Resistify - A rapid and accurate annotation tool to identify NLRs and study their genomic organisation

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Abstract

Background

Nucleotide-binding domain Leucine-rich Repeat (NLR) proteins are a key component of the plant innate immune system. In plant genomes NLRs exhibit considerable presence/absence variation and sequence diversity. Recent advances in sequencing technologies have made the generation of high-quality novel plant genome assemblies considerably more straightforward. Accurately identifying NLRs from these genomes is a prerequisite for improving our understanding of NLRs and identifying potential novel sources of disease resistance.

Results

Whilst several tools have been developed to predict NLRs, they are hampered by low accuracy, speed, and availability. Here, the NLR annotation tool Resistify is presented. Resistify is an easy-to-use, rapid, and accurate tool to identify and classify NLRs from protein sequences. Applying Resistify to the RefPlantNLR database demonstrates that it can correctly identify NLRs from a diverse range of species.

Applying Resistify in combination with tools to identify transposable elements to a panel of Solanaceae genomes reveals a previously undescribed association between NLRs and Helitron transposable elements.

Conclusion

Resistify can rapidly identify NLRs within plant genomes and provides accurate structural classifications. Its ease of use and accessibility allows easy integration into bioinformatic workflows and projects, enhancing the study of this important group of genes. Applying Resistify to a *Solanaceae* pangenome reveals an undescribed association between NLRs and transposable elements.

Availability: https://github.com/SwiftSeal/resistify

Keywords

NLRs; Plant disease resistance; transposable elements; Helitrons; Solanum

41 ## Background

42 The plant innate immune system is an essential part of plant disease resistance. A key
43 component of this are the Nucleotide-binding domain Leucine-rich Repeat (NLR) proteins –
44 an abundant and diverse family present in plant and animal genomes. NLRs act as
45 intracellular immune receptors which elicit an immune response after the detection of
46 pathogen derived factors or their activity (Biezen and Jones, 1998). Since their discovery,
47 hundreds of functional NLRs have been cloned and many sequences have been identified
48 (Kourelis and van der Hoorn, 2018). The abundance of NLRs and their sequence diversity
49 shows considerable variation across the plant kingdom (Barragan and Weigel, 2021).
50
51 One driving force of NLR diversification is thought to be the proliferation of transposable
52 elements (TEs). In *Arabidopsis*, sites of high TE insertion frequency have been previously
53 identified to contain NLRs (Quadrana *et al.*, 2016), and NLRs exhibiting elevated
54 intraspecific diversity are in close proximity to TEs (Sutherland *et al.*, 2023). Of the TEs, LTR-
55 retrotransposons are the most prolific in plant genomes and are a major factor in genome
56 expansion (Lee and Kim, 2014). Strikingly, the *Capsicum annuum* genome exhibits a marked
57 expansion of NLRs nested within LTR-retrotransposons, and this effect is seen in other
58 *Solanaceae* including tomato and potato (Seong *et al.*, 2020). Other transposable elements,
59 such as the Helitrons, can also capture and transport genes across the plant genome (Lai *et*
60 *al.*, 2005).
61
62 Canonical NLRs have a modular organisation. Central to the NLR structure is the highly
63 conserved NB-ARC domain which regulates the activity of the protein. Downstream of this is

64 the Leucine-Rich-Repeat (LRR) domain which has a role in ligand binding and auto-inhibition.
65 Upstream of the NB-ARC domain is variable. Often, there is either a coiled-coil (CC),
66 Resistance to Powdery Mildew 8-like (RPW8), or Toll/Interleukin-1 receptor/Resistance
67 protein (TIR) domain. Together, this modular structure means that NLRs can be separated
68 into different classifications: CNL, RNL, TNL, or NL if lacking a conserved N-terminal domain.
69
70 Recently, additional NLR associated signatures have been identified. For example, the post-
71 LRR C-terminal jelly-roll/Ig-like domain (C-JID) (Martin *et al.*, 2020) may be present in TNLs
72 and appears to bind with pathogen effectors, thereby contributing to the immune response.
73 Similarly, other NLRs have integrated decoys to detect pathogen activities. In terms of
74 initiating cell death through the formation of a resistosome, CNLs rely on an N-terminal
75 MADA motif, a hallmark of NRCs - NLRs which act as intermediaries in the NLR induced
76 immune response or NLRs that function independently of NRCs (Adachi *et al.*, 2019).
77
78 Due to their conservation and modular structure, NLRs lend themselves to automated
79 identification and classification. To date, several tools have been developed to achieve this
80 including DRAGO2, NLGenomeSweeper, NLR-Annotator, RGAugury, RRGPredictor, and
81 NLRtracker (table 1) (Li *et al.*, 2016; Osuna-Cruz *et al.*, 2018; Santana Silva and Micheli,
82 2020; Steuernagel *et al.*, 2020; Toda *et al.*, 2020; Kourelis *et al.*, 2021). The development
83 of the RefPlantNLR database has allowed the benchmarking of these tools against the
84 sequences of functionally characterised NLRs (Kourelis *et al.*, 2021). Performance varied
85 between tools with NLRtracker being the most sensitive and accurate. All tools perform well
86 at the identification of TIR, RPW8, and NB-ARC domains which are highly conserved, but are
87 often less accurate in predicting CC domains which are more variable and frequently missed

88 by InterProScan (Jones *et al.*, 2014). Additional to the currently available NLR classification
89 tools is NLRexpress – a set of machine learning predictors for CC, TIR, NB-ARC, and LRR
90 motifs (Martin *et al.*, 2022). Although NLRexpress does not directly identify or classify NLRs,
91 it is well suited for rapidly and accurately screening large sets of sequences for NLR-
92 associated motifs. One strength of NLRexpress is the CC-associated extended EDVID model
93 which performs strongly in identifying this challenging motif.
94
95 Table 1: A summary of the currently available NLR annotation tools. Adapted from (Kourelis
96 et al., 2021).

