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4 The extrachromosomal circular DNAs of the rice blast pathogen *Magnaporthe oryzae* contain a wide
5 variety of LTR retrotransposons, genes, and effectors

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16 **Abstract**

17 **Background**

18 One of the ways genomes respond to stress is by producing extrachromosomal circular DNAs (eccDNAs).
19 EccDNAs can contain genes and dramatically increase their copy number. They can also reinsert into the
20 genome, generating structural variation. They have been shown to provide a source of phenotypic and
21 genotypic plasticity in several species. However, whole circularome studies have so far been limited to a
22 few model organisms. Fungal plant pathogens are a serious threat to global food security in part
23 because of their rapid adaptation to disease prevention strategies. Understanding the mechanisms
24 fungal pathogens use to escape disease control is paramount to curbing their threat.

25 **Results**

26 We present a whole circularome sequencing study of the rice blast pathogen *Magnaporthe oryzae*. We
27 find that *M. oryzae* has a highly diverse circularome containing many genes and showing evidence of
28 large LTR retrotransposon activity. We find that genes enriched on eccDNAs in *M. oryzae* occur in
29 genomic regions prone to presence-absence variation and that disease associated genes are frequently
30 on eccDNAs. Finally, we find that a subset of genes is never present on eccDNAs in our data, which
31 indicates that the presence of these genes on eccDNAs is selected against.

32 **Conclusions**

33 Our study paves the way to understanding how eccDNAs contribute to adaptation in *M. oryzae*. Our
34 analysis also reveals how *M. oryzae* eccDNAs differ from those of other species and highlights the need
35 for further comparative characterization of eccDNAs across species to gain a better understanding of
36 these molecules.

37

38 **Keywords**

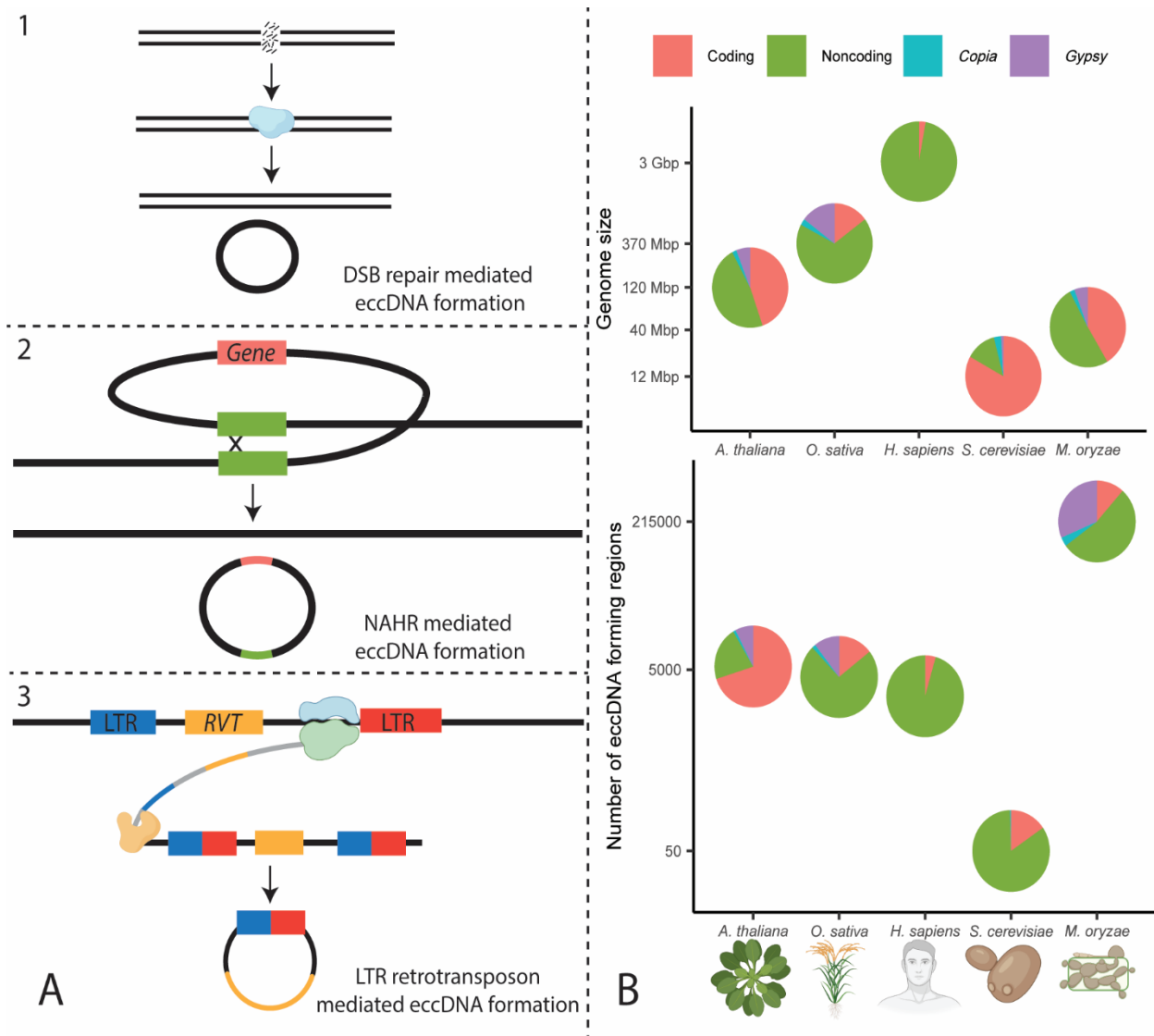
39 Extrachromosomal circular DNA; fungal plant pathogen; LTR retrotransposons; rice blast

