Pangenomic type III effector database of the plant pathogenic *Ralstonia spp.*

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Background. The bacterial plant pathogenic *Ralstonia* species belong to the beta-proteobacteria class and are soil-borne pathogens causing vascular bacterial wilt disease, affecting a wide range of plant hosts. These bacteria form a heterogeneous group considered as a "species complex"," gathering three newly defined species. Like many other Gram negative plant pathogens, *Ralstonia* pathogenicity relies on a type III secretion system, enabling bacteria to secrete/inject a large repertoire of type III effectors into their plant host cells. T3Es are thought to participate in generating a favorable environment for the pathogen (countering plant immunity and modifying the host metabolism and physiology). **Methods.** Expert genome annotation, followed by specific type III-dependent secretion, allowed us to improve our Hidden-Markov-Model and Blast profiles for the prediction of type III effectors. **Results.** We curated the T3E repertoires of 12 plant pathogenic *Ralstonia* strains, representing a total of 12 strains spread over the different groups of the species complex. This generated a pangenome repertoire of 102 T3E genes and 16 hypothetical T3E genes. Using this database, we scanned for the presence of T3Es in the 155 available genomes representing 140 distinct plant pathogenic *Ralstonia* strains isolated from different host plants in different areas of the globe. All this information is presented in a searchable database. A presence/absence analysis, modulated by a strain sequence/gene annotation quality score, enabled us to redefine core and accessory T3E repertoires.

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Ralstonia

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Abstract

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large repertoire of type III effectors into their plant host cells. T3Es are thought to participate in
generating a favorable environment for the pathogen (countering plant immunity and modifying
the host metabolism and physiology).

31 **Methods.** Expert genome annotation, followed by specific type III-dependent secretion, allowed

32 us to improve our Hidden-Markov-Model and Blast profiles for the prediction of type III

33 effectors.

35 **Results.** We curated the T3E repertoires of 12 plant pathogenic *Ralstonia* strains, representing a

36 total of 12 strains spread over the different groups of the species complex. This generated a

37 pangenome repertoire of 102 T3E genes and 16 hypothetical T3E genes. Using this database, we

38 scanned for the presence of T3Es in the 155 available genomes representing 140 distinct plant

39 pathogenic *Ralstonia* strains isolated from different host plants in different areas of the globe. All

40 this information is presented in a searchable database. A presence/absence analysis, modulated

41 by a strain sequence/gene annotation quality score, enabled us to redefine core and accessory

42 T3E repertoires.

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46 **Introduction**

47 Plant pathogenic *Ralstonia* species (Peeters et al. 2013b) were ranked among the 10 most

48 important plant bacterial pathogens (Mansfield et al. 2012). These soil-resident bacteria are

49 indeed important, as they affect many different plant species, ranging from solanaceous crops to

50 other important crops like banana and peanut, in different parts of the world. Recently, new plant

51 species have been found to be infected and present symptoms of bacterial wilt, like blueberry

52 shrubs in Florida, USA (Bocsanczy et al. 2019), ornamental roses in the Netherlands (Bergsma-

53 Vlami et al. 2018), or pumpkin in China (She et al. 2017). This bacterium has one of the largest

54 known repertoires of T3Es among all plant or animal pathogenic bacteria. The type III secretion

55 system (T3SS) of Gram negative phytopathogenic bacteria is essential for virulence, and Type

56 III-secreted effectors (T3Es hereafter) have been found to contribute in many different and

57 sometimes redundant manners to the fitness of the bacterium in interaction with its host (Buttner

58 2016).

59 Plant pathogenic *Xanthomonas* spp*.,* and animal pathogens like *Escherichia* spp*., Shigella* spp*.

60 or Yersinia* spp*.* have around 30 T3Es per strain (Dong et al. 2015; Schwartz et al. 2015).

61 Classically known strains of *Pseudomonas* spp*.* have also around 30-40 T3Es (Wei et al. 2015),

62 with some rare cases of up to 50 T3Es in a given strain (Dillon et al. 2019). It was reported that

63 *Legionella spp.* can secret in their host cells up to 300 effectors type IV effectors) (Gomez-

64 Valero et al. 2019). Plant pathogenic *Ralstonia* spp. have between 46 to 71 T3Es (Peeters et al.

65 2013a).

66 In this work, we curated the genome of 2 new phylotype I strains bringing the total number of

67 curated strains to 12 plant pathogenic *Ralstonia* strains, representing the known diversity of

68 phylotypes (Wicker et al. 2012), more recently subdivided into three species (Safni et al. 2014).

69 This generated new and updated profiles for the prediction of 102 Rips ("Ralstonia injected

70 Proteins") and 16 hypothetical Rips, to be compared with the previous 94 Rips and 16

71 hypothetical Rips (Peeters et al. 2013a). Two hypothetical Rips from the reference strain

72 CMR15, Psi07, and GMI1000 were experimentally confirmed as being *bona fide* Rips (and were

73 named RipBM and RipBO).

74 The new and improved prediction profiles were used to analyze the effector repertoires of the

75 155 genomic sequences available in genbank. This dataset represents 140 different strains spread

76 over the three newly defined species: 54 *Ralstonia solanacearum* stains(16 Phylotype IIA and

77 38 Phylotype IIB strains), 59 *Ralstonia pseudosolanacearum* strains(57 Phylotype I and 2

78 Phylotype III strains) and 27 *Ralstonia syzygii* strains(27 Phylotype IV strains). The prediction of

79 all 118 Rips (including hypothetical Rips) over the whole dataset of 155 genomes/140 different

80 strains is available as a browsable database, enabling direct comparisons between strain

81 repertoires, from presence/absence tables to multiple alignments of DNA and protein sequences.

82 This dataset was then further analyzed to evaluate how conserved the Rips are among these 140

83 strains. This analysis took into account the host of isolation as a strong (but limited) host cue, or

84 the phylogenetic group, to identify host or kinship repertoire conservation.

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88 **Materials & Methods**

89

90 *155 published whole genomes*

91   The genbank genome data repository was scanned for the presence of complete genome

92   sequences of *Ralstonia* species complex strains. The total number of genomes gathered was 155,

93   with some strains sequenced multiple times by different research groups, yielding sequence data

94   for 140 distinctive strains. Owing to the fact that for a same strain different isolates could be

95   slightly different, and also to the fact that sequence quality is important for gene repertoire

96   completeness, we decided to keep all strain duplicates (in the database duplicates and triplicates

97   are indicated as "-2" and "-3", respectively). Strains in duplicates are the following: FJAT-1458,

98   FJAT-91, PSS4, CFBP2957, K60, CFBP6783, IBSBF1503, IPO1609, Po82, and UW163. Molk2

99   strain was present in the database with three independent sequence files, and UW551 with four

100 independent sequence files. Table S1 contains all the available data on the 155 genome files.