Tool	**Input data**	**Method**	**Output**	**Distribution**
DRAGO2	Protein, Transcript	Custom HMMs	Classification, Domains	Online only, API available
NLGenomeSweeper	Transcript, Genomic	InterProScan, MUSCLE, TransDecoder, BLAST, HMMER	Classification, Genome position, GFF annotation	GitHub, Manual dependency installation
NLR-Annotator	Transcript, Genomic	MEME motifs	Classification, Genome position, GFF annotation	GitHub
RGAugury	Transcript, Genomic	InterProScan	Classification,	GitHub,

			Genome position, GFF annotation	Online or local webservice, Docker container
RRGPredictor	Protein, Transcript	InterProScan	Classification	GitHub, Manual dependency installation
NLRtracker	Protein, Transcript	InterProScan, HMMER, MEME motifs	Classification, NB-ARC sequence, Domains, GFF annotation	GitHub, Manual dependency installation
NLRexpress	Protein	HMMER, Scikit-learn	Motif position, Motif sequence	GitHub, Online or local, Conda environment provided

One drawback with the currently available tools is the reliance on InterProScan as the backend for domain annotation. InterProScan is designed as a comprehensive and generalised domain annotation tool. As a result, each input sequence is scanned against several databases each containing in total more than 180,000 protein signatures, the vast majority of which are not NLR associated. Compounding this, NLRs only represent a fraction

103 of a plant proteome resulting in unnecessary searches against non-NLR sequences. In
104 addition, domains common to NLRs are present in InterProScan databases with differing
105 levels of curation which often results in overlapping or fragmented annotations. These must
106 be parsed, particularly the LRR domain which is represented by multiple InterProScan
107 signatures. The large databases that InterProScan relies upon must also be downloaded
108 which provides an additional challenge for installation or automated deployment.
109
110 Software that is readily available with minimum manual configuration is a requirement for
111 genome assembly and annotation projects. Projects may use an array of different tools,
112 making manual installation and dependency resolution time-consuming. Dependency
113 resolution solutions such as Conda, and automated deployment of tools in pipelines with
114 Snakemake or Nextflow are becoming increasingly common (Di Tommaso *et al.*, 2017;
115 Mölder *et al.*, 2021). Additionally, bioinformatics research is often restricted to shared High-
116 Performance Clusters (HPCs) where users have limited privileges and where storage is at a
117 premium. Tools that require root privileges, are distributed as web services, or require large
118 databases are often not conducive for genomics projects.
119
120 Here, the new NLR annotation tool Resistify is presented that overcomes some of the
121 constraints of currently available tools. We show that this tool accurately predicts NLR
122 sequences from diverse plant using a range of well curated sources and can be used for
123 pangenome analysis of NLRs in *Solanaceous* genomes. In addition, we use Resistify in
124 combination with the EDTA tool to investigate the genomic organisation of NLRs and their
125 association with transposable elements.
126

Methods

Resistify is implemented in python as a command line executable. First, Resistify executes a hmmsearch of input protein sequences against a custom database of HMMs derived from curated Pfam entries (table 2). These models are used to identify CC, RPW8, TIR, and NB-ARC domains, as well as C-JID and MADA motifs. Domains of the same type are merged if they overlap or are within 100 amino acids of each other. Preliminary testing in development showed that this is necessary to overcome NB-ARC domain annotations which can become split. MADA motifs are restricted to the N-terminal of the sequence. Using this evidence, proteins are initially classified as belonging to either a CN, RN, TN, or N classification. Proteins which do not have any evidence of an NB-ARC domain are discarded at this stage.

Table 2: HMM models including in the initial hmmsearch stage of Resistify.

Domain	Source
NB-ARC	https://www.ebi.ac.uk/interpro/entry/pfam/PF00931/
TIR	https://www.ebi.ac.uk/interpro/entry/pfam/PF01582/
TIR	https://www.ebi.ac.uk/interpro/entry/pfam/PF13676/
RPW8	https://www.ebi.ac.uk/interpro/entry/pfam/PF05659/
CC	https://www.ebi.ac.uk/interpro/entry/pfam/PF18052/
C-JID	https://www.ebi.ac.uk/interpro/entry/pfam/PF20160/
MADA	https://cdn.elifesciences.org/articles/49956/elife-49956-supp2-v2.hmm

Following this, the filtered set of NB-ARC containing proteins is screened for NLR-associated motifs with the NLRexpress models. Sequences are then reclassified using the following

logic. If a protein belongs to class N (i.e., does not have any evidence of an upstream CC, RPW8, or TIR domain) it is scanned for upstream TIR or CC motifs. As motif searches can be more promiscuous, restricting motif searches to this condition prevents them from interfering with non-ambiguous NLR classification. The sequence is then screened for LxLxxL motifs to define the LRR domain. Following a previous definition, an LRR domain is annotated if four or more LxLxxL motifs are identified with inter-motif gaps of less than 75 amino acids [@martin_nlrscape_2023]. Gaps larger than 75 amino acids are predicted to be a break in the LRR domain and so the LRR annotation process is restarted from that position onwards. If less than four motifs exist across the whole sequence this process is skipped.

