EffectorO: motif-independent prediction of effectors in oomycete genomes using machine learning and lineage-specificity

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Abstract

Oomycete plant pathogens cause a wide variety of diseases, including late blight of potato, sudden oak death, and downy mildews of plants. These pathogens are major contributors to loss in numerous food crops. Oomycetes secrete effector proteins to manipulate their hosts to the advantage of the pathogen. Plants have evolved to recognize effectors, resulting in an evolutionary cycle of defense and counter-defense in plant–microbe interactions. This selective pressure results in highly diverse effector sequences that can be difficult to computationally identify using only sequence similarity. We developed a novel effector prediction tool, EffectorO, that uses two complementary approaches to predict effectors in oomycete pathogen genomes: (1) a machine learning-based pipeline that predicts effector probability based on the biochemical properties of the N-terminal amino

acid sequence of a protein and (2) a pipeline based on lineage-specificity to find proteins that are unique to one species or genus, a sign of evolutionary divergence due to adaptation to the host. We tested EffectorO on *Bremia lactucae*, which causes lettuce downy mildew, and *Phytophthora infestans*, which causes late blight of potato and tomato, and predicted many novel effector candidates, while recovering the majority of known effector candidates. EffectorO will be useful for discovering novel families of oomycete effectors without relying on sequence similarity to known effectors.

Introduction

Oomycetes are filamentous eukaryotic organisms that resemble fungi but are more closely related to brown algae (Baldauf 2003). Pathogenic oomycetes cause huge losses in agriculture and aquaculture worldwide (Derevnina et al. 2016; Haverkort et al. 2008). Oomycete pathogens and other parasites must continuously evade detection by their hosts in order to successfully infect and complete their life cycle. This is accomplished through the action of effectors, secreted proteins that interfere with host immune signaling and physiology to promote pathogen growth (Wang et al. 2019). To thwart the action of effectors and infection by the pathogen, plants have evolved receptors encoded by resistance genes (R genes) to detect effectors. Upon recognition, the receptors trigger a defense response that eventually results in localized cell death and disease resistance (Wang et al. 2019). Therefore, identification of effectors is important for both understanding how pathogens infect their hosts and for breeding disease resistance.

Most effector genes encode an N-terminal signal peptide that targets the protein for secretion outside of the pathogen and is cleaved from the mature peptide sequence.

47 However, atypically secreted effectors lacking a signal peptide have also been observed in
48 fungi and oomycetes (Liu et al. 2014), and atypical secretion has been documented in
49 protozoans at the host–parasite interface (Balmer and Faso 2021). Through conventional or
50 atypical secretion, all effectors are initially exported from the pathogen into the apoplast,
51 where they either remain (in the case of apoplastic effectors) or are translocated into the
52 plant cell (in the case of cytoplasmic effectors).

53 For some oomycetes, such as *Phytophthora* spp. and downy mildew pathogens,
54 translocation of effectors has been linked to the N-terminal sequence directly following the
55 signal peptide, which often contains one or more of several characteristic amino acid motifs.
56 These include the RXLR and EER motifs (Birch et al. 2009; Rehmany et al. 2005; Whisson et
57 al. 2007) and the LXLFLAK motif, associated with Crinkling and Necrosis (CRN) causing
58 effectors (Amaro et al. 2017). The precise mechanism of translocation is the topic of some
59 controversy (Ellis and Dodds 2011); regardless, these motifs have proven useful for effector
60 prediction in multiple pathogen genomes. Many of these cytoplasmic effectors have been
61 validated by testing the candidate proteins for recognition by resistant genotypes
62 (avirulence activity) or immune suppression (virulence activity) *in planta* (Deb et al. 2018;
63 Fabro et al. 2011; Pecrix et al. 2019; Pel et al. 2009; Stassen et al. 2013; Zheng et al. 2014).

64 While the RXLR motif is clearly a conserved effector motif in *Phytophthora* species,
65 effectors in the related downy mildew pathogens show divergence and diversification of this
66 motif (Bailey et al. 2011; Sharma et al. 2015). Due to the sequence divergence of the RXLR
67 motif in downy mildew species, both string- and Hidden Markov Model (HMM)-based
68 searches recover fewer effector candidates in these pathogens compared to *Phytophthora*
69 species (Baxter et al. 2010; McGowan and Fitzpatrick 2017). Another approach to predict

effectors in downy mildew species (as well as in *Phytophthora*) is to search for the structurally conserved WY domain, which is present in ~25–50% of RXLR effectors in *Phytophthora* and downy mildew pathogens (Bos et al. 2010; Dou et al. 2008; Win et al. 2012) and has been implicated in effector function (He et al. 2019; King et al. 2014). While HMM searches for the WY domain have a low false positive rate for predicting candidate effectors (Wood et al. 2020), they likely have a high false negative rate because not all RXLR effectors have WY domains and, furthermore, there may be *bona fide* effectors with novel N-terminal motifs and uncharacterized effector domains.

Unlike oomycetes, fungi have no widely conserved effector motifs or domains that are present across all species. Degenerate RXLR-like motifs have been found in some fungal pathogens (Gu et al. 2011; Kale et al. 2010) and others have been found to have an N-terminal Y/F/WxC motif (Godfrey et al. 2010). EffectorP, a machine learning based approach to identify effectors in fungal genomes using biochemical characteristics, successfully expanded the pool of candidate effectors in fungal species (Sperschneider et al. 2016, 2018). Machine learning-based effector prediction pipelines, such as EffectorP, work by building effector classification models trained on known effectors and secreted non-effectors from the target organisms. Once a suitable model is determined, the classifier calculates the biochemical features from the amino acid sequence of the protein and outputs a probability that the protein is an effector based on those features. At the initial submission of this paper, machine learning had not been applied to effector prediction in oomycetes. More recently, a new version of EffectorP has been published (EffectorP 3.0), which has the ability to predict oomycete effectors as well as fungal effectors and additionally classifies them as either apoplastic or cytoplasmic (Sperschneider and Dodds 2022). EffectorP 3.0 uses an ensemble

93 of machine learning classifiers trained on fungal and oomycete effectors to predict effectors
94 starting from predicted secreted proteomes (Sperschneider and Dodds 2022). In this paper,
95 we present a complementary machine learning effector prediction pipeline for oomycetes
96 that gives similar accuracy as EffectorP 3.0 and may give higher specificity, especially in
97 downy mildew species. We also present a pipeline to identify lineage-specific proteins that
98 can be combined with the machine learning pipeline to filter for effector candidates.

99 Lineage specificity is often a characteristic of effectors due to the constant
100 evolutionary cycle between pathogen and host. Pathogenicity-related proteins, such as
101 effectors (as well as their corresponding R genes), often show increased rates of sequence
102 divergence (Dodds et al. 2006; Dong et al. 2015). This is especially true for organisms that
103 have narrow host ranges, such as downy mildew pathogens. For example, the genomes of
104 the downy mildew species *Peronospora effusa* and *P. tabacina* are predicted to contain
105 ~1,800 and ~3,000 species-specific genes, respectively (Fletcher et al. 2018). In addition,
106 comparison between two isolates of *P. effusa* revealed >600 isolate-specific genes (Fletcher
107 et al. 2018). These species- and isolate-specific genes may have arisen through co-adaptation
108 to specific hosts or in response to different resistance genes of the same host, respectively.
109 We reasoned that by using lineage-specificity in combination with a machine learning-based
110 effector classifier, we would be able to expand the candidate effector repertoires of oomycete
111 pathogens, especially those with narrow host ranges, as these approaches are able to find
112 candidates that have diverged from effectors with known motifs and domains. For effector
113 prediction, we define "lineage-specific" broadly to mean a sequence that is specific to one
114 phylogenetic lineage: either at the genus, species, or isolate level. For the oomycetes studied
115 in this paper, we classified proteins as lineage-specific at the species level if there were no

116 close orthologs in any of the other sequenced oomycete genomes. The classification of a
117 protein as lineage-specific depends greatly on what genomic data is available for other
118 species, and thus this concept is a more of a heuristic tool for narrowing down lists of effector
119 candidates by removing conserved proteins, rather than a biologically-relevant
120 characteristic of any given protein.

121 Here we present EffectorO: a novel pipeline to predict effectors in oomycete genomes
122 that avoids the limitations of motif/domain-based searches. EffectorO consists of a machine
123 learning based effector classifier trained on a diverse set of oomycete effectors (EffectorO-
124 ML) combined with a lineage-specific protein sequence identification pipeline (EffectorO-
125 LSP). We used two economically important oomycete pathogens as model organisms to
126 evaluate EffectorO: *Bremia lactucae*, an obligate biotrophic downy mildew pathogen with
127 complete host-specificity to *Lactuca* spp., and *Phytophthora infestans*, which causes late
128 blight of potato and tomato. EffectorO had high accuracy for classifying protein sequences as
129 effectors, predicted thousands of novel effectors in these two oomycete genomes, and
130 recovered known effectors. Effector prediction was then expanded to the genomes of an
131 additional 26 oomycete species. One candidate effector from EffectorO was validated by
132 demonstrating the elicitation of cell death on downy mildew resistant genotypes by an
133 effector of *B. lactucae*. This candidate contained no obvious canonical sequence motifs (such
134 as RXLR or EER). Thus, this paper shows that a machine learning approach, especially when
135 combined with a lineage-specificity analysis, can be useful in gene annotation and effector
136 prediction in oomycete genomes.
137

138 **Materials and Methods**

139 *Positive and negative training sets*

140 Our positive training set consisted of oomycete effectors shown experimentally to be
141 either recognized by R genes (avirulence activity) and/or to have an immune-suppressing
142 effect on the host (virulence activity) (Supplemental Table 1). Seventy-seven of these
143 sequences were obtained from the Pathogen-Host Interaction database (PHI-base) (Urban
144 et al. 2019) and 15 effector candidates were added from *B. lactucae* that had avirulence
145 (Giesbers et al. 2017; Pelgrom et al. 2019; Stassen et al. 2013; Wood et al. 2020) or virulence
146 activity (Wood et al. 2020), resulting in an initial set of 92 sequences. We then clustered
147 sequences using CD-HIT at a sequence identity threshold of 70% and word length of five
148 amino acids in order to identify and remove closely related sequences (Fu et al. 2012; Li and
149 Godzik 2006). This resulted in a final positive training set of 88 unique and distinct oomycete
150 effectors.