40

41 **Background**

42 Extrachromosomal circular DNAs (eccDNAs) are a broad and poorly understood category of molecules
43 defined simply by the fact that they are circular and originate from chromosomal DNA. This group of
44 molecules has been referred to by many names and includes many smaller categories of molecules such
45 as episomes, double minutes, small polydisperse circular DNAs, and microDNAs. They form through
46 several mechanisms including non-allelic homologous recombination (HR), double strand break repair,
47 replication slippage, replication fork stalling, R-loop formation during transcription [1], and as a
48 byproduct of LTR retrotransposon activity [2–4] (Fig. 1A). EccDNAs can accumulate in cells through
49 autonomous replication [5–8], high rates of formation [9], or through retention in ageing cells [10].
50 EccDNAs can contain genes, and amplification of gene-containing eccDNAs has been linked to
51 adaptation to copper [9] and nitrogen [5] stress in yeast, herbicide resistance in weeds [6], and drug
52 resistance in cancer cells [11,12]. EccDNA formation is thought to sometimes cause genomic deletions
53 [5,13,14] and reinsertion of eccDNAs after their formation has also been thought to generate structural
54 variation [15,16]. Some evidence also indicates that eccDNAs could facilitate horizontal gene transfer
55 [16]. Despite their potential as important facilitators of genetic and phenotypic plasticity and presence
56 in all eukaryotes, research efforts, and especially whole circularome sequencing experiments, have been
57 limited to model organisms and human cancer. Therefore, how these molecules behave across the tree
58 of life and how different species could take advantage of these molecules to rapidly adapt to their
59 environments have remained largely unknown.

60



61

62 **Fig. 1.** Comparison of eccDNA formation in *M. oryzae* and other organisms. **A.** Examples of mechanisms of extrachromosomal
 63 circular DNA (eccDNA) formation. **1.** eccDNA formation as a result of double strand break repair. The blue enzyme represents
 64 several different types of DNA repair mechanisms **2.** eccDNA formation as a result of nonallelic homologous recombination
 65 (NAHR). The green boxes represent homologous sequences. **3.** eccDNA formation as a result of LTR retrotransposon activity.
 66 The blue and green enzyme represents RNA polymerase, and the orange enzyme represents a reverse transcriptase (RVT).
 67 Rectangles that are partly blue and partly red represent hybrid LTRs formed from 5' and 3' LTRs during retrotransposition. DNA
 68 is drawn in black and RNA in gray. **B.** Comparison of genome size and number of eccDNA forming regions for *Arabidopsis*
 69 *thaliana* [17], *Oryza sativa* [18], *Homo sapiens* [13], *Saccharomyces cerevisiae* [19], and *Magnaporthe oryzae*. The number of
 70 eccDNA forming regions are shown as called by our pipeline in an average sample. Circularome data for *A. thaliana* and *O.*

71 *sativa* leaf tissue, *H. sapiens* muscle tissue, and *S. cerevisiae* deletion collection samples are shown. The organism and protein
72 icons were created with BioRender.com.

73

74 One of the greatest threats to food security is the devastation of crops by fungal plant pathogens. These
75 pathogens secrete molecules known as effectors to modify host functions and cause disease [20]. The
76 most promising solution to these diseases is the genetic modification of crops by introducing new
77 disease resistance genes, often by allowing the crops to detect effectors and trigger immune responses
78 [21]. Unfortunately, the deployment of disease resistant crops has often had only short-term impacts as
79 some fungal pathogens have adapted to these defenses in very short time spans [22]. Similarly,
80 fungicides are often used to mitigate the devastation caused by pathogens but fungi often evolve drug
81 resistance [23]. A better understanding of how these pathogens adapt and overcome disease prevention
82 efforts so quickly is vital to implementing future strategies. Sequencing and characterization of the
83 genomes of fungal plant pathogens have implicated transposable elements [24], accessory
84 chromosomes [25,26], and horizontal gene transfer [27]. Additionally, the compartmentalized genome
85 architectures of some of these pathogens, commonly referred to as the “two-speed” genome, is thought
86 to facilitate adaptation to stress by harboring stress response genes and disease associated genes,
87 including effectors, in rapidly evolving regions of their genomes that contain few genes and many
88 repetitive elements [28]. Given the potential for eccDNAs to be a source of phenotypic and genotypic
89 plasticity, we sought to characterize the circularome of one of these pathogens to identify if eccDNAs
90 could play a role in the rapid adaptation of the fungal plant pathogen, *Magnaporthe oryzae* (syn.
91 *Pyricularia oryzae*).

92

93 *M. oryzae*, is the causative agent of the rice blast disease [29], has been described as one of the most
94 important fungal pathogens threatening agriculture [30] and is responsible for losses in rice crops

95 equivalent to feeding 60 million people each year [31]. Its ease of culture as well as the importance of
96 this pathogen for global food security have propelled it to being one of the most studied plant
97 pathogens resulting in over three hundred sequenced genomes as well as transcriptomic, and epigenetic
98 datasets in addition to genetic tools including CRISPR/Cas9 mediated genome editing [32]. The
99 availability of these extensive genomic datasets makes *M. oryzae* a prime candidate for understanding
100 the role eccDNAs may play in adaptation to stress in a fungal plant pathogen.

