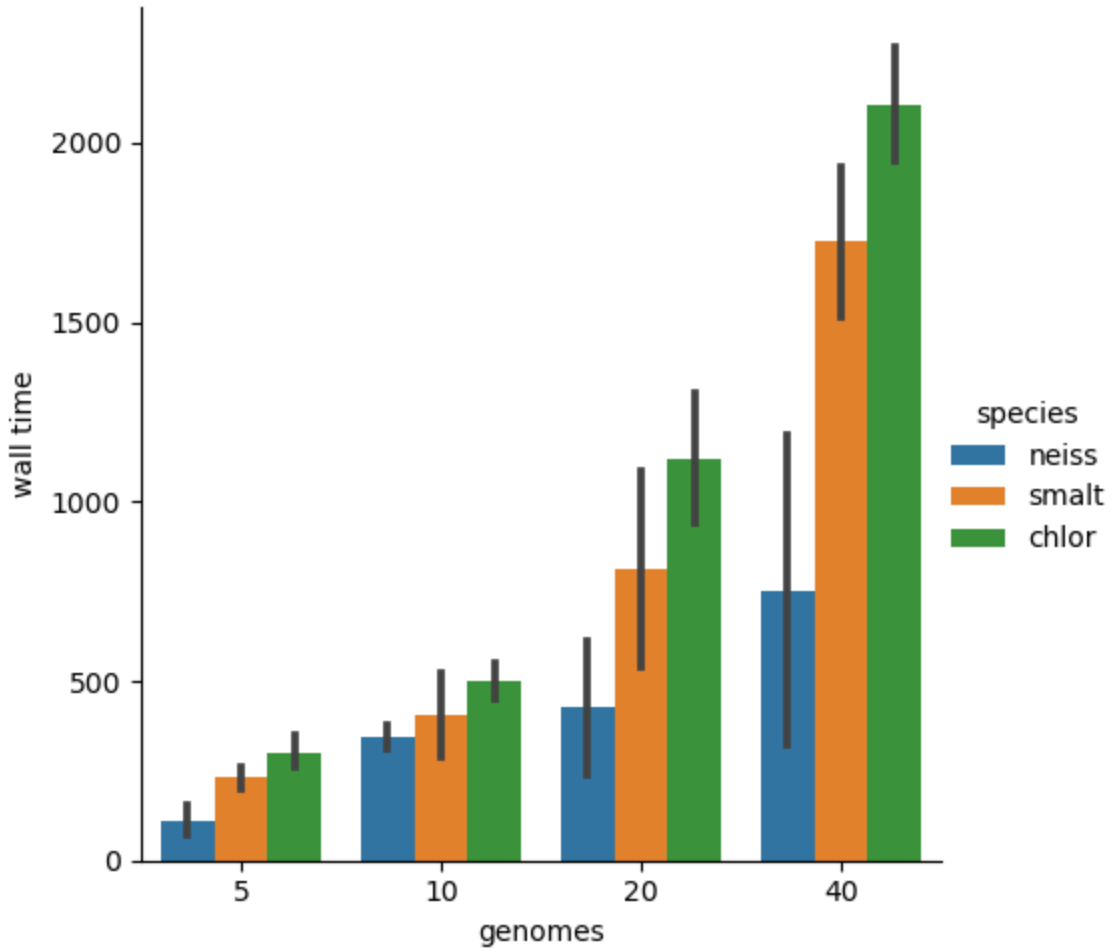
530

531 **Supplementary Figure 1. Sequence analysis shows REPIN groups are indeed associated with**
532 **monophyletic RAYTs.** Non-monophyletic or missing associations to REPIN populations identified
533 by RAREFAN were investigated in the corresponding genomes using Geneious (Kearse *et al.*
534 2012). Red boxes mark the position of the atypical RAYT that is being analyzed in detail. Mapping
535 of REPIN palindromes A-I (with zero mismatches) shows FDAARGOS_649_2 (A), NCTC10258_2
536 (B), and OUC_Est_2 (C) are linked to the wrong REPIN group because REP singlets that are
537 ordinarily linked to a RAYT sister clade are found in close proximity to the RAYT. These wrong
538 associations between REPIN and RAYT usually occur when the correct REPIN population is absent
539 from the reference genome. ISMMS2R_1 (D) and ISMMS2_1 (E) are not linked to REPIN
540 populations by RAREFAN when the maximum REPIN-RAYT distance parameter is set to 130 bp.
541 The RAYTs are linked to the correct REPIN populations when the REPIN-RAYT distance parameter
542 is set to 200 bp (default). Nucleotide sequences and positions were extracted from output files
543 generated by RAREFAN. Complete genome sequences are available in NCBI Nucleotide Database
544 using Accessions: (A) NZ_CP044092.1, (B) NZ_LS483377.1, (C) NZ_CP015612.1, (D)
545 NZ_CP011306.1, (E) NZ_CP011305.1.