151 Our negative training set consisted of representative oomycete sequences that were
152 predicted to be secreted and very highly conserved (thus, unlikely to be effectors)
153 throughout the genomes of 28 oomycete species (Supplemental Table 2). To find these
154 sequences, we first performed an Orthofinder search on the oomycete genomes at an e-value
155 cutoff of 1e-10 and a query coverage cutoff of 90% (Emms and Kelly 2015, 2019). We then
156 retrieved ortholog centroids, with the species chosen at random, that were present in at least
157 26 genomes. We predicted signal peptides using SignalP v4.1 (sensitive mode) (Petersen et
158 al. 2011). To further reduce potential model bias and redundancy in the set of non-effectors,
159 we clustered sequences using CD-HIT and removed sequences with greater than 40%

160 identity. This identified 320 sequences, from which a training set of 88 sequences was chosen
161 at random to obtain a balanced 1:1 positive to negative training set ratio.
162
163 *Feature selection and encoding for classical machine learning models*
164 To calculate biochemical characteristics of effectors and secreted presumed non-
165 effectors, we used published amino acid scales from ExPASyProtScale (ExPaSy; Expert
166 Protein Analysis System 2004) and evaluated six features that could be informative for
167 effector classification. We focused on the first 100 amino acids of the N-terminus of protein
168 sequences, with the rationale that (1) secretion and translocation signals are typically
169 encoded in the N-terminus and (2) effectors may differ in their C-terminal domains
170 depending on their specific functions (for example, not all validated oomycete effectors have
171 WY domains and RXLR effectors show high rates of positive selection in the C-terminus (Win
172 et al. 2007)). To ensure the feature scales were equivalent to each other, we transformed the
173 calculated feature distributions to zero mean and unit variance (Z-score). We calculated the
174 average values for six N-terminal biochemical features: the grand average of hydropathy
175 (GRAVY)(Kyte and Doolittle 1982), hydrophobicity (Fauchere and Pliska 1983), surface
176 exposure (Janin 1979), disorder propensity (Dunker et al. 2001), bulkiness (Zimmerman et
177 al. 1968), and residue interface propensity (Jones and Thornton 1997) of the first 100 amino
178 acids using data from ExPASyProtScale (ExPaSy; Expert Protein Analysis System 2004).
179 PONDR VSL2 (Peng et al. 2006) was used as an additional method to visualize levels
180 of intrinsic disorder for effectors and secreted non-effectors across the N-terminal sequence.
181 The average sequence disorder was calculated at each amino acid position for the first 160

182 amino acids, including the signal peptide, in order to visualize the differences between
183 candidate effectors and non-effectors.
184
185 *Machine learning architectures*
186 For the classical machine learning pipeline, we tested various parametric and non-
187 parametric models and evaluated what worked best for effector classification based on the
188 N-terminal protein sequence. We tested and compared the following models using multiple
189 cross-validation trials (resampling procedures that rotate training and testing splits):
190 Random Forest, Naïve Bayes (Bernoulli and Gaussian), Logistic Regression, and K-Nearest
191 Neighbors. To reduce overfitting, we monitored training and testing scores (accuracy,
192 sensitivity, specificity, false positive rate, and area under the precision-recall curve). Models
193 were used from the scikit-learn machine learning library in Python (Pedregosa et al. 2011).
194 We compared Logistic Regression (default parameters), Naive-Bayes models (Gaussian and
195 Bernoulli, default parameters), Random Forest (with 400 estimators and a max depth of 4),
196 and the K-Nearest Neighbors classifier (with the neighbors parameter set to 5).
197
198 *Feature encoding for Convolutional Neural Network models*
199 In order to account for signals that may be concealed through net average
200 calculations, we also tried using a convolutional neural network (CNN) to extract positional-
201 dependent differences in amino acid sequence between effectors and non-effectors. We used
202 the first 100 amino acid values for each feature and padded the ends with zeros for
203 sequences shorter than 100 aa. For each protein in our positive and negative training sets,

204 this feature encoding resulted in a 6 x 100 matrix to include every positional encoding for all
205 six biochemical features in the first 100 amino acids.
206
207 *Genome data and secretome prediction*
208 Genomic sequences from 28 oomycete species were used to test EffectorO (list of
209 species and citations in Supplemental Table 2). We then predicted open reading frames
210 (ORFs) for all genomes. ORFs were used instead of gene annotations for several reasons: (1)
211 effector genes often consist of a single exon; (2) effector genes are often missed by gene
212 annotation programs; and (3) to account for differences in gene annotation approaches used
213 in different genomes. We predicted ORFs from each genome using BioPerl's "translate_strict"
214 module, using the canonical start codon (ATG), a maximum percent X of 0, and a minimum
215 nucleotide length of 240 (Stajich et al. 2002). From translated ORFs, we then used SignalP
216 v4.1 (sensitive mode) (Petersen et al. 2011) to predict secreted proteins. These predicted
217 secreted proteins were then used in subsequent EffectorO-ML analysis and for the lineage
218 specificity pipeline.
219
220 *Lineage-specificity*
221 Proteins encoded by lineage-specific genes were obtained using the predicted
222 secretome of a species as a BLASTp query against the translated open reading frames (ORFs)
223 of other oomycete species (Supplemental Table 2). Conserved proteins were defined as
224 proteins with a BLASTp hit in another species with an e-value cutoff of <10e-7, percent
225 identity of >30%, and a query coverage of >30%. Similar BLASTp cutoffs are used in other
226 pipelines to obtain species-specific genes for numerous species, including other oomycetes

227 (Rujirawat et al. 2019; Zhou et al. 2015). Protein sequences that were below these thresholds
228 in another oomycete species were defined as lineage-specific. ORFs were used instead of
229 gene models for the reasons described above.

230 *Prediction of amino acid motifs and protein domains*

231 To predict the RXLR-EER motif, we used a string search for [RQGH]XLR, [DE][DE][KR]
232 for EER, and HMM for RXLR-EER developed by Whisson et al. (2007) for *Phytophthora*
233 species. To predict WY domains, we used an HMM built from a training set of WY domain
234 proteins from three *Phytophthora* species by Boutemy et al. (2011). To predict CRNs, we
235 used an HMM built from sequences of CRNs from *Phytophthora* species (Fletcher et al. 2018).
236 HMMer v3.1 was used for all HMM searches (Eddy 2011). Default settings were used for
237 HMMer, except we used a bitscore cutoff of >0 to classify WY domain proteins (as suggested
238 by Boutemy et al. (2011)) and CRNs, which is more inclusive than the default cutoff. To
239 predict enzymatic and other characterized functional domains, the amino acid sequences of
240 EffectorO candidates were queried for Pfam predicted protein domains using the HMMER
241 website (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan). ProfileHMM search was
242 performed with an e-value cutoff of 0.1 against the Pfam database (Mistry et al. 2020).
243 Resulting Pfam predictions were checked for accuracy of annotation and were manually
244 categorized into domain families.

245

246 *Transcriptome data*

247 Transcriptome data for *B. lactucae* were downloaded from the NCBI short read
248 archive (NCBI BioProject PRJNA523226). These reads represent Illumina HiSeq 4000

249 sequencing from RNA obtained from *B. lactucae* infecting 7-day-old lettuce seedlings and
250 represent the full lifecycle of *B. lactucae* from inoculation to sporulation (timepoints: 0h, 3h,
251 6h, 12h 24h, 3d, 5d, 7d). Read counts for all predicted ORFs were generated using samtools
252 (Li et al. 2009); resulting read counts per ORF were then normalized by ORF length and log-
253 normalized for visualization.
254
255 *Sequence analysis and protein structural prediction*
256 To search for homologous proteins and compare sequences, protein BLAST (BLASTp)
257 was used against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Sayers et al.
258 2020) with the amino acid sequence of the protein of interest as the query. ClustalW was
259 used to align amino acid sequences for comparison (Thompson et al. 1994). Protein
260 structures were predicted using the full amino acid sequences (including the predicted signal
261 peptide) using RoseTTAfold on the Robetta web server with default settings
262 (https://robetta.bakerlab.org/)(Baek et al. 2021).
263
264 *Scripts*
265 All scripts for running EffectorO-ML and EffectorO-LSP can be found on Github at
266 https://github.com/mjnur/oomycete-effector-prediction. A web app for EffectorO-ML is
267 available at https://bremia.ucdavis.edu/effectorO.php.
268
269 *Candidate gene cloning and agroinfiltration*