This combined evidence is then integrated into the domain annotation data. The domains are sorted by start position and a “domain string” is formed. For example, if the sorted domains took the order TIR, NB-ARC, LRR, C-JID, then the domain string would be TNLj. Alternatively, a canonical CNL with a MADA motif would take the form mCNL. The domain strings are searched for substrings CNL, RNL, TNL, or NL and classified accordingly. Sequences are further classified on the basis of whether they contain an N-terminal bound MADA motif or CJID domain. Additionally, a count of the unique occurrences of the conserved NB-ARC motifs is made as an indicator of NB-ARC integrity.

The primary output of Resistify is a table detailing the NLRs identified and the specific classification for each sequence. The complete domain string, classification, NB-ARC motif count, and MADA and CJID status are listed. A complete list of all annotations and NLRexpress motifs are given as separate tables. Additionally, all NB-ARC domain sequences are extracted and provided as a FASTA file.

166

167 Resistify is provided as a single executable available for download on GitHub, PyPi, and
168 Conda. All databases and models are distributed with the executable so that manual setup is
169 not required. All output files are placed in a nameable output directory for easy integration
170 into automated workflows. Temporary files are also handled internally to reduce output
171 clutter. Resistify requires scikit-learn v0.24.2 and hmmer v>=3.0.

172

173 RefPlantNLR benchmarking

174 Protein sequences of the RefPlantNLR database members were retrieved and used as input
175 for Resistify with default settings (Kourelis *et al.*, 2021). Resistify classifications were
176 compared directly in R v4.3.2 with tidyverse v2.0.0. Any sequence where the Resistify
177 classification did not exactly match with the provided RefPlantNLR structure was taken as a
178 potential misclassification.

179

180 Araport11 benchmarking

181 The latest release of Araport11 representative gene model protein sequences were
182 downloaded from TAIR and used as input for Resistify with default settings(Lamesch *et al.*,
183 2012). A phylogenetic tree was built from the Resistify-extracted NB-ARC domain sequences
184 with mafft v7.52.0 and fasttree v2.1.11 with default settings.

185

186 ## Pangenome pipeline

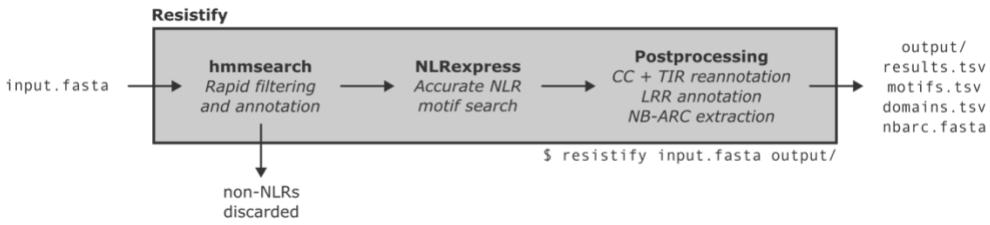
187 A Snakemake workflow was developed to predict genes and NLRs in a Solanum pangenome
188 comprised of chromosome-scale genomes. Snakemake was executed in a mamba v1.4.2
189 environment with snakemake v7.32.3 and cookiecutter v1.7.3 (Mölder *et al.*, 2021). Genes
190 were predicted *de-novo* from the genome alone using Helixer v0.3.2 with the
191 land_plant_v0.3_a_0080.h5 model (Holst *et al.*, 2023). Protein sequences were extracted
192 using AGAT v1.2.0 and used as input for Resistify (Dainat *et al.*, 2023). Ortholog analysis was
193 carried out using OrthoFinder v2.5.5 using all predicted protein sequences (Emms and Kelly,
194 2019). Transposable elements were annotated with the latest GitHub release of EDTA (Ou *et*
195 *al.*, 2019).
196
197 Gene models were translated to bed format using AGAT v1.2.0 and overlaps with intact
198 transposable elements were identified using bedtools v2.31.1 with the command bedtools
199 intersect using the options -f 0.9 -wo (Quinlan and Hall, 2010). To identify previously
200 characterised NLRs, protein sequences of a subset of *Solanum*-originating NLRs in the
201 RefPlantNLR database were queried against each genome with blastp v2.15.0 (Camacho *et*
202 *al.*, 2009). The full pipeline and all post-hoc analysis are available at
203 https://github.com/SwiftSeal/pangenomics/.
204

205 # Results

206

207 ## Performance against RefPlantNLR

208 To evaluate the performance of Resistify (figure 1), it was applied against the RefPlantNLR
209 database - a curated set of 415 previously cloned NLRs from a diverse range of species.
210 NLRtracker was also applied to the RefPlantNLR database for comparison.
211



212

213 Figure 1: The Resistify program.
214

215 In the default mode, only three RefPlantNLR entries were not identified by Resistify -
216 *AtNRG1.3* which carries only an LRR domain and *Pb1* and *RXL* which have significantly
217 truncated NB-ARC domain that are not listed in the RefPlantNLR database. However, if
218 required, these genes can be identified with Resistify using –ultra mode which skips the
219 initial filtering stage and reports and sequence which contains at least one NLRexpress motif.
220 Sequences are reported as an unmerged string of NLR motifs. Consequently, *AtNRG1.3*, *Pb1*,
221 and *RXL* are reported as NNLLLLLLL, CNNNLLLLLLLLLLL, and CNNNLLLLLLLLL respectively.
222