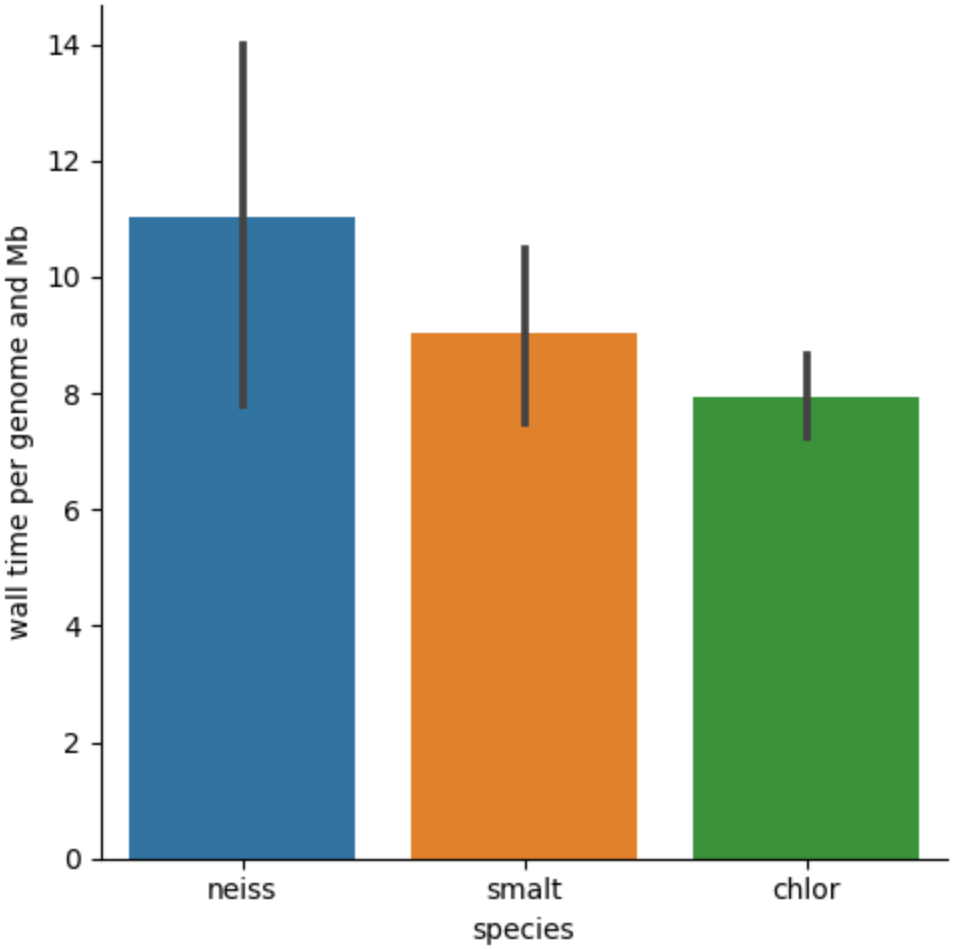
546

547 **Supplementary Figure 2. RAYT gene ISMMS3_1 cannot be linked to a REPIN population.** The
548 sequence of the RAYT gene ISMMS3_1 and its flanking sequences were analysed in Geneious
549 (Kearse *et al.* 2012). The inset shows the location of ISMMS3_1 in the RAYT phylogeny (grey box).
550 When mapping all of the identified palindromes to the RAYT region and allowing up to four
551 mismatches (yellow annotations), various mutants of palindrome C were found in close proximity
552 of the RAYT gene. However, we could not identify a corresponding REPIN population, which may
553 indicate that the population has not yet expanded in the genome.



554
555 **Supplementary Figure 3.** Average time (in seconds) it takes RAREFAN to complete for different
556 genome numbers from three bacterial species (*N. meningitidis, N. gonorrhoeae*, *S. maltophilia*,
557 *Pseudomonas chlororaphis*). Black bars indicate the 95% CI across four runs, where two runs
558 share the same query RAYT. For each run reference and query strains were randomly selected.
559 All measurements were performed on 4CPU cores with 16 GB of shared memory.

560



Supplementary Figure 4. Approximate elapsed run time per megabase sequence length calculated from the same runtime data generated in **Supplementary Figure 3**.