270 The effector candidate *BLE01* was cloned from *B. lactucae* genomic DNA using
271 Gateway cloning into pEG100 (35S promoter) as described previously (Wood et al. 2020).
272 The signal peptide was excluded from the coding sequence to ensure expression inside the
273 plant cell. Two distinct alleles were obtained. *Agrobacterium tumefaciens* strain C58Rif+ was
274 transformed with pEG100-BLE01 and infiltrations were performed on 3- to 5-week-old
275 lettuce plants as described previously (Wood et al. 2020). Leaves were scored for necrosis
276 4–5 days after infiltration. *A. tumefaciens* containing pEG100 (empty vector) and pEG100-
277 GFP were used as negative controls; *A. tumefaciens* containing pBAV139-35S:HopM1 and
278 pBAV139-35S:AvrPto were used as positive controls for induction of cell death (Wroblewski
279 et al. 2009).
280
281 **Results**
282 *Effector and non-effector proteins differ in their biochemical characteristics*
283 To determine whether various N-terminal amino acid characteristics would be
284 sufficient to distinguish effectors from non-effector proteins, we compared predicted
285 biochemical parameters between validated oomycete effectors and secreted, conserved
286 proteins (presumed non-effectors). We found that validated effectors had higher intrinsic
287 disorder and had lower scores for GRAVY, hydrophobicity, exposed residues, bulkiness, and
288 interface, compared with secreted, conserved proteins (Fig. 1A). The intrinsic disorder score
289 was significantly higher in validated effectors, starting from just after the signal peptide (first
290 ~20 aa) until approximately 80 aa after the start codon (Fig. 1B).
291

292 *Evaluation of machine learning effector classifier models*

293 To find the effector classifier with the best performance, we evaluated six machine
294 learning models (Random Forest, Linear SVCs, Bernoulli and Gaussian Naïve Bayes, Logistic
295 Regression, and K-Nearest Neighbors) as well as a convolutional neural network model that
296 takes into account positional information for classification. The Random Forest classifier
297 initially outperformed other models, so we attempted several combinations of parameters,
298 adjusting the number of estimators (n_estimators) and maximum tree depth (max_depth),
299 while monitoring the cross-validation accuracy. The best receiver-operator curve (ROC) was
300 achieved by the Random Forest classifier (with parameters n_estimators = 400 and
301 max_depth = 4) with an area under the curve (AUC) of 0.89 (Figure 2) and an overall 5-fold
302 cross-validation accuracy of 84.26% (Table 1). The convolutional neural network was found
303 to perform similarly but not better than the most accurate Random Forest model. Therefore,
304 we used the Random Forest model for our EffectorO-ML pipeline to classify effectors based
305 on the biochemical characteristics of the first 100 amino acids.

306

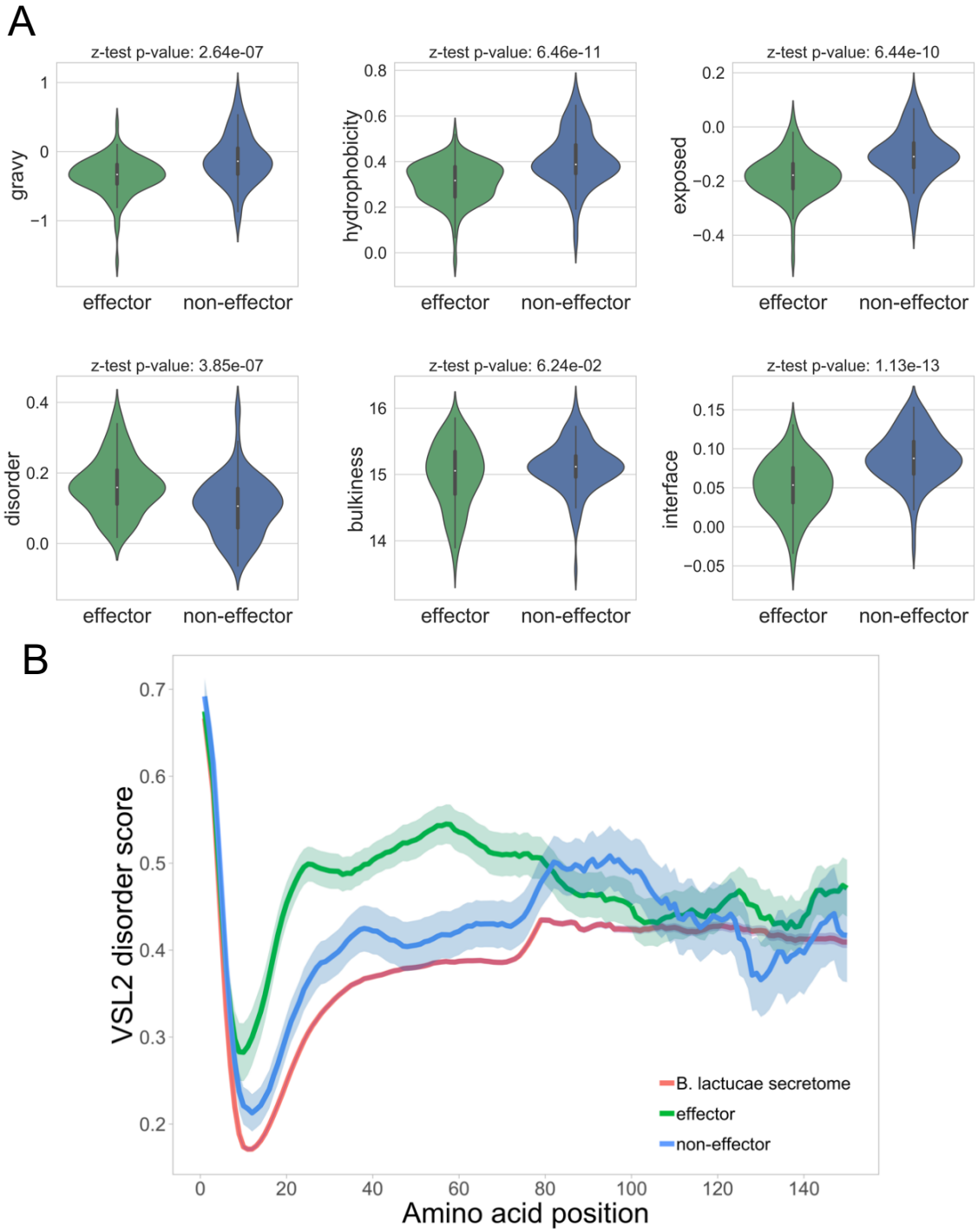
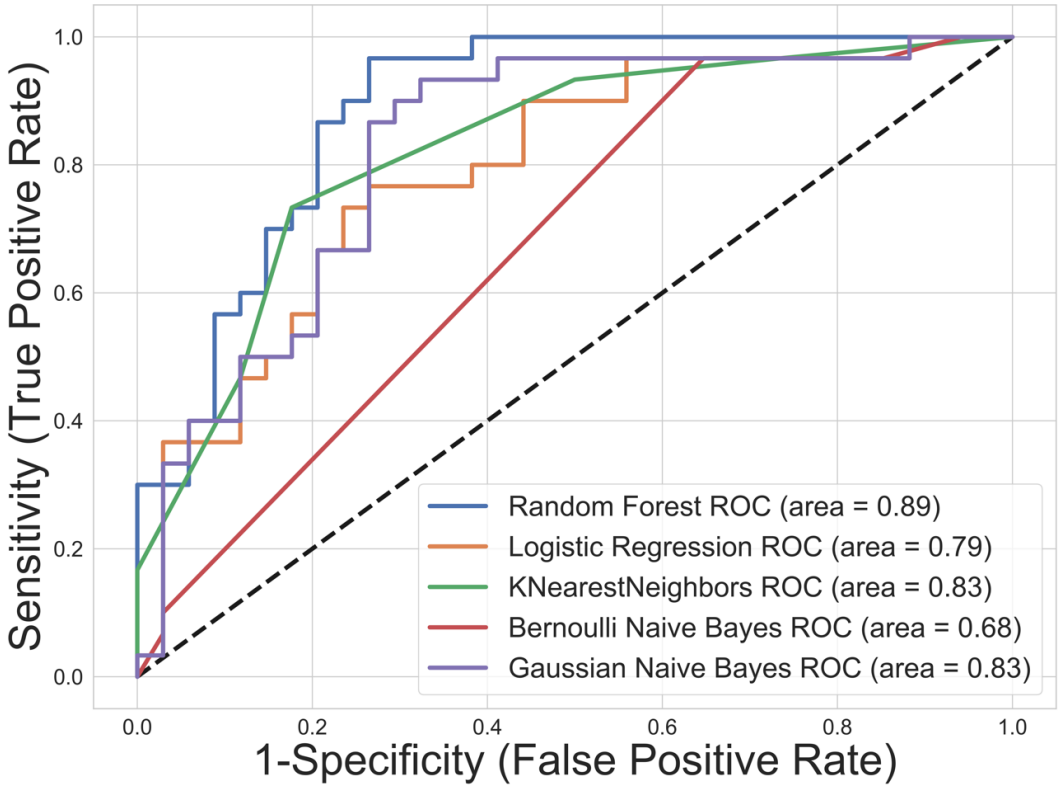


Figure 1. (A) Amino acid biochemical characteristics for validated oomycete effectors and secreted non-effectors as calculated by the ExPASyProtScale and used as features for the machine learning classifier. Violin plot shows the distribution of the N-terminal Z scores for each group of proteins, with the p-value shown above the plot. **(B)** Intrinsic disorder as calculated by PONDR VSL2 for validated oomycete effectors and secreted non-effectors with the *B. lactucae* secretome for reference. The average and standard error are shown for each group of proteins at each amino acid position.