101

102 We present here our analysis of circularome sequencing data for *M. oryzae* and identify eccDNA forming
103 regions in its genome. We describe the high diversity of eccDNA forming regions that we found in the
104 rice blast pathogen and compare it to previously sequenced circularomes. We find that most of the *M.*
105 *oryzae* circularome is made up of LTR retrotransposon sequences and that genes on eccDNAs tend to
106 originate from regions of the genome prone to presence-absence variation. Additionally, our
107 characterization of the genes found on eccDNAs shows that many genes are never found on eccDNAs
108 under the conditions we tested and suggests that selection may shape which genes are found on these
109 molecules. Finally, our analysis reveals that many disease-causing effectors are found on eccDNAs in the
110 pathogen.

111 **Results**

112 **Identification of eccDNA forming regions in *Magnaporthe oryzae***

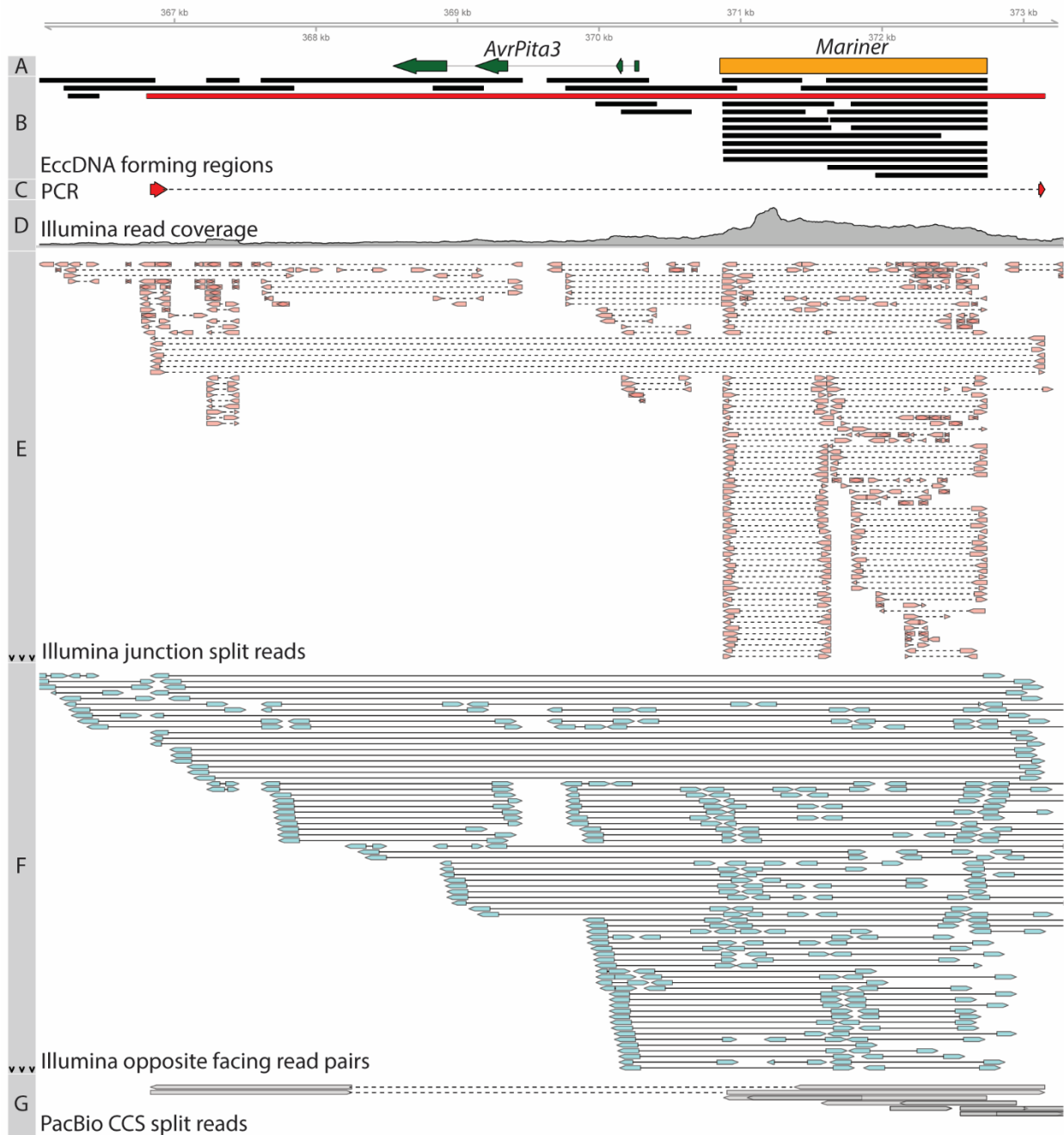
113 To characterize the circularome of *M. oryzae*, eccDNAs were purified and sequenced from pure cultures
114 of *M. oryzae* Guy11 using a protocol adapted from previously published methods [18]. Briefly, after total
115 DNA extraction of 3 biological replicates, linear DNA was degraded from 3 technical replicates for each
116 biological replicate using an exonuclease and the remaining circular DNA was amplified using rolling
117 circle amplification (RCA). Depletion of linear DNA was verified with qPCR using markers to the *M.*