101 Whenever available, data for the following fields were also recorded : strain synonym; pubmed

102 ID of reference articles; Species name (Safni et al. 2014); Phylotype; Geographical origin

103 (isolation site); Plant isolated from; Genome assembly size; Assembly score; Number of contigs;

104 Number of scaffolds; Bioproject. Figure S1 provides a *mutS* phylogeny (Wicker et al. 2012)

105 indicating the strain relatedness.

106

Genome quality

108 Some genome sequences deposited by their authors were of insufficient quality to be included in

109 the Refseq database. Among the different quality critera used by refseq one can find low contig

110 N50, low gene count, or absence of essential rRNA, robosomal proteins and tRNA genes; for a

111 complete list of criteria see https://www.ncbi.nlm.nih.gov/assembly/help/anomnotrefseq/. This

112 is the case for the genomic entries FJAT-452, FJAT-462, T110, T12, T25 and UW700. These

113 sequences were left on the complete database but were excluded for the further analysis in this

114 work.

115 We then devised an assembly score in order to sort all the strains and to distinguish draft from

116 complete and "polished" genomes. This score is Log_{10}(assembly size/number of contigs, with

117 contigs being the N-free scaffolds or assembled pseudomolecules that were spliced by us on N

118 stretches), and is a good general score to assess the overall completeness of the genome

119 sequence. One exception to this is the strain CFBP2957, with a high score (6.287), but for which

120 only the chromosome was available (and not the megaplasmid, see the bipartite nature of plant

121 pathogenic *Ralstonia* genomes (Salanoubat et al. 2002)), and thus artificially increased the

122 quality score.

123 We then used a known metric for genome quality called BUSCO ("Benchmarking Universal

124 Single-Copy Orthologs") based on the comparison of predicted ORF (we used PRODIGAL

125 (Hyatt et al. 2010)) with an adequate set of conserved single-gene orthologs (Waterhouse et al.

126 2017). We used the beta-proteo-bacteria lineage derived set of 582 BUSCO genes. The

127 completeness metric (C%) represents the presence and not-frameshifted BUSCO ORFs in a

128 given strain genome. This data was added in Table S1. This metric was not sufficient to remove

129 the poorly sequenced genomes as 5 out of the 6 genomes excluded from the Refseq database had

130 a high completeness score (T12: 98.8%; T110: 98.6%; UW700: 98.6%; FJAT-462: 98.6%; T25:

131 96.2%).

132 Neither the assembly score, the BUSCO metric (nor a combination of both) was efficient enough

133 to weed out poorly sequenced genomes. We thus felt the need to indirectly rate the gene

134 annotation and prediction if it were to be further used in T3E repertoire comparisons. This is why

135 we decided to generate two stringency cutoffs using the total T3E gene prediction performed by

136 our prediction pipeline: for each strain the content in multicopy paralogous genes ("MULTI"),

137 the single defined genes ("OK"), the frame-shifted genes ("FS"), and the pseudogenes ("PG"),

138 were computed for the 102 T3Es. We applied two levels of stringency. For "stringency 1", we

139 kept only the strains for which the total number of pseudogenes plus frame-shifted genes is lower

140 than 10: (PG+FS) < 10 ; this yielded a total of 123 genomes corresponding to 114 different

141 strains. For "stringency 2", we only kept the strains that also had more than 50 T3Es in total ;

142 this yielded to a set of 88 genomes corresponding to 84 different strains. Table S1 contains two

143 columns identifying the 123 "stringency 1" and 88 "stringency 2" strains.

144 This "stringency" ranking is an artificial cutoff, but we believe this is a valid method to further

145 compare the complete gene repertoires. The two strains T110 and UW700 have high genome

146 assembly scores (6.45 and 5.06 respectively), but performed badly in this stringency test, with

147 only 30 and 21 well-predicted T3Es (excluding them from "stringency 2" group) and 35 and 25

148 frameshifted and pseudogenes (excluding them from "stringency 1" group).

149

150 *Gene presence/absence*

151   For each strain, the Table S1 contains the presence/absence scoring for all the 102 Rips and 16

152   hypothetical Rips. We used the prediction data for each strain (see further) as highlighted on the

153   database website (https://iant.toulouse.inra.fr/T3E). Frameshifted genes are rare in well-

154   sequenced genomes. Indeed, out of the 67 strains reported with two scaffolds (corresponding to

155   the expected chromosome and megaplasmid (Salanoubat et al. 2002)), 52 have no frame-shifted

156   genes, and nine only contain one frameshifted gene (see Table S1 for the data). We thus

157   hypothesized that a frameshift is more due to sequencing errors than representing true genomic

158   data. As a consequence, when making a binary scale for scoring the presence/absence of T3Es,

159   we considered all "MULTI" (recently duplicated genes), "OK" (single gene) and "FS"

160   (frameshifted) as "1" (or "present"); when absence "NO" and pseudogene ("PG") were

161   considered as "0" (or "Absent"), this same reasoning was used previously (Peeters et al. 2013a).

162

163   *T3E prediction improvements*

164   We have improved our first T3E prediction pipeline (Peeters et al. 2013a), by adding databases

165   of confirmed T3Es from *Xanthomonas* spp. (www.xanthomonas.org/t3e.html) and *Pseudomonas*

166   spp. strains (www.pseudomonas-syringae.org/T3SS-Hops.xls). In order to capture more distantly

167   related Rips, we lowered the tblastN/blastX thresholds (Query coverage per subject 60 %, and

168   Percentage of identical matches 60 %), this was well exemplified by the RipBN case, an

169   AvrRpt2 ortholog clearly present in the CMR15strain (Eschen-Lippold et al. 2016) and also

170   detectable by blast, but not without slightly lower thresholds. We also rewrote some parts of the

171   pipeline in order to speed up the prediction engine. The updated pipeline is outlined in Fig. 1.

172

173   *T3Edb v3 specificities*

174   The new database version is very similar to the previous version (Peeters et al. 2013a). In this

175   new version a set of curated strains ("curated repertoire") is listed on a tab, with a comparison of

176   their T3E repertoire. This set is composed of the following strains: 244 (phylotype I) (Ramesh et

177   al. 2014); GMI1000 (I) (Salanoubat et al. 2002); YC45 (I) (She et al. 2015); CFBP2957 (IIA)

178   (Remenant et al. 2010); CMR15 (III) (Remenant et al. 2010); IPO1609 (IIB) (Gonzalez et al.

179   2011); Molk2 (IIB) (Remenant et al. 2010); Po82 (IIB) (Xu et al. 2011); UW551 (IIB) (Gonzalez

180   et al. 2011); PSI07 (IV) (Remenant et al. 2010); BDBR229 (IV) (Remenant et al. 2011), and R24

181   (IV) (Remenant et al. 2011).

182   In order to "build profiles" of Rip prediction in different strains to compare the strains and/or to

183   generate multifasta files (of nucleotide or protein sequences of specific Rips), one can now sort

184   the whole set of 155 complete genomes on the different headers available, namely these are:

185   "status (curated or not); "code" (abbreviated name); "synonym", "species name" (Safni et al.