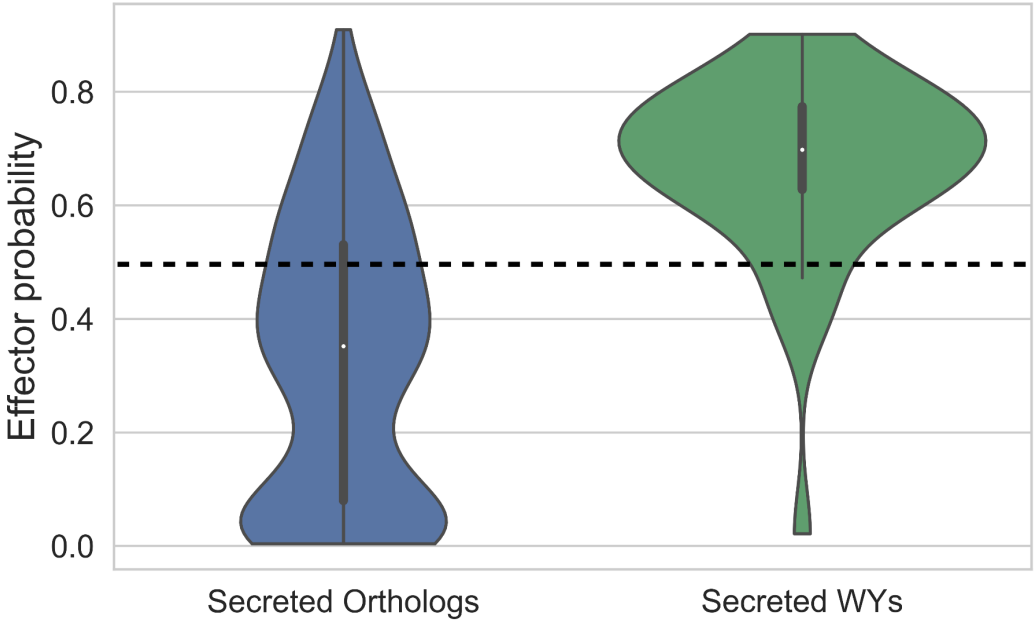
316



317
318 **Figure 2.** Receiver-operator curves (ROCs) for the various machine learning models tested
319 on a cross validation fold using 1:1 effector to non-effector testing and training sets.
320

321 The Random Forest effector classifier uses a default probability threshold of 0.5 to
322 classify a protein as an effector; this probability threshold can be modified depending on
323 preference for either inclusivity/sensitivity or specificity. For the top performing Random
324 Forest model, we calculated and averaged accuracy, specificity, sensitivity (true positive
325 rate), and false positive rate along the five cross-validation folds for different probability
326 thresholds, illustrating the tradeoffs between rates of false positives and true positives when
327 varying probability thresholds (Table 2). To obtain another estimate of the false positive and
328 false negative rates, we ran EffectorO-ML on a set of 634 predicted secreted proteins that
329 were highly conserved (presumed non-effectors) in at least 24 of the 28 sequenced oomycete

330 species used for EffectorO-LSP, as well as on the 41 secreted *B. lactucae* WY effectors
331 (presumed true effectors). We found that a probability cutoff of 0.5 resulted in
332 approximately 28% of these conserved proteins being classified as effectors while capturing
333 90% of the WY effectors (Figure 3 and Table 3). At a probability cutoff of 0.6, only 16% of the
334 conserved proteins were classified as effectors, with 85% of the WY proteins classified as
335 effectors (Table 3). Since we presume that most of the conserved secreted proteins are not
336 effectors due to lack of lineage-specificity, 16% would be a good estimate of the false positive
337 rate at the 0.6 threshold, which is similar to the false positive rate for string searches for the
338 RXLR motif (Wood et al. 2020). The false positive rate can be decreased further if desired by
339 increasing the probability threshold, but at the loss of capturing true effectors.



340
341 **Figure 3.** Violin plots of effector probabilities for conserved secreted oomycete orthologs
342 and secreted *B. lactucae* WY domain containing proteins, generated by the top-performing
343 Random Forest model. Dotted line at 0.5 shows the suggested inclusive cutoff for effector
344 classification.
345
346
347

348 **Table 1. Average cross validation metrics for various machine learning models for**
349 **classifying proteins as effectors or non-effectors**

	Random Forest	Logistic Regression	K-Nearest Neighbors	Bernoulli Naïve Bayes	Gaussian Naïve Bayes
Accuracy	84.3%	65.9%	70.2%	63.6%	67.9%
Specificity	83.0%	63.3%	69.1%	31.0%	61.1%
Sensitivity	87.0%	68.7%	71.1%	96.1%	74.6%
False Positive Rate	17.0%	36.7%	30.9%	69.1%	38.9%

350

351 **Table 2. Metrics at different probability thresholds for the most accurate Random**
352 **Forest model**

	Random Forest Model Effector Probability Threshold =		
	0.5	**0.6**	**0.7**
Accuracy	84.3%	79.0%	76.2%
Specificity	83.0%	85.1%	89.8%
Sensitivity	87.0%	73.0%	62.7%
False Positive Rate	17.0%	14.9%	10.2%

353

354 **Table 3. Number and percent of oomycete conserved secreted orthologous proteins**
355 **(634 total) and *B. lactucae* secreted WY proteins (41 total) classified as an effector by**
356 **the EffectorO-ML Random Forest model at different effector probability thresholds**

	Random Forest Model Effector Probability Threshold =				
	0.5	**0.6**	**0.7**	**0.7**	**0.9**
Secreted Orthologs	180 (28%)	103 (16%)	54 (8.5%)	12 (1.9%)	2 (0.3%)
Secreted WY Proteins	36 (90%)	34 (85%)	20 (50%)	5 (12.5%)	1 (2.5%)

357

358

359

360

361 *EffectorO predicts novel effectors in oomycete species and recovers known effectors*

362 To find novel effectors from *P. infestans* and *B. lactucae*, we used our EffectorO-ML
363 and LSP pipelines on the predicted secreted ORFs of these genomes. For *P. infestans*, we
364 found 8,685 proteins (41% of the secretome) to be lineage-specific and 5,814 proteins (30%)
365 to be classified as candidate effectors by machine learning, with 1,235 proteins (6%) present
366 in both categories (Figure 4). For *B. lactucae,* we found 2,478 proteins (34% of the
367 secretome) to be lineage-specific and 1,777 proteins (24%) to be classified as candidate
368 effectors by machine learning, with 597 proteins (8%) present in both categories (Figure 4).
369 EffectorO was largely able to recover proteins containing known effector signatures, such as
370 the RXLR and EER motifs and the WY domain (Figure 4), which were not used as classifiers
371 in our analysis but were contained in some proteins in the positive training set.