118 *oryzae* actin gene (MGG_03982, Additional File 1: Fig. S1). This gene was used as a marker for linear DNA
119 since increased copies of the ACT1 gene are thought to be deleterious in yeast [19,33]. Isolated eccDNAs
120 were then sequenced using both paired-end Illumina sequencing and PacBio circular consensus
121 sequencing (CCS). In total, we sequenced 8 samples as one technical replicate failed quality checks
122 during library preparation. Illumina sequencing yields were 6.5 Gbp per sample, on average, and PacBio
123 sequencing yields were 8 Gbp (subreads) and 500 Mbp (CCS) per sample, on average.

124

125 To identify specific breakpoints indicating eccDNA formation in our Illumina sequencing data, we
126 developed a pipeline inspired by previously published methods [13]. In circularome sequencing data,
127 split mapping reads originate from sequencing circularization junctions of eccDNAs. Additionally, read
128 pairs in the data that map in the opposite direction represent sequencing from paired-end sequencing
129 fragments that span these circularization junctions. Our pipeline uses split reads in combination with
130 opposite facing read pairs to find evidence of eccDNA formation (Fig. 2). This allowed us to identify, with
131 high confidence, genomic sequences belonging to eccDNAs, which we will hereafter refer to as “eccDNA
132 forming regions.” We will refer to split reads associated with these eccDNA forming regions simply as
133 “junction split reads.” Our analysis was limited to these eccDNA forming regions, rather than the fully
134 resolved structure of each eccDNA molecule, because of the complexity of eccDNAs as well as the
135 techniques used into sequenced them in this study. For example, eccDNAs can sometimes contain
136 multiple copies of the same sequence [34] and our use of RCA, which generates long DNA fragments
137 containing hundreds of tandem repeats of each circular molecule [35], prevents determination of
138 whether a sequence is repeated many times on an eccDNA molecule or just present once. Additionally,
139 eccDNAs have also been shown to assemble with others, forming complex structures [36]. While our
140 long-read PacBio sequencing may have been able to address this issue, our attempts at reference-free
141 assembly of complete eccDNAs were unsuccessful, likely due to insufficient coverage of each molecule.

142 While only eccDNA forming regions could be described in this study, these regions still enable a detailed
143 description of the *M. oryzae* circularome. Across all 8 sequenced samples, our pipeline identified
144 1,719,878 eccDNA forming regions using Illumina paired-end sequencing data (Additional File 3). We
145 validated 8 of these eccDNA forming regions using outward PCR and Sanger sequencing (Fig. 2 and
146 Additional File 1: Fig. S2). These regions were chosen for validation as they fully contained genes of
147 interest to the rest of the study, including well-known effectors.



148

149 **Fig 2.** Summary of evidence supporting an eccDNA forming region of interest in the *M. oryzae* genome. **A.** Location of effector
150 *AvrPita3* and *Mariner* transposon. **B.** Location of eccDNA forming regions. The eccDNA forming region in red was chosen for
151 validation using outward PCR. This eccDNA forming region was considered to fully encompass *AvrPita3*. **C.** Sanger sequencing
152 read generated from outward PCR (Additional File 1: Fig S2) that supports eccDNA forming region highlighted in red in track B.
153 **D.** Overall Illumina sequencing read coverage. **E.** Junction split reads obtained from Illumina data. Split reads are joined by a
154 dashed line. Black arrows indicate not all reads were shown in areas with high counts. **F.** Opposite facing read pairs obtained

155 from Illumina data. Read pairs are joined by a solid line. Black arrows indicate that not all reads were shown in areas with high
156 counts. **G.** Split reads obtained from PacBio CCS data. Overlapping arrows indicate single reads mapped to the same location
157 more than once. Split reads are joined by a dashed line. All data was obtained from a single sequenced sample (biological
158 replicate 1, technical replicate A).

159

160 To determine how similar our technical and biological replicates were to each other, we compared the
161 coordinates of eccDNA forming regions found in each sample. Overall, we found little overlap in eccDNA
162 forming regions between technical replicates (14.16%, 10.09%, and 23.77%, for biological replicates 1, 2
163 and 3, respectively) and between biological replicates (9.41%) when comparing the exact start and end
164 coordinates of these regions (Additional File 1: Fig. S3). Rarefaction analysis showed that these
165 differences could be, at least partially, attributed to under sequencing, though this data could also be
166 evidence of many low copy number eccDNAs being produced by the *M. oryzae* genome (Additional File
167 1: Fig. S4). However, principal component analysis using the coverage of junction split reads throughout
168 the genome showed that technical replicates were more likely to be similar to other technical replicates
169 within the same biological replicate than across biological replicates in the content of their eccDNA
170 forming regions (Additional File 1: Fig. S5). Additionally, while exact coordinates of eccDNA forming
171 regions did not have much overlap between samples, considering eccDNA forming regions whose start
172 and end coordinates were within 100 bp of each other in two different samples to be the same
173 increased this overlap greatly between technical replicates (48.46%, 45.55%, and 58.29% for biological
174 replicates 1, 2 and 3, respectively) and between biological replicates (42.89%) (Additional File 1: Fig. S6).
175 We performed a permutation analysis to simulate random formation of eccDNAs throughout the
176 genome to verify that this result was meaningful and observed little overlap between replicates in this
177 simulated scenario when increasing our overlap tolerance up to 100bp (Additional File 1: Fig. S6). All
178 together, these results, as well as others presented throughout this study suggested that while the exact

179 breakpoints of eccDNA forming regions were not identical across samples, the genomic loci, or hotspots,
180 of eccDNA formation were highly similar.