186   2014); "phylotype"; "plant isolated from"; "assembly size"; "number of contigs"; "number of

187   scaffolds"; "assembly score" (see definitions above).

188

189   *Type III secretion dependence*

190   The type III-dependent secretion of Hyp15 and Hyp16, two hypothetical T3Es previously

191   identified in strains GMI1000, CMR15, and PSI07 (Peeters et al. 2013a) was demonstrated in

192   this work. The coding sequences of PSI07_1860 and CMR15v4_mp10184 (both formerly

193   Hyp15), were ordered as DNA synthesis from Sangon (Shanghai, China). RSc3174, from the

194   reference strain GMI1000 (formerly Hyp16) was amplified in two steps. The first PCR was

195   performed using the following primers: Forward : 5'

196   GGAGATAGAACCATGAAAGTCGGCAACCAATC-3' and Reverse 5'

197   CAAGAAAGCTGGGTCTCCACGTGATAAGTTGTAGCG-3', using proof-reading Phusion

198   polymerase using high GC buffer (New England Biolabs). The second PCR was performed using

199   1 µl of the first PCR as matrix and attB universal primers (oNP291

200   5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3' and

201   oNP292 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC-3', using the same polymerase as

202   the previous PCR with a two step annealing temperature: 10 cycles at 45°C and then 25 cycles at

203   55°C. Then, Rsc3174, PSI07_1860 and CMR15v4_mp10184 were cloned into pDONR207

204   vector using a BP reaction and in pNP329 using a LR reaction following the instructions of the

205   manufacturer (LifeTechnologies). The final expression vectors were transformed into the *R.*

206   *pseudosolanacearum* GMI1000 strain and in the *hrcV* mutant (type III secretion defective

207   mutant, used as a negative control) as previously described (Perrier et al. 2018). In-vitro

208   Secretion assays and Western blot analysis were performed as previously described (Lonjon et

209   al. 2018).

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218 **Results**

219 *Curation of 2 new Phylotype I strains and identification of 8 New Rips*

220 Because strain GMI1000 was the only curated *R. pseudosolanacearum* strain in the former

221 RalstoT3Edb (Peeters et al. 2013a), we conducted a manual curation of the Type III effectome in

222 two other *R. pseudosolanacearum* strains, both differing in host range from GMI1000. Strain Rs-

223 10-244 was isolated from chilli pepper (*Capsicum annuum*) on the Andaman Islands (India)

224 (Ramesh et al. 2014) and strain YC45 was isolated from a monocotyledoneous host, aromatic

225 ginger (*Rhizoma kaempferiae*) in Southern China (She et al. 2015). Manual curation identified 73

226 Rip genes (+1 candidate) in strain YC45 and 77 Rip genes (+3 candidates) in Rs-10-244. Novel

227 Rip effectors and candidates were identified in these strains (Table 1).

228 RipBJ was identified by secretome analysis of the GMI1000 strain (*R. pseudosolanacearum*)

229 (Lonjon et al. 2016). RipBK and RipBL were identified in the process of curation of the strain

230 YC45 (*R. pseudosolanacearum)*, owing to their similarity to HopAM1 (Chang et al. 2005; Goel

231 et al. 2008) and HopAO1 (Chang et al. 2005; Macho et al. 2014). RipBM and RipBO, formerly

232 known as Hyp15 and Hyp16, respectively (Peeters et al. 2013a), were experimentally confirmed

233 to be secreted by the T3SS in GMI1000 (Fig. 2, Fig. S2). RipBN was identified by sequence

234 homology in the strain CMR15 (*R. pseudosolanacearum*) (Eschen-Lippold et al. 2016). RipBP

235 (homolog to HopW1 (Zumaquero et al. 2010)) was identified in the strain OE1-1 (*R.*

236 *pseudosolanacearum*), and RipBQ (homolog to HopK1 (Chang et al. 2005)) in the strain

237 KACC10722 (*R. syzygii*). RipBP and RipBQ are considered here as Rips by applying the rule of

238 similarity with a known T3E (Peeters et al. 2013a). These two latter Rips have been highlighted

239 in the curated list of strains although none of these curated strains harbor these effectors. This is

240 the same for RipBE which is specific to strain RS1000 (Mukaihara & Tamura 2009; Peeters et

241 al. 2013a). Table 1 mentions the reference sequences for new Rip genes. Two new hypothetical

242 Rips were also identified; named Hyp17 and Hyp18 with two associated reference sequences

243   (See Table 1). Considering that in the previous database (Peeters et al. 2013a), some Rips were

244   only represented by pseudogenes, we corrected this by attributing new reference sequences to

245   RipBA (strain Rs-10-244, sequence RS244_c002320), RipBE (strain YC40-M, sequence YC40-

246   M_00170) and RipP3 (strain Rs-10-244, sequence RS244_c031810).

247

248   *Improved Rip-scanning pipeline*

249   Thanks to the increased dataset of 102 total Rips (and 16 hypothetical T3Es), on a total of 155

250   genomes (totaling 140 different strains), we were able to generate new effector profiles for the

251   improved prediction of these Rips and candidate Rips in newly available *Ralstonia* genomes. The

252   "scan your genome" tool is available on the database website. For large dataset analysis, we

253   prefer to be contacted directly (RalstoT3E-toulouse@inra.fr), to prevent server overload. A Blast

254   tool, as well as all the files and results of predictions for the 155 genomes are also available on

255   our website. A convenient tool is the availability of multifasta files for the nucleotide or protein

256   sequences for a given Rip, containing the genome/strain sequences that one queried for

257   comparison in the first place.

258

259   *Core effectors*

260   We wanted to have a new look at the number of conserved Rips among this new diverse set of

261   strains. As a principle, as more strains are compared, the smaller the core set of Rips will

262   become. In order to have a pertinent set of strains to compare, we decided to limit the core

263   comparisons to the "stringency 2" set of strains (the 84 distinct strains having less than 10

264   pseudogenes or frameshifted genes and, at the same time, more than 50 predicted Rip genes).

265   Fig. 3 shows a phylogenetic tree built using the *mutS* gene sequence of these 84 strains to be able

266   to judge the relatedness of this set of strains. We are aware of the risk of excluding some strains

267   based on this stringent selection. This could in particular be the case for the known strains that

268   have seen genome reduction and hence have fewer T3Es. This is the case for the Moko disease,

269   or blood disease bacterium BDBR229 (Remenant et al. 2011), and the *R. syzygii* clove-tree

270   infecting and insect-transmitted R24 strain (Remenant et al. 2011), which each have respectively

271   54 and 48 Rip genes (as defined under the "stringency 2" criteria). Moreover, both strains are

272   already left out under "stringency 1" criteria, for having more that 10 (respectively 20 and 12),

273   frameshifted and pseudo genes.