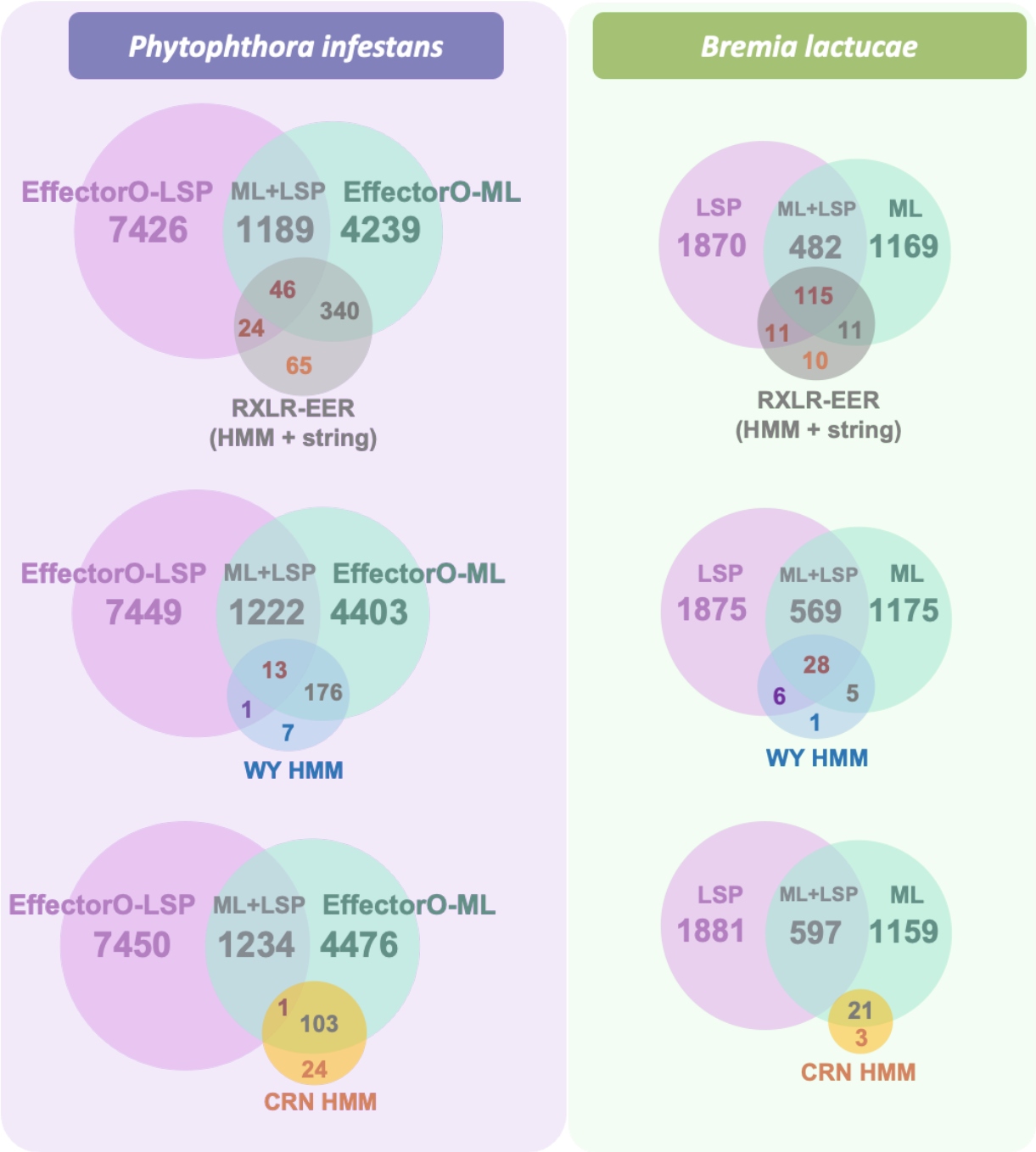
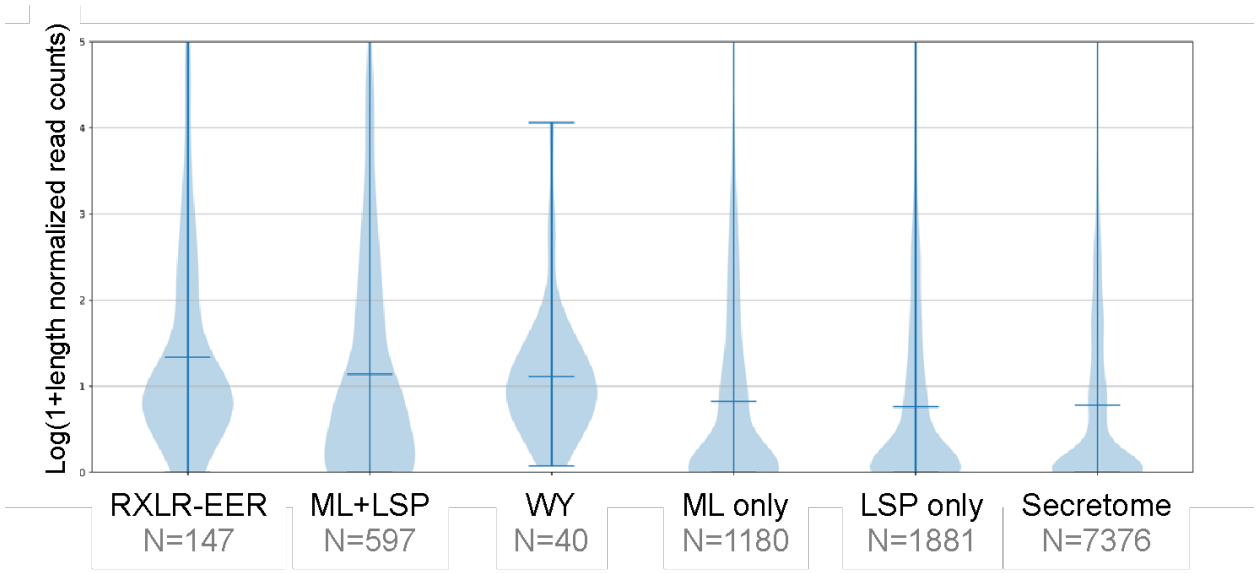


Figure 4. Venn diagrams showing the overlap between EffectorO predictions and canonical RXLR, WY, and CRN effector predictions for *P. infestans* and *B. lactucae*. Numbers from the predicted secretomes that are lineage-specific proteins (EffectorO-LSP) and/or predicted to be an effector by the machine learning classifier (EffectorO-ML, at probability score threshold of 0.5). RXLR-EERs were predicted using HMM and string search. WY domain proteins and CRNs were predicted using HMMs. Circle sizes are relatively proportional for each set but not precisely to scale.

381 *The majority of EffectorO candidates are expressed in B. lactucae*

382 To investigate whether EffectorO candidates are expressed at the transcript level, we
383 used an available RNA-seq dataset of *B. lactucae* grown on lettuce seedlings from 0 to 7 dpi
384 (from inoculation to sporulation). We found that 73% of transcripts that encoded proteins
385 predicted to be effectors by EffectorO-ML and EffectorO-LSP were expressed. The EffectorO-
386 ML+LSP candidates had similar mean expression levels as transcripts encoding proteins
387 containing WY domains and slightly lower expression than those containing RXLR-EER
388 motifs (Figure 5). Transcripts that encoded proteins predicted to be effectors by either the
389 ML or LSP pipeline, but not both, had lower mean expression levels and more proteins with
390 zero reads present in the RNA-seq dataset (Figure 5). The full secretome had the lowest
391 mean expression and also had 1,716 predicted transcripts with zero reads, suggesting that
392 many of the predicted ORFs may not be expressed (Figure 5).
393

394



395

396 **Figure 5.** RNA-seq expression levels in *B. lactucae* (log of length-normalized read counts) for
397 candidate effector transcripts encoding proteins with the RXLR-EER motif (RXLR-EER), WY
398 domain (WY), EffectorO-ML-LSP (ML and LSP), EffectorO-ML only (ML only), EffectorO-LSP
399 only (LSP only), or the full secretome. RNA was derived from a time course of *B. lactucae*
400 infecting lettuce from 0–7 dpi. The number of ORFs in each category is given below the name.
401
402 *The majority of EffectorO-ML candidates have no predicted functional domains*

403 In order to investigate whether known functional domains were predicted to be
404 present in the EffectorO-ML effector candidates, we queried EffectorO-ML candidates (with
405 a score of 0.5 or higher) against the Pfam protein domain database. Most EffectorO
406 candidates (88%) had no predicted Pfam domains (Table 4). There were 205 proteins that
407 contained a total of 246 Pfam domains (with some proteins predicted to have multiple
408 domains). The largest class of functional domains predicted was reverse transcription, or
409 transposon-related domains, suggesting that these candidates may actually be pseudogenes
410 disrupted by an insertion event (Table 5). Of the remaining EffectorO predictions with non-
411 transposon-related domains, the most common domains predicted were enzymatic
412 (including cell-wall degrading), protease, protein kinase, histidine phosphatase, lectin,

413 cysteine-rich chitin binding, glycosyl hydrolase, and necrosis inducing protein domains

414 (Table 5). All predictions are given in Supplemental Table 3.

415

416 **Table 4. Pfam domains in *B. lactucae* EffectorO-ML candidates**

417

Category	Number of proteins
1572	No Pfam domains
205	With Pfam domains
1777	**Total EffectorO-ML candidates**

418

419

420 **Table 5. Top Pfam domain classifications for EffectorO-ML candidates (with at least**
421 **six domains represented per family)**

422

Domain classification	# of Domains
RT related	**61**
Reverse Transcriptase	39
RNase H-like domain found in reverse transcriptase	13
LTR copia-type	7
Integrase zinc binding domain	2
Enzymatic	**35**
Carbohydrate metabolism and/or Cell-wall degrading	24
Other activity	11
Protease	**17**
Protein kinase domain	**15**
Histidine phosphatase	**13**
Lectin	**10**
Cysteine-rich chitin binding	**9**
Chaperone	**9**
Necrosis inducing protein	**8**
Kazal-type serine protease inhibitor	**7**
Calmodulin-binding motif	**7**
Ribosomal protein	**6**
Elicitin	**6**

423

424

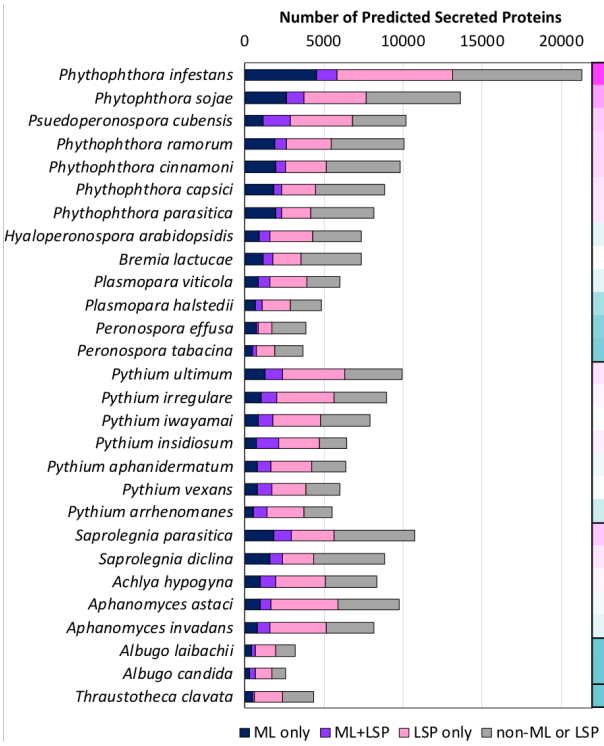
425

426

427 *EffectorO predicts candidate effectors in diverse oomycete pathogens*

428 To determine whether EffectorO could predict effectors in other pathogens across the
429 oomycete phylum, we tested EffectorO-ML and -LSP on a total of 28 published oomycete
430 genomes. We found that even though EffectorO was trained primarily on effectors from
431 downy mildew and *Phytophthora* pathogens, the algorithm predicted hundreds to thousands
432 of candidate effectors in distantly related oomycetes infecting animals (e.g., *Achlya hypogyna*,
433 *Aphanomyces* spp., *Saprolegnia* spp.), as well as oomycetes from the genera *Albugo* and
434 *Pythium* (Figure 6). Critically, EffectorO-ML predicted a similar number of effectors in the
435 free-living oomycete species *Thraustotheca clavata* (14.6% of the genome) as the false
436 positive rate for EffectorO (17.0%, Table 1).