181

182 Likely due to the great number of different eccDNAs in *M. oryzae*, the coverage of our PacBio
183 sequencing data was too low to enable *de novo* assembly of eccDNA molecules. Therefore, we used our
184 long read data to infer eccDNA forming regions by mapping them to the *M. oryzae* Guy11 genome and
185 comparing these regions to those called using our short read data. This was done using a similar pipeline
186 to the Illumina data with less stringent criteria which was better adapted to the lower read depth of the
187 long read data. Our long read data allowed us to identify 147,335 eccDNA forming regions across all
188 samples (Additional File 4). We compared these eccDNA forming regions to those called using Illumina
189 data, allowing for up to a 10 bp difference between breakpoints to account for mapping ambiguity, and
190 found that, on average, 81.42% of eccDNA forming regions called using PacBio data for one sample were
191 also found in our eccDNA forming regions called using Illumina reads in the same sample (Additional File
192 1: Fig. S7). We were able to attribute much of this discrepancy to our stringent criteria for calling
193 eccDNA forming regions since simply looking for split reads in our Illumina data increased this rate to
194 90.36% (Additional File 1: Fig. S7). The remaining differences are likely due to Illumina reads not being
195 long enough to properly be mapped as split reads in certain regions of the genome. Such strong overlap
196 between eccDNA forming regions called by long reads and short reads demonstrates the robustness of
197 our short read data analysis. Aside from this validation, we chose not to include the PacBio data in our
198 final analyses due to the low read depth.

199

200 Next, we quantified the potential false positive rate of our pipeline that could have originated from any
201 undigested genomic DNA in our samples by running the pipeline on previously published whole genome
202 sequencing data from *M. oryzae* Guy11 [32,37,38]. Based off the number of eccDNA forming regions

203 called from this data, we estimated this false positive rate to be approximately 3 junction split reads per
204 million sequencing reads (Additional File 2: Table S1). In comparison, we found 41,873 junction split
205 reads per million reads in our eccDNA enriched samples, on average, indicating a very low false positive
206 rate from our pipeline. Additionally, we could not completely rule out the presence of eccDNAs in the
207 whole genome sequencing samples we analyzed. This validation showed that any remaining linear DNA
208 in our samples after linear DNA degradation were unlikely to be called as eccDNA forming regions by our
209 pipeline.

210

211 Finally, we benchmarked our pipeline on previously published eccDNA data in human tissue [13]
212 (Additional Files 5 and 6). We found that, on average, 74.62% of eccDNA forming regions called by our
213 pipeline were also described in the published dataset (Additional File 1: Fig. S8A). This number was even
214 higher for eccDNA forming regions associated with 10 or more junction split reads (85.63%). The small
215 fraction of eccDNA forming regions called by our pipeline that did not appear in the published list could
216 not be attributed to how our pipeline handled multi-mapping reads (Additional File 1: Fig. S8A, see
217 Methods) and were likely due to differences in sequence data processing and different criteria for
218 selecting split reads between the two studies [13]. However, the two lists significantly differed in the
219 number of eccDNA forming regions identified, with our pipeline identifying substantially less (Additional
220 File 1: Fig. S8B). This difference can be attributed to our stricter evidence to call eccDNA forming
221 regions. In our method, eccDNA forming regions were only called if split reads mapped to the region.
222 This is in contrast to other methods of calling eccDNA forming regions which rely at least partly on peaks
223 in sequencing coverage [13,19,39]. This meant that our pipeline could not detect eccDNAs formed from
224 HR between identical repeats which do not result in split reads. We chose this method for *M. oryzae*
225 because it showed circularome sequencing coverage throughout the entire genome in our samples and
226 very few clear coverage peaks, which indicates that many low copy number eccDNAs were present in

227 our samples. The high degree of overlap between our called eccDNA forming regions and those
228 described by Møller *et al.* makes us confident that the eccDNA forming regions we called using our
229 pipeline are robust.

230

231 **The *M. oryzae* circularome is more diverse and contains more noncoding sequences than the** 232 **circularomes of other organisms**