274 We then decided to use the host of isolation as an interesting criterion to compare strains. Of

275 course there are numerous examples of strains isolated on one host and later shown in laboratory

276 settings to be able to infect other host plants. GMI1000, isolated from tomato (Salanoubat et al.

277 2002), was shown to be very well capable of wilting *Medicago truncatula* (Vailleau et al. 2007)

278 or *Arabidopsis thaliana* (Deslandes et al. 1998). As laboratory settings are hard to compare

279 between labs, and as thorough host-compatibility has been done only for a handful of strains, we

280 preferred to stick with the host of isolation information, without excluding that the host range

281 might be much wider for some strains, and restricted for others. We decided to compare the

282 conservation of Rip repertoires among the 84 "stringency 2" strains, classifying them into hosts

283 of isolation; *Solanaceae* strains ("SOL"), tomato strains ("TOM", 15 strains), Eggplant ("EGG",

284 9 strains), potato ("POT", 30 strains) and banana ("BAN", 15 strains). The larger, encompassing

285 category being the Solanaceae group, with 58 strains (for the list of strains see Table S1). Table 2

286 indicates the list, per host-of-isolation category of the core set of Rip genes. For a set of n total

287 strains, we decided to still consider core, the Rip genes present in the interval (n; n-5%) number

288 of strains. For instance, for the 58 "SOL" strains, the core Rip genes are the ones present in 58 to

289 55 strains. Obviously, the larger the number of strains, the lower the number of conserved Rips.

290 For instance, for the 9 "EGG" strains, there are 44 strictly conserved Rips (in all 9 strains),

291 whereas there are only 27 conserved Rips in the 15 "BAN" strains (in 14 to 15 strains). Figure 4

292 show two Venn diagram comparing these sets of conserved Rip between host-of-isolation

293 groups.

294

295 The set of 140 strains is evenly spread over the three newly defined *Ralstonia* species (see

296 Fig. S1 for a *mutS* phylogeny (Wicker et al. 2012) of the 140 strain/155 genome sequences of

297 this study). One possible caveat is the small number of phylotype III strains (only two strains:

298 CMR15 and CFBP3059), now classified with phylotype I strains among the *R.*

299 *pseudosolanacearum.* One interesting way to look at the conservation of Rips is to make specific

300 species groups. The 84 "stringency 2" strains are well spread over the three phylogenetic groups:

301 38 are *R. pseudosolanacearum* strains (phylotypes I and III), 25 are *R. solanacearum* strains

302 (phylotypes IIA and IIB), and 21 are *R. syzygii* strains (phylotype IV), see Fig. 3 for the *mutS*

303 phylogeny of these 84 strains (Wicker et al. 2012). Table 3 represents the Rip distribution among

304   these three species, together with the conservation in the total set of 84 strains. Figure 5 displays

305   the Venn diagram corresponding to this triple comparison.

306

307

Discussion

309   In this work, we significantly updated the *Ralstonia* type III secretion effector database (T3Edb)

310   (Peeters et al. 2013a). This latter work, providing a new nomenclature for these essential

311   virulence proteins was widely accepted and cited by the community. Here, we reported on the

312   curation of new plant pathogenic *Ralstonia* strains, adding new T3Es to this database. These are

313   represented by the new series from RipBJ to RipBQ, among which both RipBM and RipBO were

314   shown in this work to be indeed secreted by the GMI1000 (*R. pseudosolanacearum)* T3SS. One

315   of these newly defined Rips, RipBN, was identified for being an ortholog of the *Pseudomonas*

316   *syringae* AvrRpt2 T3E (Eschen-Lippold et al. 2016), and recently shown to function similarly in

317   triggering resistance in Ptr1-tomato lines (Mazo-Molina et al. 2019).

318   The newly defined Rip profiles (102 Rips and 16 Hypothetical Rips) were then used to predict

319   the T3E repertoire of the 155 genome sequences available, representing a total of 140 different

320   strains, compared to the 12 genomes previously available. This large set of strains allows us to

321   provide an updated database with a better representation of each of the three phylogenetic clades

322   of this species complex. These are: Phylotypes I and III, or the newly proposed species named

323   *R. pseudosolanacearum (Safn*i et al. 2014), phylotypes IIA and IIB, or *R. solanacearum* and

324   phylotype IV, or *R. syzygii*. For a better view of strain relatedness, *mutS* phylogenies are

325   displayed in Fig. 3 (set of 84 strains), and in Fig. S1 (all 140 strains). In order to understand the

326   contribution of these T3Es to the virulence of these bacteria on their host plant, it is particularly

327   interesting to analyze which T3Es are conserved among the different strains. Our comparison

328   results highlight two ways to explore these repertoires: either by host plant or by phylogenetic

329   relatedness.

330

331   Ideally, each of the deeply-sequenced and well-annotated strains (the "stringency 2" list of 84

332   strains) should be tested on a panel of host plants in order to define their actual host range. As

333   these data are not available we focused on the host of isolation as a limited but natural host

334   definition factor. This is a strong limitation in this comparison, as it is reported or known (and

335   shared through personal communications) that some strains are also compatible with other, and

336   sometimes distantly related hosts. We decided to add the published information on the

337   compatibility on other host plants in the Table S1. A few research groups added a significant

338   amount of host-compatibility information for a set of strains (Ailloud et al. 2015; Cho et al.

339   2018; Lebeau et al. 2011). Other groups have performed host-compatibility experiments and

340   shared this information with us, *e.g.* tobacco strain CQPS-1 (Liu et al. 2017) is also mildly

341   pathogenic on tomato (Personal communication Prof. Ding W.), while the blueberry strains

342   P816, P822 and P824 (Bocsanczy et al. 2019), are very aggressive on tomato (personal

343   communication Dr Norman DJ). In our repertoire comparison, we allow a tolerance of presence

344   for the Rip in the interval of strains between the total number of strains n, and n-5%, this allows

345   to compensate the effect of unequal set of strains to compare. Table 2 and Fig. 4A show that,

346   unsurprisingly strains isolated from Eggplant "EGG", tomato "TOM" and potato "POT", share a

347   significative number of their conserved T3Es (22 shared out of 44 "EGG", 44 "TOM" and 30

348   "POT"), this number is probably largely underestimated as we know that some of these

349   *Solanaceae*-isolated strains are compatible with other *Solanaceae* (Lebeau et al. 2011). Another

350   comparison shown (Fig. 4B) is between the "TOM", "POT" and banana "BAN" strains. Here,

351   we can see that there could be more T3Es shared between "BAN" and "TOM" (21 out of 27

352   "BAN" strains) than between "BAN" and "POT" (15 out of 27 "BAN" strains). To evaluate this

353   potential difference, one has to keep in mind that, although banana and *Solanaceae* are distantly

354   related, it has been shown that 9 out of the 27 "BAN" strains are also compatible with tomato

355   and potato, when only one strain (BDBR229) was shown to be incompatible on these two

356   *Solanaceae* hosts (Ailloud et al. 2015). When considering all *Solanaceae* (SOL) as host of

357   isolation (58 strains from the "stringency 2" set of 84 strains), the core set of T3Es (as defined to

358   be present in 55 to 58 strains) is represented by a list of 27 T3Es (see Table 2). It is only when

359   host-compatibility is compared in detail with T3E repertoires that we can start to potentially

360   associate the presence (or the presence of specific alleles) to be required (or deleterious) for

361   specific host-compatibility (Cho et al. 2019; Wang et al. 2016).