223 The largest source of variation between RefPlantNLR and Resistify classification was 29 NLRs
224 which had an NL structure according to RefPlantNLR but CNL according to Resistify. All of
225 these belonged to CNL-associated subclasses which are known to have CC domains that are
226 challenging to identify. This included 15 CC_{G10}-NLRs including *Pvr4*, *Tsw*, *RPS2*, *RPS5*, *SUT1*
227 and *SUMM2* which have previously been noted for their lack of the CC-associated EDVID

228 motif (Lee *et al.*, 2021). Others included six members of the *Pm5* locus which despite not
229 containing a CC domain in RefPlantNLR, have previously been identified to contain CC-like
230 domains (Xie *et al.*, 2020). This analysis demonstrates that Resistify is highly sensitive at
231 retrieving canonical NLRs and accurately describing their structure.
232
233 As NLRtracker does not exclude NLRs without NB-ARC domains, *AtNRG1.3*, *Pb1* and *RXL*
234 were identified and classified as "CC-NLR or CCR-NLR or CCG10-NLR", "CC-NLR", and "CC-
235 NLR" respectively. NLRtracker relies on the NB-ARC associated RNBS-D motif to classify NLRs
236 as "CC-NLR or CCR-NLR or CCG10-NLR". As a result, 26 of the conflicting CNLs identified by
237 Resistify were classified as "CC-NLR or CCR-NLR or CCG10-NLR" despite failing to identify a
238 CC domain. The exception of this was for *SpNBS-LRR*, *Rpp1-R1*, and *Rpp4C4* which were
239 classified as "UNDETERMINED".
240 Reliance on RNBS-D also led to classification of *NtTPN1* as "CC-NLR or CCR-NLR or CCG10-
241 NLR". Whilst *NtTPN1* is a CNL ortholog, it lacks any upstream domain and is structurally an
242 NL. Unexpectedly, the TNL *DSC1* was misclassified as "CC-NLR" despite having a domain
243 structure of "(TIR)(NBARC)(LRR)(CJID)" according to NLRtracker.
244
245 In summary, Resistify performs well at identifying canonical NLRs from a diverse range of
246 species. In default mode, it does not assign genes as NLRs with extremely truncated or
247 entirely absent NB-ARC domains, unlike NLRtracker which reports any sequence with NLR-
248 associated domains. However, this can be replicated in Resistify with the –ultra mode. Thus,
249 Resistify's structure-based classification method is well suited for correctly classifying NLRs,
250 including members of challenging CNL subclasses.
251

Performance against the Araport11 proteome

To assess the performance of Resistify across a well characterised and annotated genome, the representative gene models of Araport11 were analysed (Cheng *et al.*, 2017). In total, Resistify identified 166 NLRs - the majority of which were TNLs and CNLs (figure 2a). Of the CNLs, 25% had a MADA motif, and 44.4% and 41.2% of NLs and TNLs had C-JID domain respectively. Partial NLRs either without an N-terminal or LRR domain were also identified.

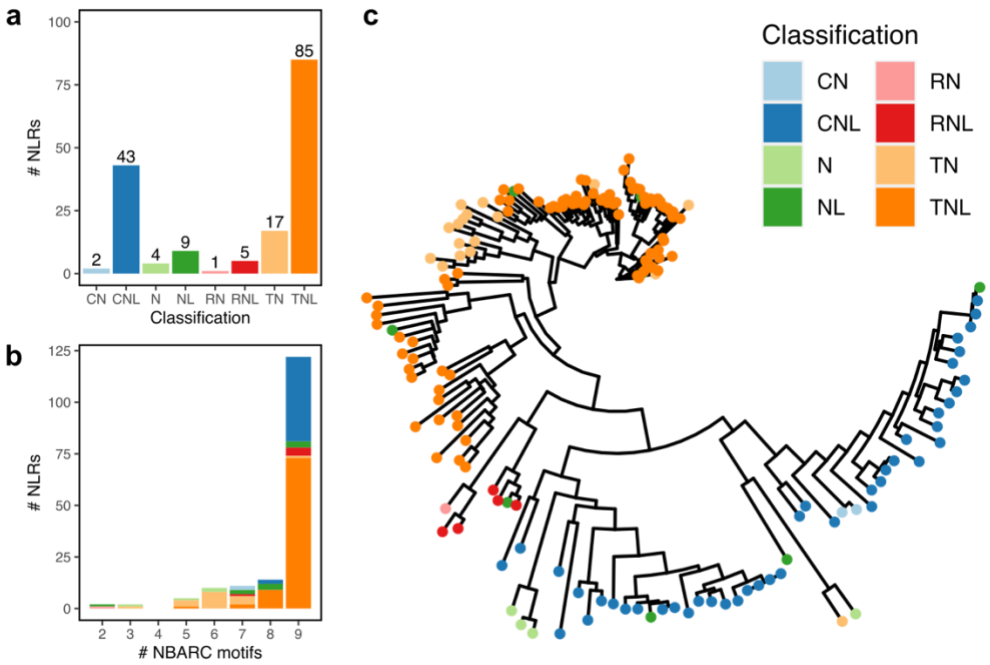


Figure 2: Resistify applied to Araport11.

a) The number of NLRs belonging to each identified classification. b) The number of NLRs grouped by number of identified unique NB-ARC associated motifs. c) A phylogenetic tree of NLRs based on the Resistify extracted NB-ARC domain sequences.