233 We were first interested in comparing the circularome of *M. oryzae* to those of other previously
234 characterized organisms. To compare these datasets across different organisms, we gathered
235 sequencing data from several previous studies [13,17–19] and reanalyzed them using our pipeline
236 (Additional Files 5-20). Our analysis revealed a very large number of eccDNA forming regions in *M.*
237 *oryzae* compared to other previously sequenced organisms (Fig. 1B). We also looked at the percentage
238 of the genome that was found in eccDNA forming regions and found that, while most organisms had 1-
239 10% of their genome in eccDNA forming regions, our samples showed an average of 74.48% of the *M.*
240 *oryzae* genome in eccDNA forming regions (Additional File 1: Fig. S9A). The difference in the number of
241 eccDNA forming regions between organisms was still striking after normalizing for genome size and
242 sequencing library size (Additional File 1: Fig. S9B). These results supported the idea that the low
243 amount of overlap in eccDNA forming regions between our samples could be explained partly by the
244 great number of eccDNAs produced by the *M. oryzae* genome. While the difference in the number of
245 called eccDNA forming regions could be attributed to differences in the methods used for eccDNA
246 purification (Additional File 2: Table S2), we extracted and sequenced eccDNAs from *Oryza sativa* and
247 found similar levels of diversity to previously published samples (Additional File 1: Fig. S9B). We also
248 found that *M. oryzae* had more eccDNA forming regions made up of noncoding sequences relative to
249 the percentage of noncoding sequence in its genome than other organisms aside from *S. cerevisiae* (Fig.
250 1B, Additional File 1: Fig. S9C).

251

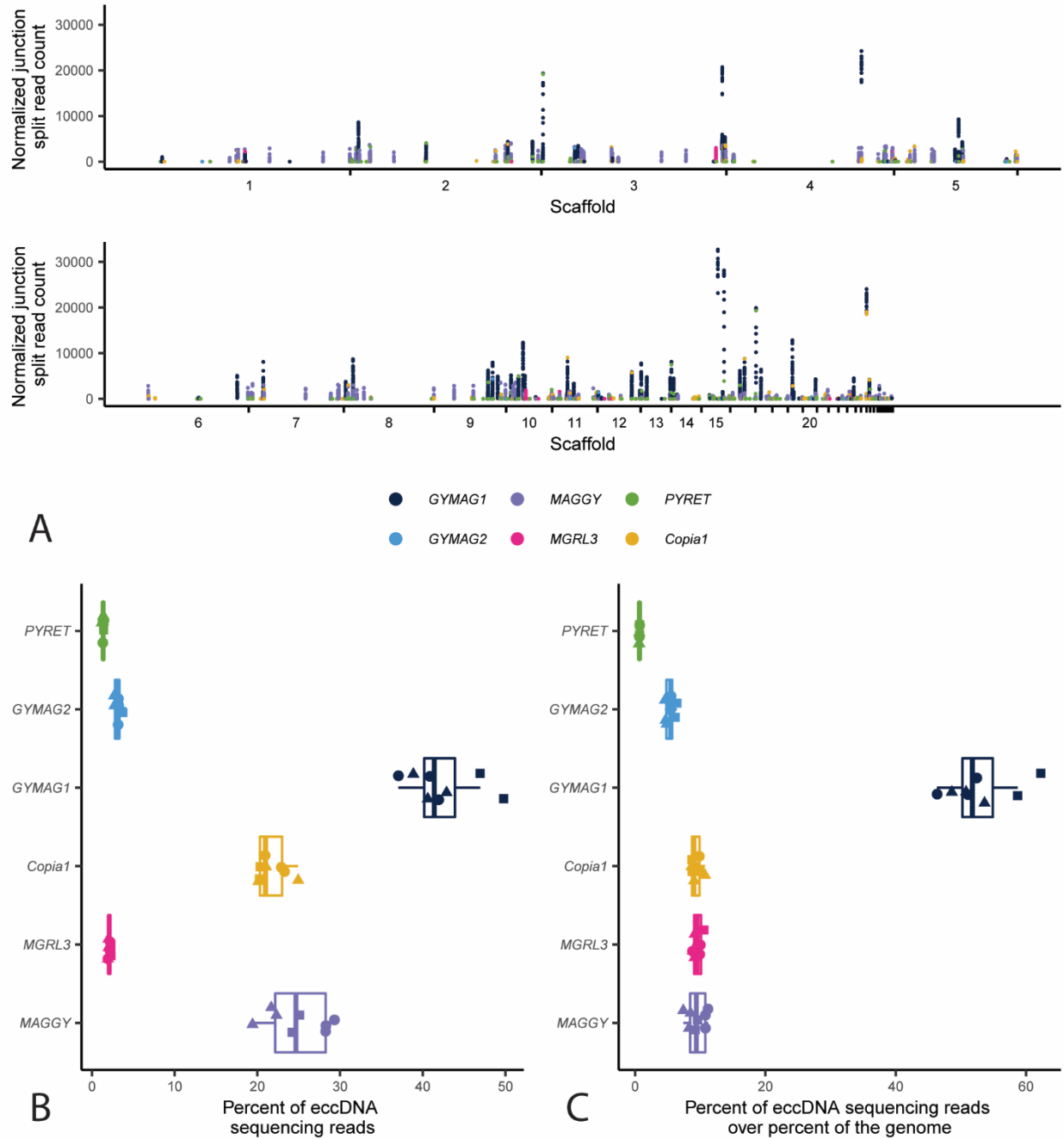
252 **LTR retrotransposon sequences make up most of the *M. oryzae* circularome**

253 *Gypsy* and *Copia* LTR retrotransposons frequently generate eccDNAs through several mechanisms [2–4],
254 so we looked for the presence of these sequences in the *M. oryzae* circularome. Our analysis revealed
255 that 54.12% of the eccDNA forming regions we identified seemed to be composed of more than 90%
256 LTR retrotransposon sequence indicating that these elements made up a large portion of the pathogen’s
257 circularome despite only making up a small fraction of its genome (Fig. 1B, Additional File 1: Fig. S10).
258 Further comparative analysis revealed that a much higher proportion of the *M. oryzae* circularome was
259 made up of these LTR retrotransposon sequences than in other organisms (Fig. 1B, Additional File 1: Fig.
260 S9D and S9E).