362   A second and maybe less ambiguous way to compare lists of conserved T3Es is to group the

363   strains by their phylogenetic origin. Table 3 summarizes the T3Es conserved within each of the

364   three phylogenetic groups of strains (Wicker et al. 2012). These groups are: the 38 strains from

365   phylotypes I and III, or *R. pseudosolanacearum;* the 25 strains from phylotypes IIA and IIB, or

366 *R. solanacearum;* and the 21 strains from phylotype IV or *R. syzygii* ; Fig. 3 displays the *mutS*

367 phylogeny of these 84 strains. Some strong phylogenetic associated presence/absence can be

368 highlighted, like the systematic presence in *R. pseudosolanacearum* and *R. syzygii* and

369 systematic absence in *R. solanacearum* of the conserved T3Es RipA2, RipG5 and RipZ. Some

370 T3Es are systematically associated with only one of these phylogenetic groups, like RipC1, RipI,

371 RipAC, RipD with *R. solanacearum*; RipL, RipQ, RipS4 with *R. pseudosolanacearum* and

372 RipA5, RipM, RipS5, RipAQ with *R syzygii.* Sixteen (16) T3Es are conserved among the

373 phylogenetic groups (Fig. 5 and Table 3). Eight of them are conserved in the different species:

374 RipB (absent only in *R. pseudosolanacearum* CQPS-1 (Liu et al. 2017)); RipH2 (absent only in

375 *R. pseudosolanacearum* RSCM isolated from Cucurbita maxima in China (She et al. 2017));

376 RipR (absent only in *R solanacearum* UW181, a plantain banana strain (Wicker et al. 2012), and

377 *R. syzygii* BDB_RUN1347, no host of isolation reported); RipW (absent only in

378 *R. pseudosolanacearum* strain SL3822 isolated form potato in Korea (Cho et al. 2018); RipAB

379 (absent only in *R. pseudosolanacearum* strain YC40-M, no host of isolation reported, and

380 *R. solanacearum* strain MolK2 (Remenant et al. 2010)); RipAI (absent only in

381 *R. pseudosolanacearum* strain HA4-1 a Chinese peanut strain); RipAO (absent only in

382 *R. pseudosolanacearum* strain SL3755 isolated form potato in Korea (Cho et al. 2018)). The only

383 strictly conserved T3E among these 84 strains is RipAJ. Eight others are slightly

384 underrepresented in one species out of the three (number of strains in which the T3E is present is

385 indicated in brackets in Table 3). Among these two (2) are less conserved in

386 *R. pseudosolanacearum* (Phylotype I and III): RipAN and RipAY; four (4) are less conserved in

387 *R. solanacearum* (Phylotype II): RipA2, RipG5, RipG6, RipAM; two (2) are less conserved in

388 *R. syzygii* (Phylotype IV): RipU and RipV1.

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Conclusions

394 This work describes the methods and strains used to build a comprehensive database of the type

395 III effectors (T3Es) from the *Ralstonia solanacearum* Species Complex (*R. solanacearum,*

396 *R. pseudosolanacearum* and *R. syzygii*). Representing a resource to both study and identify new

397   allelic versions of specific T3Es, the database contains all the specific T3E sequences (102 T3Es

398   and 16 hypothetical T3Es in over 155 strains), but also allows to identify new T3E orthologs by

399   scanning DNA sequences (partial, shotgun or complete genomes) from original isolates.

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581

582   **Figure legends**

583

584   **Figure 1. New T3E prediction pipeline.**

585

586   **Figure 2. RipBM and RipBO are secreted through the T3SS.**

587   The wild-type strain and the *hrcV* mutant were transformed to express a RipBM$_{CMR15}$-3HA,

588   RipBM$_{Psi07}$-3HA **(A)** or a RipBO-3HA **(B)** fusion protein. Secretion assays were performed and

589   total proteins from bacterial pellets and proteins in the supernatants were detected by Western-

590   Blot.

591

592   **Figure 3. *mutS* alignment and phylogenetic tree of the 84 different strains ("stringency 2").**

593   A neighbor-joining tree was built using the *mutS* from *Ralstonia pickettii* as an outgroup.

594   Bootstrap were performed on 100 replicates, only support higher than 50% displayed in the

595   consensus tree.

596

597   **Figure 4. Venn diagram of conserved T3Es among different sets of "host-of-isolation"**

598   **defined strains. (A)** comparisons of conserved T3Es among "TOM" (host of isolation : tomato),

599   "EGG" (Host of isolation: eggplant) and "POT" (host of isolation : potato). **(B)** Comparison

600   between "TOM", "POT" and "BAN" (host of isolation: banana). The lists of compared T3Es are

601   visible in Table 2.

602

603   **Figure 5. Venn diagram of conserved T3Es among the different phylogenetic clades of**

604   **strains.** Comparison of conserved T3Es between *R. pseudosolanacearum* (phylotypes I and III),

605   *R. solanacearum* (phylotypes IIA and IIB), and *R. syzygii* (Phylotype IV) strains. The lists of

606   compared T3Es are visible in Table 3.

607

608   **Supplemental Figure S1. *mutS* alignment and phylogenetic tree on the set of 140 different**

609   **strains.** A neighbor-joining tree was build using the *mutS* from *Ralstonia pickettii* as an

610   outgroup. Bootstrap were performed on 100 replicates, only support higher than 50% displayed

611   in the consensus tree.

612

613   **Supplemental Figure S2. Uncropped western-blot version of Figure 2.**

614

8 new T3E and 2 new Hypothetical T3E identified

1 **Table 1. 8 new T3E and 2 new Hypothetical T3E identified**

Proposed T3E family name	Representative gene member	Hop/Xop homologues	Functional domain	Evidence for T3SS-dependent secretion or translocation
RipBJ	GMI1000 RSp0213	none		Lonjon et al. 2016
RipBK	YC45_c025370	HopAM1		Chang et al. 2005
RipBL	YC45_m001910	HopAO1	Protein-tyrosine phosphatase	Chang et al. 2005
RipBM	Psi07 RSPsi07_1860 (former Hyp15)		Protein-Ser/Thr kinase	This work
RipBN	CMR15v4_30917	AvrRpt2	cysteine protease	Eschen-Lippold et al. 2016
RipBO	GMI1000 RSc3174 (former Hyp16)	none		This work
RipBP	OE1-1_24290	HopW1 + homologs in Xantho	N-term domain=HopW1 and C-term= uncharacterized protein ABJ99_3552 [Pseudomonas syringae pv. cilantro]	Zumaquero et al. 2010
RipBQ	KACC10722_38580	HopK1/XopAK		Chang et al. 2005
Hyp17	RS244_m000380	none		This work
Hyp18	CMR15v4_mp10535	none		This work

4

List of Type III effectors conserved according to the host of isolation.