266 Manual inspection of the motifs within these sequences confirmed that they were not due
267 to a failure to identify these elements. The majority of NLRs carried all nine conserved NB-
268 ARC motifs - those with fewer increasingly belonged to partial NLR classifications (figure 2b).
269 NLRs with as few as two of the conserved NB-ARC motifs were successfully identified. A
270 phylogenetic tree of the Resistify-identified NB-ARC domains independently validated the
271 classifications and allowed the placement of ambiguous NL or N classified sequences into
272 subclasses (figure 2c).
273
274 NLRtracker identified an additional 48 sequences, however these sequences did not contain
275 any NB-ARC domain annotation according to either tool. Three sequences - AT4G19060.1,
276 AT4G19060.1, and AT5G45440.1 - were not identified by NLRtracker. According to Resistify
277 these contained a single NB-ARC domain each, with 5, 6, and 6 NB-ARC motifs respectively.
278 Overall, Resistify performs well at identifying and classifying NLRs from whole proteomes
279 and successfully retrieves NB-ARC domain annotations for phylogenetic analyses.
280

281 Application against an example workflow

282 To demonstrate how Resistify might be implemented to identify novel resistance genes, a
283 pangenome experiment was performed. Eighteen contiguous *Solanum* genomes were
284 downloaded and processed with a simple workflow which predicts genes de novo, identifies
285 orthologues, and classifies NLRs with Resistify (Tang *et al.*, 2022; Li *et al.*, 2023). For gene
286 annotations, the recently developed tool Helixer was selected for its near reference quality
287 predictions and lack of requirement for repeat-masking which is known to result in false-
288 negative NLR annotations (Bayer, Edwards and Batley, 2018; Holst *et al.*, 2023). The

289 workflow also predicts transposable elements with EDTA. The *C. annuum* genome was
290 included as an outgroup, but also because recent analysis has suggested a substantial
291 expansion of NLRs in this species associated with LTR transposable elements (Kim *et al.*,
292 2017).
293
294 Predicted gene content varied between genomes from 29,223 in *S. habrochaites* to 61,015 in
295 the larger *C. annuum* genome (table S1). Transposable element content ranged from 55.6%
296 in *S. chmielewskii* to 76.8% in *C. annuum*. Whilst there was no significant difference in total
297 TE content between tuber-bearing and non-tuber-bearing genomes ($p = 0.644$), there was a
298 significant increase in the number of intact TEs reported by EDTA in the genomes of tuber-
299 bearing species ($p = 0.008$).
300
301 In total, 8144 NLRs were identified across all genomes (figure 3). CNLs were the most
302 abundant classification of NLR identified, ranging from 84 in *S. habrochaites* to 422 in *S.*
303 *tuberosum* (group tuberosum RH10-15). There was a notable expansion of NLRs in tuber-
304 bearing *Solanum* species. This is in agreement with the previous observation that potato-
305 bearing *Solanum* species have an expansion of tuber expressed NLRs (Tang *et al.*, 2022).
306 This effect does not correlate with the proportion of genome occupied by transposable
307 elements of any classification (figure S1).
308