261

262 All six LTR retrotransposons identified in *M. oryzae* Guy11 formed eccDNAs (Fig. 3A). However, the
263 elements *MAGGY*, *GYMAG1*, and *Copia1* made up the majority of the eccDNA sequencing data (Fig. 3B).
264 When this data was normalized to the proportion of the genome made up by each transposon, *GYMAG1*
265 stood out as making up a much greater percentage of the sequencing data than expected (Fig. 3C,
266 Additional File 1: Fig. S11).

267



268

269 **Fig. 3.** The majority of eccDNAs in *M. oryzae* are made up of LTR retrotransposons. **A.** Manhattan plot showing the number of
 270 junction split reads per million averaged across biological replicates for all 100 bp bins that overlap an LTR retrotransposon in
 271 the *M. oryzae* Guy11 genome. Each point represents one of these bins. **B.** Boxplot showing the percentage of sequencing reads
 272 that map to LTR retrotransposons. Each point represents one sample, and the shape of the points represent the biological
 273 replicate that sample was taken from. **C.** Boxplot showing the ratio of the percentage of sequencing reads that map to LTR

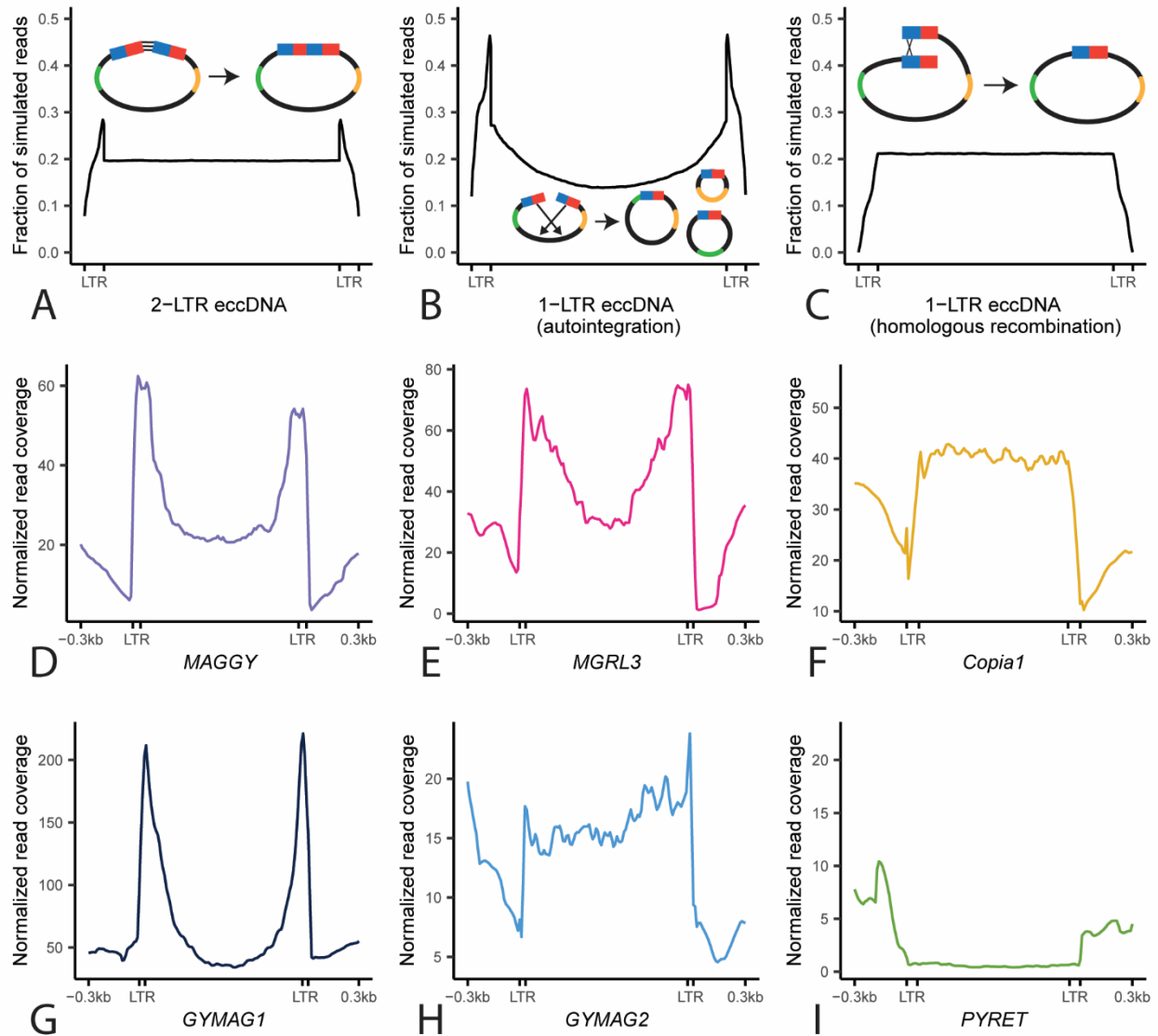
274 retrotransposons to the percentage of the *M. oryzae* Guy11 genome that is made up by that retrotransposon. Each point
275 represents one sample, and the shape of the points represent the biological replicate that sample was taken from.