437 We compared effector predictions from EffectorO-ML for the 28 oomycete genomes
438 to EffectorP 3.0 predictions. We found that EffectorP 3.0 predicts larger numbers of effectors
439 in each genome (Table 6), with about 42% overlapping between the sets of predictions. For
440 example, for *B. lactucae*, EffectorP 3.0 predicts 3111/7376 (42.2%) proteins in the
441 secretome as effectors, while EffectorO predicts 1777/7376 (24.0%). In the free-living
442 oomycete *T. clavata*, EffectorP 3.0 predicts 1918/4352 (44.1%) as effectors, while EffectorO
443 predicts 635/4352 (14.6%).



	EffectorO-ML	ML+LSP	LSP	RXLR-EER	WY	Total Secreted
Phythophthora infestans	5814	1275	8619	475	197	21320
Phytophthora sojae	3749	1115	5031	343	118	13605
Psuedoperonospora cubensis	2899	1755	5638	131	48	10192
Phythophthora ramorum	2654	737	3569	270	131	10043
Phythophthora cinnamoni	2601	645	3193	238	96	9847
Phythophthora capsici	2348	534	2662	226	76	8820
Phythophthora parasitica	2328	394	2219	336	144	8160
Hyaloperonospora arabidopsidis	1563	657	3402	98	45	7383
Bremia lactucae	1777	603	2409	147	40	7376
Plasmopara viticola	1562	701	3038	110	214	5997
Plasmopara halstedii	1087	447	2218	58	34	4863
Peronospora effusa	856	154	983	99	67	3840
Peronospora tabacina	761	300	1438	73	37	3671
Pythium ultimum	2380	1093	5042	87	0	9928
Pythium irregulare	2013	957	4587	71	0	8953
Pythium iwayamai	1751	880	3918	80	0	7907
Pythium insidiosum	2132	1374	3994	72	0	6467
Pythium aphanidermatum	1646	831	3420	50	0	6407
Pythium vexans	1743	938	3053	63	0	6019
Pythium arrhenomanes	1388	829	3190	34	0	5523
Saprolegnia parasitica	2922	1066	3816	112	0	10767
Saprolegnia diclina	2365	798	2764	63	0	8835
Achlya hypogyna	1984	1026	4114	52	0	8370
Aphanomyces astaci	1667	680	4881	44	0	9747
Aphanomyces invadans	1569	792	4377	33	0	8175
Albugo laibachii	664	261	1582	20	0	3200
Albugo candida	643	329	1393	16	0	2572
Thraustotheca clavata	635	144	1904	11	0	4352

Figure 6. Number of predicted secreted proteins (translated ORFs) classified as effectors using EffectorO-ML only (cutoff = 0.5) (dark blue bars), EffectorO-LSP only (pink bars), EffectorO-ML and LSP (purple bars), and those classified as non-effectors (grey bars) in 28 species of oomycetes, including Peronosporales (*Phytophthora* and downy mildew species), *Pythium* species, animal pathogens (*Achlya, Saprolegnia*, and *Aphanomyces* spp.), *Albugo* spp., and a free-living oomycete (*Thraustotheca clavata*).

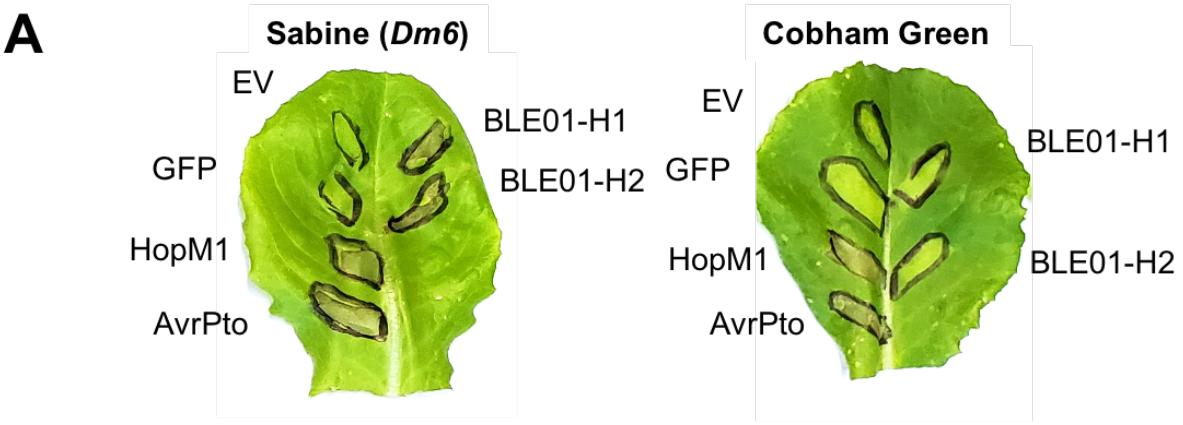
452 **Table 6. Number of effectors in 28 oomycete genomes predicted by EffectorO-ML and**
453 **EffectorP 3.0.**
454

Species	Unique to EffectorO-ML	Predicted by both	Unique to EffectorP 3.0
Achlya hypogyna	1210	774	1317
Albugo candida	195	448	753
Albugo laibachii	177	487	953
Aphanomyces astaci	886	781	1945
Aphanomyces invadans	797	772	1456
Bremia lactucae	618	1159	1945
Hyaloperonospora arabidopsidis	582	981	1694
Peronospora effusa	315	541	858
Peronospora tabacina	254	507	953
Phythophthora capsici	1058	1290	1597
Phythophthora cinnamoni	1313	1288	1739
Phythophthora infestans	2841	2973	4080
Phythophthora parasitica	945	1383	1548
Phythophthora ramorum	1303	1351	1744
Phytophthora sojae	1936	1813	2183
Plasmopara halstedii	340	747	1297
Plasmopara viticola	498	1064	1521
Psuedoperonospora cubensis	1316	1583	1618
Pythium aphanidermatum	884	762	1039
Pythium arrhenomanes	791	597	814
Pythium insidiosum	1152	980	1069
Pythium irregulare	884	1129	1926
Pythium iwayamai	843	908	1477
Pythium ultimum	1111	1269	2043
Pythium vexans	1039	704	901
Saprolegnia diclina	1367	998	1236
Saprolegnia parasitica	1664	1258	1484
Thraustotheca clavata	163	472	1444

455

456 *An EffectorO predicted effector candidate causes cell death when expressed in lettuce*

457 To test whether any EffectorO candidates were recognized effectors (i.e., have
458 avirulence activity), we cloned a gene from *B. lactucae* that was within the mapping region
459 for *Avr6,* as determined by sequence-based mapping of segregating sexual progeny of *B.*
460 *lactucae* isolates (R.J. Gil, unpublished data). This region contained no genes encoding RXLR
461 or EER motifs nor WY domains, but did contain one ORF encoding a protein that was
462 classified as a probable effector by both EffectorO-ML and Effector-LSP. The ORF encoding
463 this protein was not found in the gene models (i.e., was not predicted to be a gene by the
464 annotation software). The candidate effector, named BLE01 for (*Bremia lactucae* EffectorO
465 candidate 1), caused cell death when transiently expressed in the lettuce cultivar Sabine,
466 which contains *Dm6*, but not Cobham Green, which does not contain any known *Dm* genes
467 (Fig 7A). When we screened a larger panel of lettuce cultivars with various *Dm* genes, we
468 found that it also elicited cell death in RYZ2164, but not in the other seven cultivars with
469 other *Dm* genes or in the non-host plant *Nicotiana benthamiana* (Fig 7B).