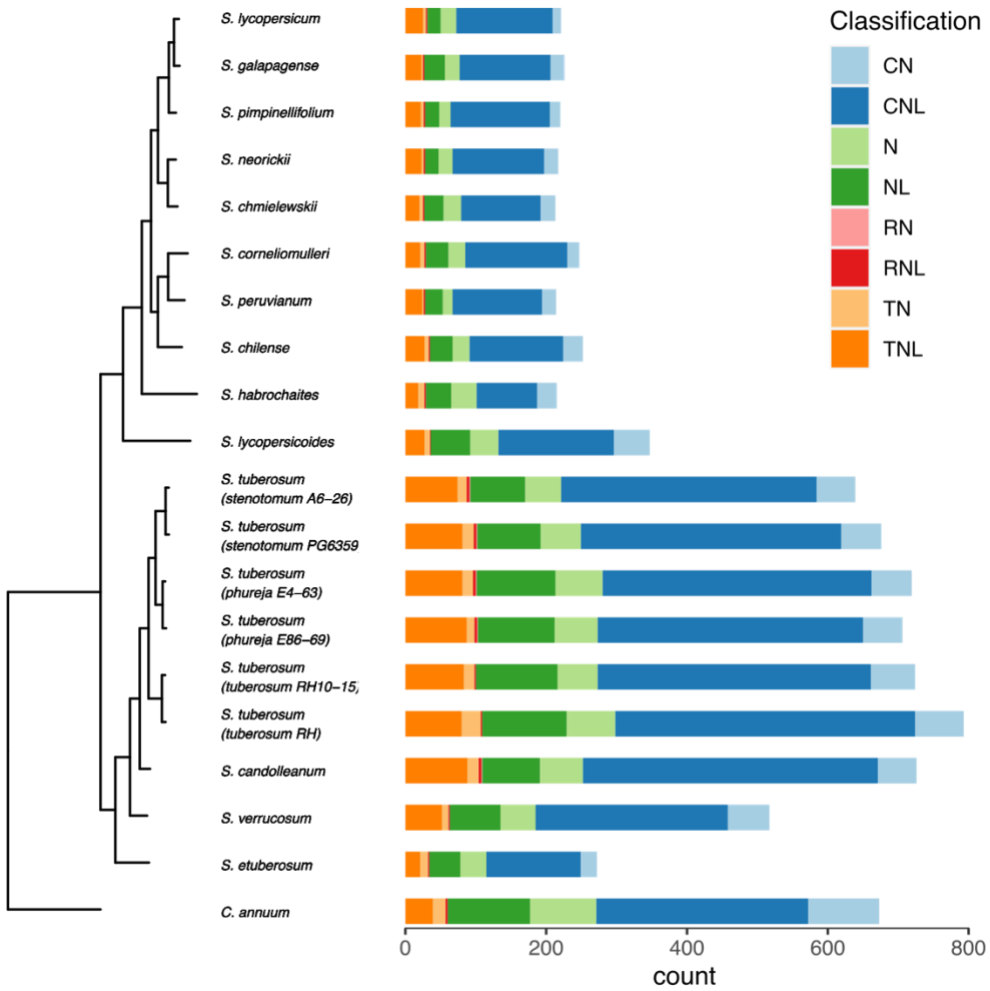


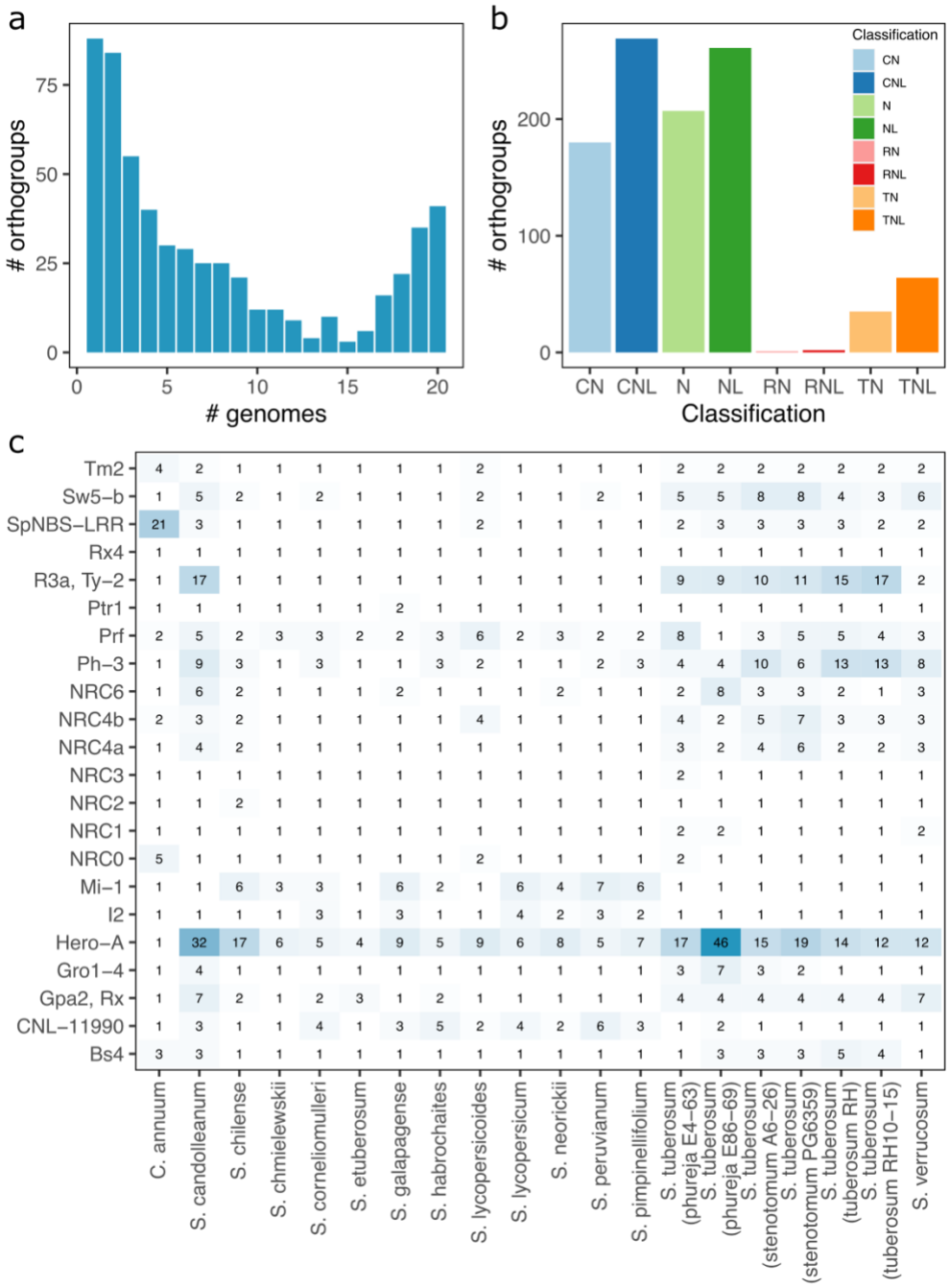
Figure 3: NLRs identified across the *Solanum* pangenome.

In total, 38,590 orthogroups were identified, of which 687 (1.8%) contained NLRs. The distribution of orthogroups showed a clear divide between core and shell/cloud orthogroups within the pangenome with the majority of NLR orthogroups existing within the shell/cloud (fig 4a). This reflects the relatively large width of the pangenome, which captures NLR variation over a Genus level. Species specific NLRomes also exhibit an abundance of cloud orthographs, reflecting the high variability of NLRs in genomes (Van de Weyer *et al.*, 2019). Many (48.1%, n = 371) orthogroups were classified as containing N or NL NLRs (figure 4b).

319 Whilst these classifications are less abundant (24.6%, n = 2081), they were often associated

320 with smaller orthogroups contributing to this inflation.