The # strains, indicate the total number of strains analysed and the 5% tolerance. The numbers in each cell indicate how many strains actually harbor the cognate effector. Grey scale according to conservation between columns.

Table 2. List of Type III effectors conserved according to the host of isolation. The # strains, indicate the total number of strains analysed and the 5% tolerance. The numbers in each cell indicate how many strains actually harbor the cognate effector. Grey scale according to conservation between columns.

	Eggplant	Tomato	Banana	Potato	Solanacea
# strains	9	14-15	14-15	28-30	55-58
RipA2	9	15		30	58
RipA3		15		28	55
RipA4		14			
RipA5		14	14	30	55
RipB	9	15	15	30	57
RipC1		14	15		
RipD			15		
RipE1	9	14	15		
RipE2			15		
RipF1		15	14	29	
RipG2	9	14			
RipG3			14		
RipG4	9				
RipG5	9	15	14	30	58
RipG6	9	14	15	30	57
RipG7		15		28	55
RipH1	9	14	14		
RipH2	9	15	15	30	58
RipH3	9	15		28	56
RipI		14	15		
RipJ	9				
RipL	9				
RipM	9	15		28	55
RipN	9	15			55
RipO1	9				
RipQ	9				
RipR	9	15		30	58
RipS1	9				
RipS2		15			
RipS3		15			

RipS4		14			
RipS5	9	14	14	29	55
RipS6	9				
RipU	9	15		30	58
RipV1	9	15	15	28	56
RipW	9	15	15	29	57
RipX	9	15		29	57
RipY	9	14			
RipZ	9	15		29	57
RipAA	9	14		29	55
RipAB	9	15	14	30	58
RipAC	9	15	15		
RipAD		14		29	
RipAE	9	15			
RipAF1	9				
RipAI	9	15	15	30	58
RipAJ	9	15	15	30	58
RipAL				30	
RipAM	9	15		30	58
RipAN		15	15	30	57
RipAO	9	15	15	29	57
RipAP	9				
RipAQ	9	15		28	56
RipAR		14	14		
RipAS	9				
RipAT			15		
RipAU			14		
RipAV	9				
RipAW		14			
RipAX1			14		
RipAY		14	15	29	
RipAZ1	9				
RipBA	9				
RipBM	9				
RipTAL	9				
RipTPS	9	14		29	55
Total CoreT3Es	44	44	27	30	27

List of Type III effectors conserved according to the phylogenetic origin.

The # strains, indicate the total number of strains analysed and the 5% tolerance. The numbers in each cell indicate how many strains actually harbor the cognate effector. Grey scale according to conservation between columns.

Table 3. List of Type III effectors conserved according to the phylogenetic origin. The # strains, indicate the total number of strains analysed and the 5% tolerance. The numbers in each cell indicate how many strains actually harbor the cognate effector. Grey scale according to conservation between columns.

	Phylotype I and III	Phylotype IIA and IIB	Phylotype IV	"stringency 2"
# strains	36-38	24-25	20-21	80-84
RipA2	38	(21)	21	80
RipA3	36		21	
RipA5			21	
RipB	37	25	21	83
RipC1		25		
RipD			20	
RipE1		24		
RipE2		24		
RipF1		25	20	
RipF2		24		
RipG2	36			
RipG4	38	24		
RipG5	38	(23)	21	82
RipG6	37	(23)	21	81
RipG7	37	24		
RipH1			20	
RipH2	36	25	21	82
RipH3	36			
RipH4			20	
RipI		25		
RipL	38			
RipM			21	
RipN			20	
RipO1		24		
RipQ	38			
RipR	38	24	20	82
RipS2	36			
RipS4	38			
RipS5			21	
RipS6	37			

RipU	38	25	(18)	81
RipV1	38	25	(19)	82
RipV2			20	
RipW	37	25	21	83
RipX			21	
RipY			20	
RipZ	38		21	
RipAB	37	24	21	82
RipAC		25		
RipAD		25		
RipAE		24		
RipAF1	36			
RipAI	36	25	21	82
RipAJ	38	25	21	84
RipAK	36			
RipAM	38	(23)	21	82
RipAN	(35)	25	21	81
RipAO	36	25	21	82
RipAP	36	25		
RipAQ			21	
RipAS	36			
RipAU			20	
RipAV	36			
RipAY	(35)	25	20	80
RipAZ1	36		20	
RipBF			20	
T3E_Hyp1			20	
Total Core T3Es	31	25	32	16

Figure 1

T3E prediction pipeline

Pipeline: T3E v3
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LIPM INRA/CNRS
March 2019
https://framagit.org/LIPM-BIO INFO/RalstoT3E.git

Blastx vs. EffectorDB
Database = Curated *R. solanacearum*
T3E + *P. syringae* T3SS + *Xanthomonas* sp. T3E
Program = USEARCH
Parameters = evalue 1e-5

Genome

Assembly analysis
(contigs, N50, N content)

Assembly metrics

ORFs and Pseudogenes Prediction
Program = FrameD
--frameshift_penalty=999
--matrix = Curated T3E CDS

Frameshifted genes prediction
Program = FrameD
--matrix = Curated T3E CDS

CDS and CDS fragments From blastx hits
qcov > 60
%identity > 60

Remove Redundancy

Predicted Effectome

RalstoT3E proteins

RalstoT3E protein HMM Profiles

RalstoT3E CDS

Family analysis
Program= OrthoMCL 1.4
--pmatch_cutoff=90
--pmatch_cutoff=80
--pmatch_cutoff=70
--pmatch_cutoff=60

Extract Blastp Best Hits
qcov > 40

HMM Scan
-E 1e-5

tblastn
--evalue 1e-5 seq no --num_alignments 1
qcov > 60
%identity > 60

Consolidate assignation

Assigned T3E

Extract Unassigned proteins

Blastp vs. EffectorDB
Database = Curated *R. solanacearum*
T3E + *P. syringae* T3SS + *Xanthomonas* sp. T3E
Program = USEARCH
Parameters = evalue 1e-5