B

Cultivar	*Dm* genes	N plants	EV	GFP	BLE01-H1	BLE01-H2	HopM1	AvrPto
N. benthamiana	n/a	3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.0	0.0 ± 0.0
Cobham Green	n/a	4	0.5 ± 0.6	0.75 ± 0.5	0.5 ± 0.6	0.5 ± 0.6	4.0 ± 0.0	3.8 ± 0.5
UCDM2	*Dm2*	4	0.5 ± 0.6	0.3 ± 0.5	0.0 ± 0.0	0.3 ± 0.5	3.8 ± 0.5	3.5 ± 0.6
Dandie	*Dm3*	4	0.5 ± 0.6	0.5 ± 0.6	0.3 ± 0.5	0.3 ± 0.5	4.0 ± 0.0	3.3 ± 0.6
Sabine	*Dm6*	6	0.8 ± 0.4	0.8 ± 0.4	2.7 ± 0.8	3.5 ± 0.6	3.7 ± 0.5	3.0 ± 0.0
LSE57/15	*Dm7*	4	0.5 ± 0.6	0.5 ± 0.6	0.3 ± 0.5	0.3 ± 0.5	3.3 ± 1.5	3.8 ± 0.5
Capitan	*Dm11*	4	1.0 ± 0.8	1.0 ± 0.8	0.8 ± 1.0	0.8 ± 0.5	3.3 ± 0.5	2.8 ± 0.5
Ninja	*Dm3,4,11,36*	4	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	4.0 ± 0.0	4.0 ± 0.0
Discovery	*Dm7, 37*	4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.8 ± 0.5	3.5 ± 0.6
RYZ2164	*Dm25*	4	0.8 ± 1.0	0.5 ± 0.6	3.0 ± 0.0	3.3 ± 0.5	4.0 ± 0.0	2.5 ± 1.7
ViAE	R20	4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 0.6	3.3 ± 0.5

Figure 7. (A) Example transient expression results from lettuce infiltrated with *A. tumefaciens* containing two candidate effector alleles from *B. lactucae* (BLE01-H1 and BLE01-H2); the negative controls, empty vector (EV) and green fluorescent protein (GFP); and the positive controls for cell death, *Pseudomonas syringae* pv. *tomato* effectors HopM1 and AvrPto, which cause strong and medium necrosis in lettuce, respectively. Leaves are representative of four replicates and photos were taken 4 days after infiltration. (B) Necrosis scores four days post-agroinfiltration of the candidate effectors and controls in a panel of lettuce cultivars and the non-host *Nicotiana benthamiana*. Necrosis was scored as follows: 0 = no necrosis or chlorosis, 1 = mild chlorosis, 2 = chlorosis, 3 = chlorosis and necrosis, 4 = full necrosis. Number of replicates (N), averages, and standard deviations are shown for each line and effector combination.

BLE01 is similar in sequence and structure to WY domain proteins

To investigate potential protein domains of BLE01, the amino acid sequence of the protein was used as a BLAST query against the *B. lactucae* genome. One of the top BLAST hits

486 was a previously characterized WY domain containing protein, BSW13 (Wood et al. 2020).
487 There was 61% amino acid identity in the overlapping region, which consists of the entire
488 amino acid sequence for BSW13 and the N-terminal half of the sequence for BLE01 (Figure
489 8). Since BSW13 was predicted to contain a WY domain, we performed a search using the
490 HMM for the WY domain on the BLE01 sequence to see if there might be a low scoring WY
491 domain predicted for BLE01 that had been previously missed. We found that BLE01 was
492 predicted to have a low scoring WY domain in the same region as BSW13, with a domain e-
493 value of 0.029 and a bitscore of 1.8. We discovered that this protein had been originally
494 filtered out from our HMM search for WYs by the default bias filter in HMMer.

495 To predict three-dimensional protein structures for BLE01 and BSW13, we used the
496 RoseTTAfold web server (https://robetta.bakerlab.org/). Both proteins were predicted to
497 have six alpha helical bundles (Figure 8B), which resemble the repeated WY domains as seen
498 in other effector proteins that have been characterized by x-ray crystallography (Chou et al.
499 2011; Boutemy et al. 2011; Leonelli et al. 2011; Win et al. 2012; He et al. 2019).

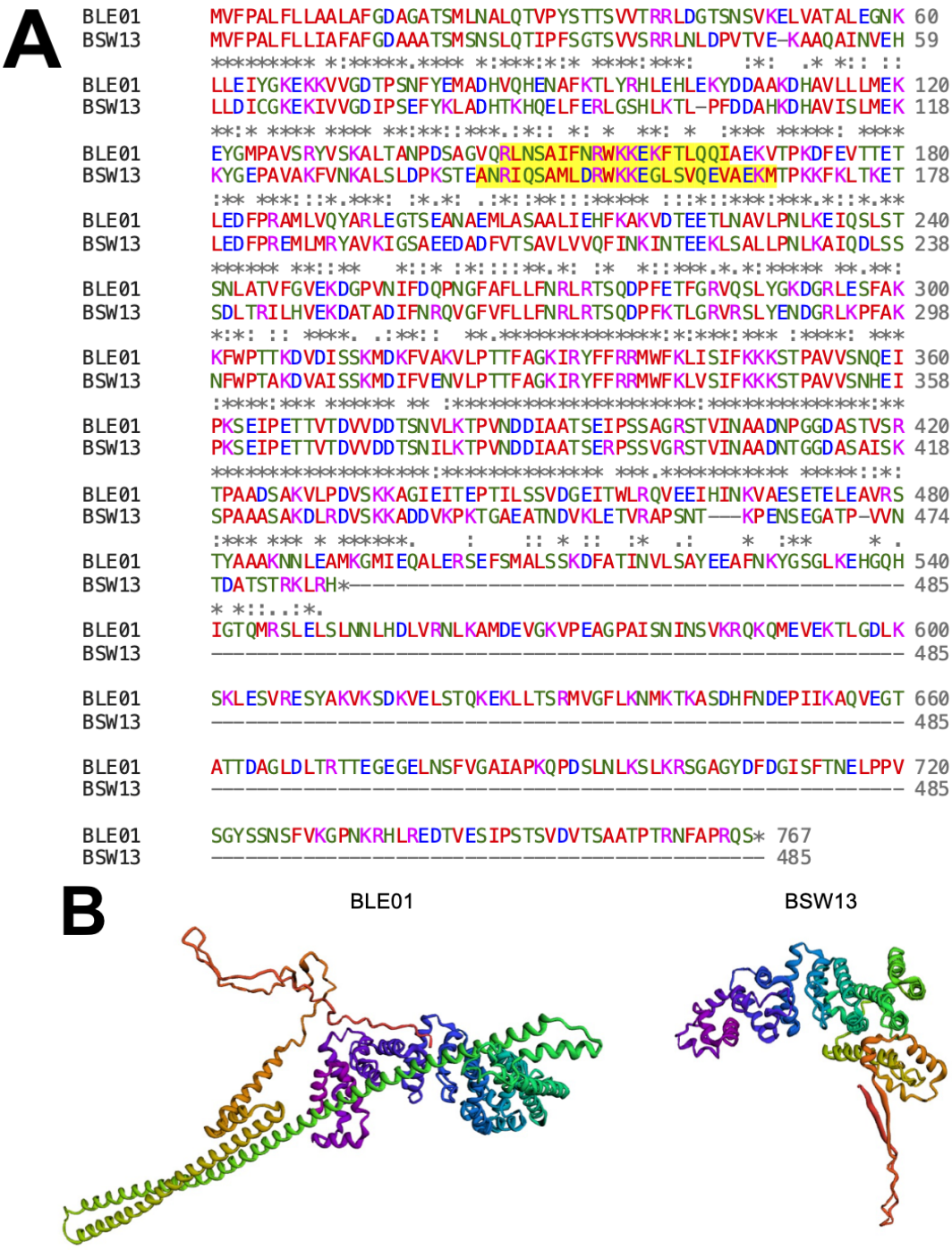


Figure 8. (A) Pairwise alignment between amino acid sequences of BLE01 and BSW13. Amino acids are colored red for hydrophobic, blue for negatively charged, magenta for positively charged, and green for all other amino acids. Similarity is indicated below the alignment with an asterisk (*) for matching, a colon (:) for strongly similar, and a period (.) for weakly similar amino acids. The predicted WY domains for each protein are highlighted in yellow. (B) Protein structure predictions from RoseTTAfold for BLE01 and BSW13. The protein structure is rainbow color coded from N-terminus (purple) to C-terminus (red).

509 **Discussion**

510 We have demonstrated that our EffectorO pipeline can predict novel candidate
511 effectors using a machine learning-based approach combined with a lineage-specificity
512 criterion for oomycete effector classification. EffectorO can be used to mine oomycete
513 genomes to expand the set of predicted effectors without relying on canonical N-terminal
514 motifs (such as RXLR, EER, and LXLFLAK) or C-terminal domains (such as the WY domain);
515 thus, it can reveal effectors that were previously missed by only searching for these features.
516 EffectorO predicts an additional ~600 ML+LSP candidate effectors from *B. lactucae* and
517 ~1,200 additional ML+LSP candidate effectors from *P. infestans*. The genome of *P. infestans*
518 *is* larger than the genome of *B. lactucae (150 Mb vs 240 Mb)*, which may in part explain why
519 more effectors were predicted in that genome (as well as in other *Phytophthora* species). The
520 percentage of effectors predicted in the secretome for both species by EffectorO-ML was
521 approximately 25%, which is similar to what was predicted for fungal secretomes with
522 EffectorP (Sperschneider et al. 2018). The number of potentially novel effectors in *B. lactucae*
523 and *P. infestans* that were identified is much larger than previous reports of effector
524 repertoires from these two species (Fletcher et al. 2019; Haas et al. 2009). These predicted
525 effector repertoires are larger because EffectorO-ML uses N-terminal biochemical
526 characteristics rather than specific amino acid motifs and thus finds effector candidates that
527 would have been missed by prediction pipelines that rely on specific sequences. As with any
528 prediction, some of the candidates will be false positives; therefore, functional
529 characterization is necessary to confirm that they are effectors. The large size of these sets
530 of effector candidates should not preclude functional analysis when combined with

531 additional data from the pathogen of interest such as avirulence gene mapping, population
532 genomics, and gene expression data.