321

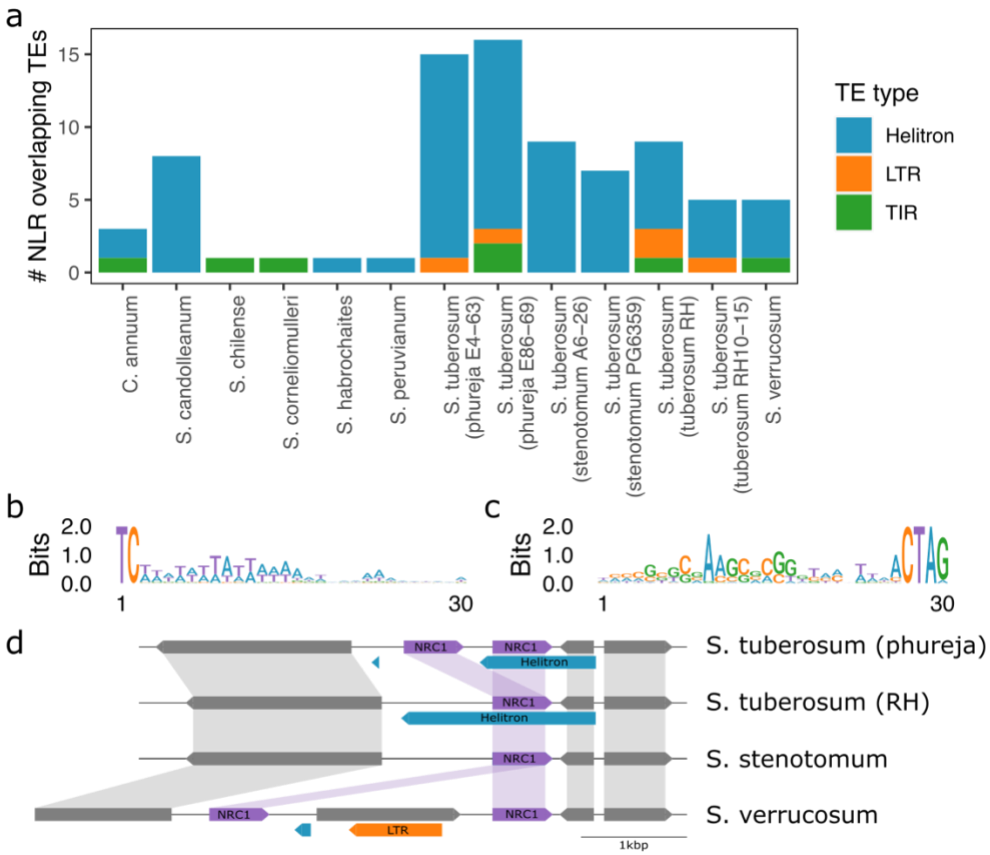


322

323 Figure 4: NLR-containing orthogroups.

324 a) The number of orthogroups shared between genomes. b) The number of orthogroups
325 according to NLR classification. c) The number of orthologs of known *Solanaceae* NLRs
326 identified in each genome.
327
328 The distribution of previously identified *Solanaceae* NLR orthologs across the pangenome
329 was examined (figure 4c). In agreement with previous findings, members of the NRC group
330 remained relatively stable across the pangenome except for NRC4a, NRC4b, and NRC6 which
331 were expanded in the tuber-bearing genomes. By contrast, an expansion of *Mi-1* and *Hero*
332 was evident in the non-tuber bearing and tuber bearing genomes respectively. At least one
333 ortholog was identified in each genome for all genomes.
334
335 It has previously been reported that there is a vast expansion of NLRs in the *C. annum*
336 genome due to retroduplication; NLRs nested within LTRs represented a large proportion
337 (~13%) of NLRs within the genome, and this effect is also seen in tomato (8%) and potato
338 (18%) (Kim *et al.*, 2017). To explore whether this effect could be linked to the expansion of
339 NLRs within the tuberising members of *Solanum*, a similar analysis was repeated. Intact TEs
340 were identified and considered to interact with NLRs if they covered >90% of the gene
341 annotation.
342
343 Unexpectedly, the effect could not be replicated and across all genomes only five intact LTRs
344 were identified as containing NLRs (figure 5a). None were identified in *C. annuum* but
345 instead in the *S. tuberosum* group Phureja and clone RH. The putative retrotransposed NLRs
346 within these all belonged to the same orthogroup (OG0000639) which was expanded in both
347 Phureja and RH, but not *S. stenotomum*.

348



349

350 Figure 5: Helitrons associated with NLRs.

351 a) The number of intact TEs overlapping with NLRs across the pangenome. b) The 5' motif of

352 NLR overlapping Helitrons. c) The 3' motif of NLR overlapping Helitrons. d) The *NRC1* locus

353 and its association with Helitrons across four genomes. Helitron (blue) and LTR (orange)

354 transposable elements are highlighted.

355

356 By contrast, a proliferation of Helitron-associated NLRs was identified in the tuber-bearing

357 genomes. All predicted Helitrons carried the expected 5’-TC…CTRR-3’ signature as well as a

358 GC-rich region in the 3’ terminal (figure 5b,c). An example of an NLR which may have

359 undergone Helitron-associated duplication is *NRC1* (figure 5d) which is present as a single

360 copy except in both *S. tuberosum* group Phureja genomes and *S. verrucosum*. Close

361 examination of the *NRC1* locus reveals a duplication event in *S. tuberosum* group Phureja
362 where one *NRC1* gene is nested entirely within a predicted Helitron. In *S. tuberosum* clone
363 RH which only has one copy of *NRC1*, *NRC1* is similarly nested within a Helitron although this
364 appears to be extended to a distant Helitron terminator past the site of duplication in group
365 Phureja. In *S. stenotomum*, only one *NRC1* is present and no Helitron predicted.
366 Interestingly, *S. verrucosum* has two copies of *NRC1* and whilst it does not have a nested
367 Helitron copy, the leftmost *NRC1* copy does have a short predicted Helitron upstream of its
368 start position in the same orientation as the other Helitron elements. In this case, the two
369 copies of *NRC1* appear to have been further separated by an intact LTR insertion which has
370 resulted in an additional gene prediction.
371