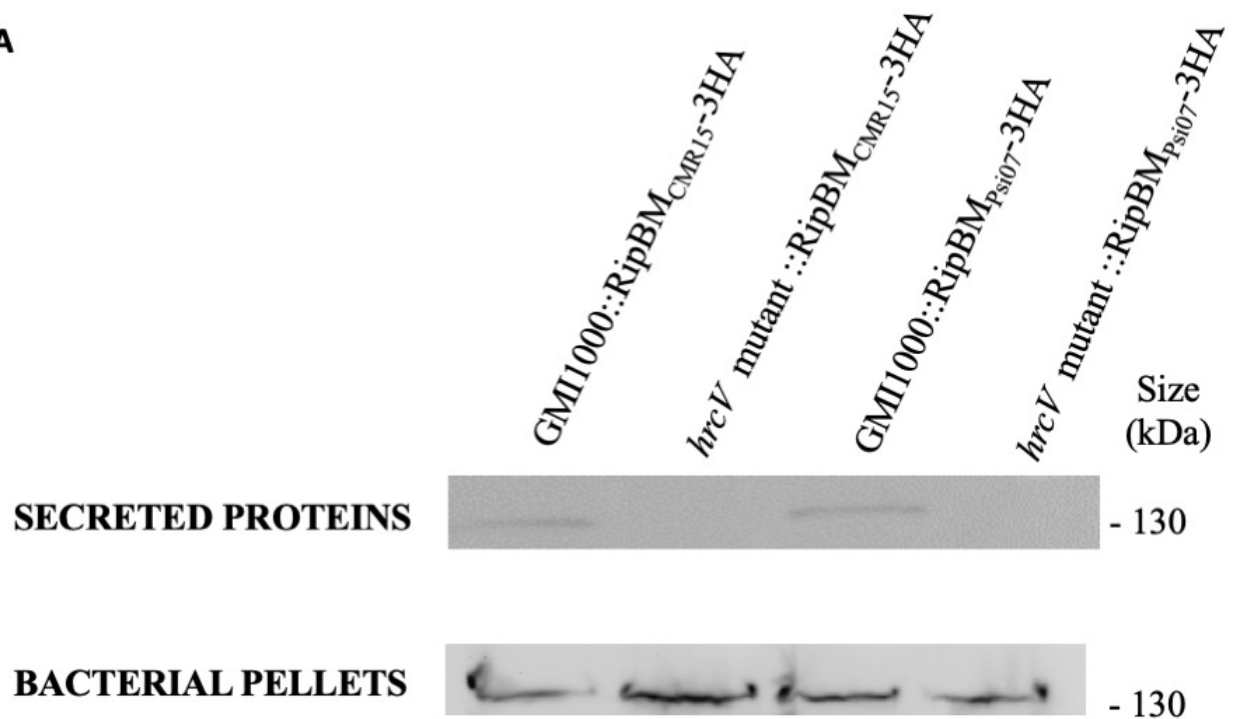
Unassigned hypothetical T3E

```
P
R
I
O
R
I
T    | ##LEVEL 1 : orthoMCL pmatch_cutoff between 100-90%
Y    | ##LEVEL 2 : orthoMCL pmatch_cutoff between 90-80%
     | ##LEVEL 3 : orthoMCL pmatch_cutoff between 80-70%
     | ##LEVEL 4 : orthoMCL pmatch_cutoff between 70-60%
     | ##LEVEL 5 : Best Hit Blastp
     | ##LEVEL 6 : Best Hit HMM
     | ##LEVEL 7 : Best Hit tBlastN
     |
     |
```

Figure 2

RipBM and RipBO are secreted through the T3SS

The wild-type strain and the *hrcV* mutant were transformed to express a $RipBM_{CMR15}$-3HA, $RipBM_{Psi07}$-3HA **(A)** or a RipBO-3HA **(B)** fusion protein. Secretion assays were performed and total proteins from bacterial pellets and proteins in the supernatants were detected by Western-Blot. Uncropped western-blot are displayed on Fig. S2

A

B

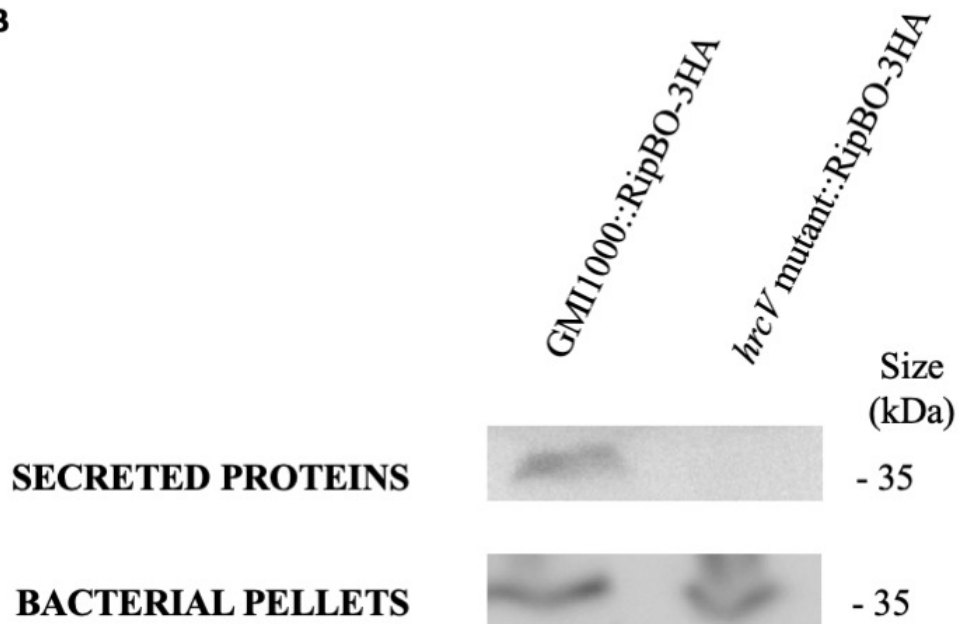



Figure 3

mutS alignment and phylogenetic tree on the set of 84 different strains.

A neighbor-joining tree was build using the *mutS* from *Ralstonia pickettii* as an outgroup.
Bootstrap were performed on 100 replicates, only support higher than 50% displayed in the
consensus tree.