533 EffectorO-ML was trained on the N-terminal sequences of validated effectors
534 compared to conserved secreted proteins (presumed non-effectors), which, as we show,
535 differ in several biochemical characteristics. Sequences in the N-terminus of effector
536 proteins are known to be important for secretion and translocation of effectors; here we
537 demonstrate that they have distinct biochemical signatures that can be used to predict the
538 probability that a given sequence is an effector. Our positive training set showed increased
539 N-terminal intrinsic disorder compared to conserved proteins, which has also been observed
540 for oomycete RXLR effectors (Marin et al. 2013; Shen et al. 2017). Intrinsically disordered
541 regions are flexible segments of proteins that may adopt changeable conformations upon
542 binding to interacting partners or in changing conditions, such as pH (Marin et al. 2013). It
543 has also been observed that the N-terminus of cytoplasmic effectors are enriched for
544 positively charged amino acids (Sperschneider and Dodds 2022). These observations are
545 intriguing and should be followed up by mutational studies *in vitro* and *in vivo* to clarify their
546 role in effector translocation and/or function.

547 EffectorO-ML was able to recover the majority of predicted RXLR-EER and WY
548 domain-containing proteins from *B. lactucae* and *P. infestans* without relying on using those
549 motifs for prediction. By focusing on the N-terminus for effector classification, our pipeline
550 allows for a diversity of C-terminal domains, which are typically the functional region of
551 effector proteins. Because EffectorO does not rely on known motifs, it may help to uncover
552 new effector families. Interestingly, the majority of EffectorO-ML candidates had no
553 functional domains predicted using Pfam. This does not mean that the effectors do not

554 contain domains with biochemical activities, but rather their domains have yet to be
555 characterized. Functional validation followed by structural characterization using cryo-EM
556 or x-ray crystallography can be done on these predicted effector proteins, as has been done
557 for WY-containing effectors (Boutemy et al. 2011). The predicted effector repertoires should
558 also be analyzed with the latest machine learning structural prediction algorithms such as
559 trRosetta (Yang et al. 2020), AlphaFold (Jumper et al. 2021), or RoseTTAfold (Baek et al.
560 2021). Recently, trRosetta was used to predict effectors based on structure for the fungal
561 species *Magnaporthe oryzae* (Seong and Krasileva 2021) and a similar analysis should be
562 done with EffectorO candidates.

563 The most accurate model for EffectorO-ML was the Random Forest classifier. Random
564 Forest classifiers work by building many decision trees and then taking the consensus of all
565 of the decision trees to calculate an overall probability for a given classification (Pavlov
566 2000). Because the final output is the average of many independent models, Random Forest
567 classifiers are robust to individual errors in any given single model. The fungal and oomycete
568 effector classifier, EffectorP 3.0, uses a similar approach by utilizing an ensemble classifier
569 that takes the consensus predictions of multiple Naive-Bayes and decision trees to obtain a
570 balanced accuracy of 85% for predicting apoplastic and cytoplasmic effectors. While
571 EffectorP is trained on a larger set of effectors than EffectorO and relies on additional
572 biochemical characteristics (15 features vs. our six), we achieved a similar accuracy to
573 EffectorP 3.0 in predicting oomycete effectors. Our Random Forest model had an estimated
574 84% accuracy and 82% specificity at a probability cutoff of 0.5. The new EffectorP 3.0
575 classifies 86/92 (93.5%) of the oomycete effectors used in our positive training set as
576 effectors and 19/88 (21.6%) of our negative training set as effectors, giving it similar

577 sensitivity and specificity to our pipeline. However, predictions did not completely overlap
578 between the two methods; therefore, the two methods could be used as complementary tools
579 to predict high confidence effectors. For example, EffectorP 3.0 does not predict BLE01 from
580 B. lactucae to be an effector, however in this paper we show that it causes cell death in lettuce
581 and is an Avr candidate. EffectorP 3.0 also predicts many more effectors compared to
582 EffectorO, especially in downy mildew genomes (with 40–50% of the secretome predicted
583 as effectors), which could be indicative of a higher number of false positives than EffectorO-
584 ML.

585 The false positive rate for EffectorO-ML can be decreased by increasing the
586 probability cutoff for classification of an effector; however, this will also result in more false
587 negatives (missed true effectors). For inclusive searches (especially if combined with other
588 lines of evidence), we recommend 0.5 as the threshold for analyses of oomycete genomes.
589 For a slightly less inclusive but more accurate threshold, we recommend a threshold of 0.6.
590 The choice of threshold cutoff depends on downstream effector prediction steps and
591 throughput for effector validation. It may also be possible to further improve the accuracy
592 and specificity of EffectorO by adding additional proteins to the training set or by adding
593 more biochemical and structural characteristics. Gene models may be used instead of ORFs
594 to reduce false positives, although gene prediction algorithms may miss true effectors (as
595 was the case for the *Avr6* candidate *BLE01*). We also used predicted secreted proteins as our
596 input (predicted using the most sensitive version of SignalP 4.1); however, since some
597 effectors may have non-conventional secretion signals, total proteomes could also be used
598 for EffectorO-ML, although there may be an increased false positive rate using this approach
599 and candidates should be evaluated using additional lines of evidence to find true effectors.

600 Similar to EffectorO-ML, EffectorO-LSP will predict proteins other than effectors, not
601 only due to false positives but also due to the biological reality that there may be lineage-
602 specific proteins that have other functions. Thus, EffectorO-LSP predictions are most useful
603 in combination with other lines of evidence of effector activity (such as EffectorO-ML,
604 expression during infection, RXLR motifs and/or WY domains). It is important to note that
605 CRN effectors do not appear to be lineage-specific (they are conserved across species), so the
606 LSP pipeline may filter out CRN type effectors. Approximately 41% of the *P. infestans*
607 secretome and 34% of the *B. lactucae* secretome were predicted to be lineage-specific. Some
608 of these predicted proteins may not actually be lineage-specific; some may be encoded by
609 pseudogenes or result from failure to detect homology (Weisman et al. 2020). We found that
610 many were expressed in *B. lactucae* during infection, suggesting that some represent true
611 genes. In addition to effectors, these lineage-specific secreted proteins could also be related
612 to the lifestyles of these organisms, their sexual reproduction, or may be products of co-
613 evolution with other microbes rather than co-evolution with the host.

614 The number of lineage-specific genes detected for a given organism will depend on
615 whether or not closely related organisms are included in the analysis. The user can decide
616 which closely related species to include in order to adjust the stringency of the filter. We
617 recommend that BLAST results from very closely related species not be included in order to
618 reduce the false negative rate. The researcher can choose their own trade-off between false
619 negatives and false positives. If closely related species are included in the analysis, then some
620 true effectors will likely be filtered out due to the presence of effector orthologs in closely
621 related genomes. On the other hand, when only distantly related species are present in the
622 database, there may be more false positives. Note that for species that do not have complete

623 and well annotated genomes compared to closely related organisms, sequences may be
624 inaccurately classified as "species-specific;" however, they can still be considered "lineage-
625 specific" to the other genomes in the database used for comparison. Therefore, we consider
626 the lack of orthologs in other oomycete genomes as a useful characteristic to help predict
627 effectors, even if its accuracy may be limited by the contemporary genomic resources for a
628 given species.

629 EffectorO was validated for a predicted effector from *B. lactucae,* which was within
630 the region co-segregating with the *Avr6* phenotype, but did not have RXLR or EER motifs or
631 WY domains. We showed that the gene, *BLE01*, induced cell death when expressed in lettuce
632 cultivars expressing the *Dm6* gene but not on cultivars containing other known *Dm* genes,
633 making it a good candidate for *Avr6.* Expression of *BLE01* also caused cell death on RYZ2164.
634 The background of RYZ2164 is proprietary, so it is unknown whether it has *Dm6* in its
635 lineage or another *Dm* gene that recognizes the effector. Bioinformatic analysis of BLE01
636 revealed that it was similar in sequence and structure to the WY domain containing protein
637 BSW13. Subsequently, when the bias filter was turned off in HMMer, BLE01 was predicted
638 to be a WY domain protein, albeit a low scoring one. Since the filters in HMMer exist to
639 increase the speed of the search at the expense of false negatives, we recommend that
640 HMMer users who are not concerned with speed turn off the bias filter using the option (--
641 nobias) or all filters using the option (--max).

642 EffectorO-ML and -LSP can expand the set of predicted effectors for identification of
643 genes for resistance to agriculturally important oomycetes. These predictions can be used to
644 choose candidates for "effectoromics" screens (Vleeshouwers and Oliver 2015) to identify
645 effectors with avirulence and virulence activities. For pathogen species without as extensive

646 genetic resources as *B. lactucae*, other lines of evidence can be used to narrow down the list
647 of effectors for functional characterization, including transcriptomic analyses to identify
648 proteins expressed during infection and population genomics to identify loci under selection.
649 Effectors encoded by avirulence genes are valuable for discovering, mapping, and
650 characterizing host resistance genes. Knowledge of R gene–effector interactions will enable
651 the stacking and maintenance of multiple R genes during introgression from wild relatives
652 and can help prevent redundancy of effector recognition specificities within a breeding
653 program. In addition, more accurate annotation of pathogen genomes will enable pathogen-
654 informed R gene deployment and sequence-based monitoring of pathogen populations
655 (Michelmore et al. 2013).
656

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663

664 **Supplementary Files**

665

666 **Supplemental Table 1**: Validated oomycete effector sequences used for the EffectorO-ML
667 positive training set

668 **Supplemental Table 2**: Information on and citations for the 28 oomycete genomes used in
669 this paper
670 **Supplemental Table 3**: Protein family domain predictions for the B. lactucae EffectorO-ML
671 predictions
672
673 **Literature Cited**

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