372 Discussion

373 Resistify is presented as a highly accurate and easy-to-use tool to aid the identification of
374 novel resistance genes. Applying Resistify against the RefPlantNLR database demonstrates
375 that it is effective at identifying NLRs from a diverse range of plant genomes. It is highly
376 sensitive at classifying challenging CNL families which often evade other NLR classification
377 tools due to the inclusion of the performant NLRexpress motif models. Members of the
378 CC_{G10}-NLR clade have previously been described as lacking the CC-associated EDVID motif
379 and are often predicted as having an NL structure in the RefPlantNLR database (Lee *et al.*,
380 2021). By utilising NLRexpress' highly sensitive extended EDVID motif model, Resistify
381 successfully classifies these elusive NLR clades, highlighting it as the most sensitive NLR
382 classifier to date.
383

384 Resistify can be easily integrated into workflows and is scalable to large pangenome
385 experiments. As high-quality genome assemblies become more common, providing
386 accessible tools for the study of NLRs will be crucial for fully appreciating their diversity and
387 identifying novel sources of resistance. The recent releases of chromosome level assemblies
388 for potato and tomato pangenomes are a valuable resource for understanding the diversity
389 of NLRs within *Solanum* (Tang *et al.*, 2022; Li *et al.*, 2023). An expansion of NLRs including
390 essential families such as the NRCs is apparent in tuber-bearing species.
391
392 As Resistify relies on an initial NB-ARC domain search to reduce the search space prior to
393 NLRexpress motif identification, NLRs with very truncated or entirely absent NB-ARC
394 domains can be missed. To resolve this, an additional mode is provided which does not
395 perform initial NB-ARC domain filtering which identifies sequences with any NLR motifs.
396 Unlike NLRtracker, Resistify does not search for noncanonical integrated domains (Kroj *et al.*,
397 2016). As integrated domains are widespread and often critical to NLR function, their
398 identification and analysis are an important factor in studies of NLR diversity (Barragan and
399 Weigel, 2021). Pairing Resistify with a secondary sweep for these domains with NLRtracker
400 or InterProScan would permit this analysis whilst also benefiting from a reduced search
401 space and high-quality NLR classifications from Resistify.
402
403 An unexpected finding of this study was the failure to replicate the previous observation of
404 an abundance of LTR associated NLRs across *Solanaceae* (Kim *et al.*, 2017). Differences in
405 gene and TE annotation and NLR classification likely contributed to this. Although Helixer
406 predicted ~70% more genes in *C. annuum* than were identified in the previous study, this did
407 not translate to an increase in NLRs identified. Instead, only 673 NLRs were identified here in

408 comparison to the previous estimate of 835. Previously, a tBLASTn search of the NB-ARC
409 domain followed by ORF identification and BLASTP searches against GenBank to classify
410 NLRs was used to identify NLRs in the genome (Seo *et al.*, 2016). This method may result in
411 more false positives in comparison to identifying NLRs directly from gene annotations. Here,
412 EDTA was selected for identifying TEs which has an improved sensitivity and selectivity for
413 identifying intact LTRs in comparison to LTRHarvest which was used previously. Although
414 Helixer does not require repeat masking which can result in false negative NLR annotations,
415 annotations used in training the model (which may have relied upon repeat masking) might
416 introduce TE-avoidant behaviour (Bayer, Edwards and Batley, 2018). However, the
417 identification of several LTR-associated NLRs indicates that this is unlikely to be the source of
418 the difference in the result.
419
420 The identification of Helitron-associated NLR expansion has not been previously reported.
421 Helitrons are challenging to identify due to their lack of structural elements and as a result
422 the pipeline used in this study is known to overestimate Helitron density (Baril, Imrie and
423 Hayward, 2022). However, a large part of this overestimation is likely due to EDTA
424 reannotating the genome with HelitronScanner predictions, which itself suffers from a high
425 false positive rate, leading to a proliferation of fragmented Helitron annotations across the
426 genome. For this study, only intact Helitrons which passed EDTAs stringent filter were
427 considered. As a result, all predicted Helitrons had the required structural elements for
428 activity. Further validation would be required to determine if Helitron association is a valid
429 mechanism of NLR expansion. The Helitron/*NRC1* relationship highlighted here would be a
430 good starting point for unpicking this mechanism.

Conclusions

Resistify is presented as a new tool to aide in the study of NLRs in plant genomes. It provides high-quality structural classifications for canonical NLRs. Applying Resistify to the RefPlantNLR database demonstrates that it can correctly classify NLRs from a diverse range of plant species. Applying Resistify to a panel of *Solanaceae* genomes demonstrates its suitability for studying NLRs in large datasets. This analysis revealed functionally important NLR families undergoing expansion in tuberising *Solanum* species. Unexpectedly, combining this with transposable element annotations did not identify a previous description of NLRs embedded within LTR retrotransposons in *Solanaceae* genomes. Instead, an undescribed association between NLRs and Helitron transposable elements in genomes with expanded NLR inventories was revealed.

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Availability of data and materials

Resistify is available at https://github.com/SwiftSeal/resistify. All data analysis workflows and scripts are available at https://github.com/SwiftSeal/pangenomics. The RefPlantNLR database was accessed from its source publication (Kourelis *et al.*, 2021). The genomes used in this study were accessed from their source publications (Tang *et al.*, 2022; Li *et al.*, 2023).

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