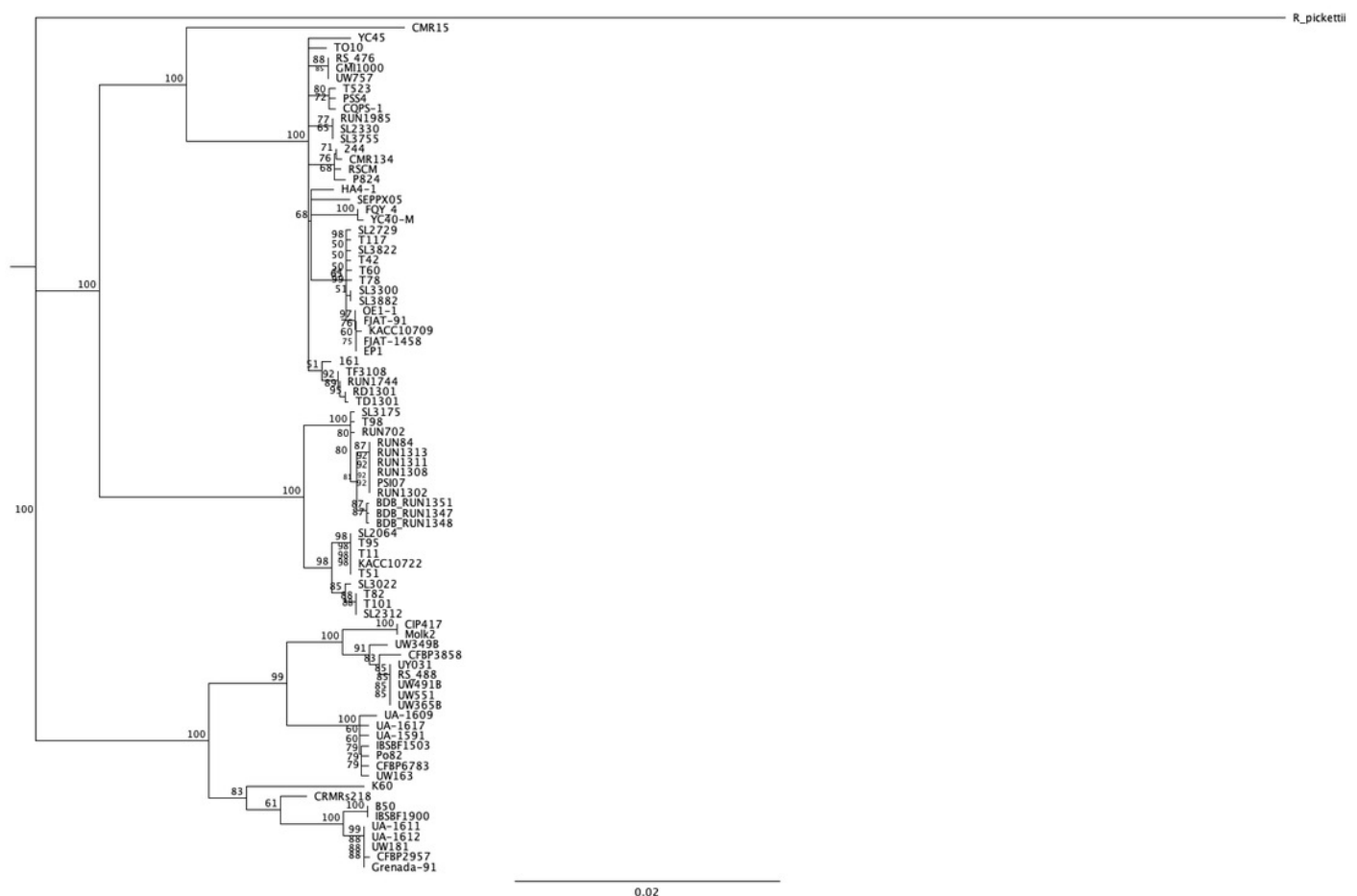



Figure 4

Venn diagram of conserved T3Es among different sets of "host-of-isolation" defined strains

(A) comparisons of conserved T3Es among "TOM" (host of isolation : tomato), "EGG" (Host of isolation: eggplant) and "POT" (host of isolation : potato). **(B)**Comparison between "TOM", "POT" and "BAN" (host of isolation: banana). The lists of compared T3Es are visible in Table 2.

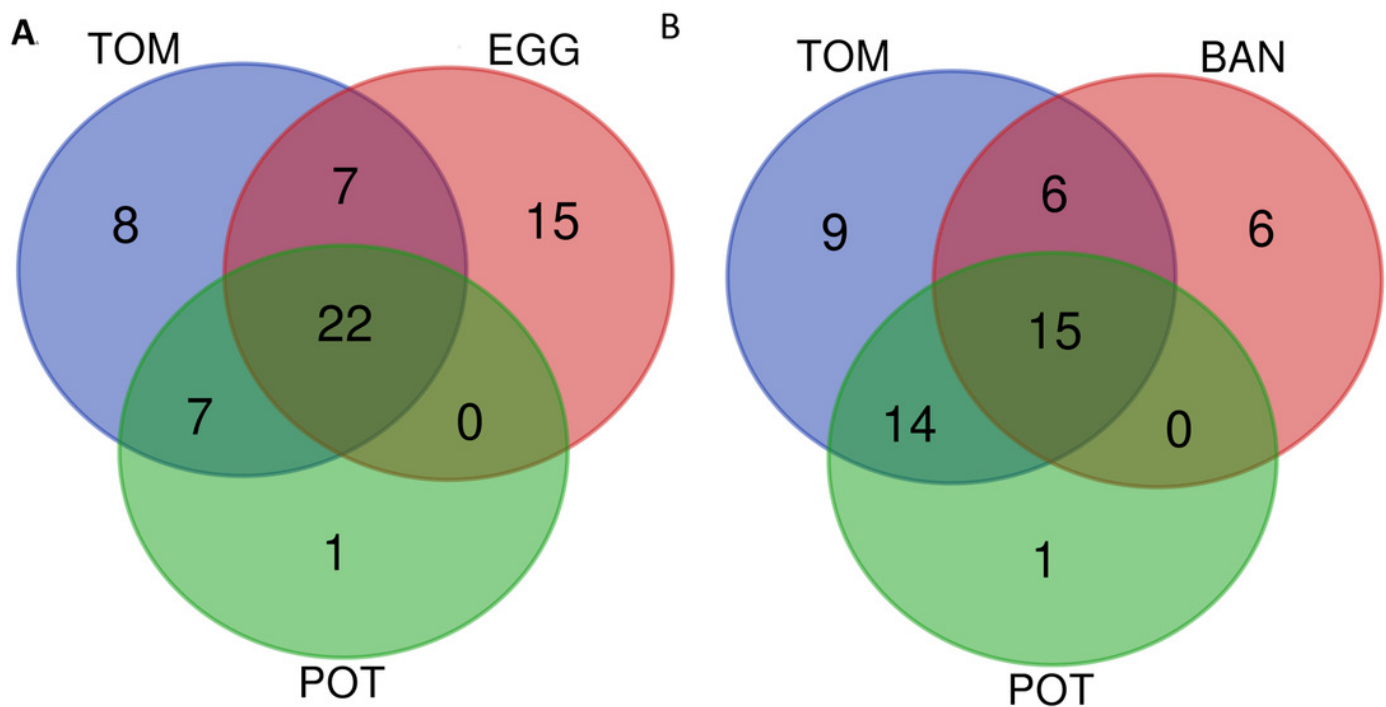



Figure 5

Venn diagram of conserved T3Es among the different phylogenetic clades of strains.

Comparison of conserved T3Es between *R. pseudosolanacearum*(phylotypes I and III), *R. solanacearum* (phylotypes IIA and IIB), and *R. syzygii* (Phylotype IV) strains. The lists of compared T3Es are visible in Table 3.