276

277 **LTR retrotransposons in *M. oryzae* form eccDNAs through a variety of mechanisms**

278 LTR retrotransposons can form eccDNAs through a variety of mechanisms [2–4]. EccDNA formation
279 commonly occurs after transcription and reverse transcription of the transposon which results in a linear
280 fragment of extrachromosomal DNA [40] (Fig. 1A). Then, the most common circularization mechanisms
281 are nonhomologous end joining (NHEJ) of the two LTR ends to form eccDNAs containing 2 LTRs (scenario
282 1, Fig. 4A), autointegration of the retrotransposon forming single LTR eccDNAs of various lengths,
283 depending on where in the internal sequence of the transposon the autointegration event happens
284 (scenario 2, Fig. 4B), and HR between the two LTRs to forming single LTR eccDNAs (scenario 3, Fig. 4C).
285 Finally, LTR retrotransposon sequences can also become part of eccDNAs by other eccDNA formation
286 mechanisms that do not rely on retrotransposition activity, such as intrachromosomal HR between solo-
287 LTRs or between multiple copies of the same transposon [4,5,19]. Given this diversity of mechanisms,
288 we wanted to evaluate which of them contributed to eccDNA formation in *M. oryzae*. To do this, we first
289 simulated the expected read coverage for each of the three active LTR eccDNA formation mechanisms
290 under ideal conditions where only one mechanism of formation was occurring (Fig. 4A-C). Then, we
291 measured the prevalence of scenarios 1 and 2 by identifying specific split read variants in our data. LTR
292 eccDNAs formed through NHEJ result in split reads that map to one end of an LTR and the other which
293 we will refer to as LTR-LTR split reads (Additional File 1: Fig. S12 and S14A). Autointegration results in
294 split reads that map to one LTR and to the internal region of the transposon which we will refer to as
295 LTR-internal split reads (Additional File 1: Fig. S13 and S14B). HR between two identical LTRs (scenario 3)
296 would not result in a split read so we could not find this type of evidence in our data.

297



298

299 **Fig. 4.** LTR retrotransposons in *M. oryzae* form eccDNAs through a variety of mechanisms. **A-C.** Profile plots showing expected
300 sequencing read coverage for each LTR retrotransposon eccDNA formation scenario as well as graphical representations of the
301 scenario. In the graphics, blue and red rectangles represent hybrid LTRs formed from 5' and 3' LTRs during retrotransposition
302 and green and orange lines represent areas of the internal region of the retrotransposon with distinct sequences. **D-I.** Profile
303 plots showing observed sequencing read coverage for each LTR retrotransposon found in the *M. oryzae* Guy11 genome.

304

305

306 Comparisons between simulated and observed read coverage plots revealed contributions of several
307 eccDNA formation mechanisms that varied by transposable element. For *MAGGY*, our analysis indicated
308 that it forms eccDNAs primarily through autointegration (Fig. 4D). This was supported by a high
309 correlation between the number of sequencing reads and LTR-internal split reads (Additional File 1: Fig.
310 S13A) and a low correlation between sequencing reads and LTR-LTR split reads (Additional File 1: Fig.
311 S12A). The data also pointed to *MGRL3* and *GYMAG1* forming eccDNAs primarily through
312 autointegration (Fig. 4E and 4G, Additional File 1: Fig. S12BD and S13BD). *Copia1*, on the other hand
313 showed a clear pattern of read coverage corresponding to eccDNA formation through HR (Fig. 4F),
314 though the high correlation between sequencing reads and LTR-internal split reads mapping to this
315 element hinted that a small, but proportional, fraction of *Copia1* elements formed eccDNAs through
316 autointegration (Additional File 1: Fig. S13C). In the case of *GYMAG2*, its sequencing read coverage
317 resembled a pattern expected for LTR-eccDNAs formed through NHEJ (Fig. 4H). The large amount of LTR-
318 LTR split reads per million mapped reads found corresponding to *GYMAG2* elements compared to other
319 retrotransposons supported this inference (Additional File 1: Fig. S14A). *PYRET*'s distinct sequencing
320 read coverage profile likely indicated that it mostly formed eccDNAs by other eccDNA formation
321 mechanisms that do not rely on retrotransposition activity such as intrachromosomal HR (Fig. 4I). A low
322 correlation between sequencing read coverage and both LTR-LTR split reads and LTR-internal split reads
323 as well as the fragmented nature of *PYRET* elements, which is a sign of low recent retrotransposon
324 activity, supported this inference (Additional File 1: Fig. S12F and S13F). Finally, to determine whether
325 the results we obtained were caused by bias in the length and completeness of the retrotransposon
326 sequences in the *M. oryzae* genome, we generated profile plots for each retrotransposon using
327 previously generated whole genome sequencing data [32,37,38]. The results from this analysis ruled out
328 this possibility (Additional File 1: Figure S15). In conclusion, it is clear that a variety of eccDNA formation

329 mechanisms contributed to eccDNAs containing LTR retrotransposon sequences and that these
330 mechanisms varied by element.

331

332 **MicroDNAs are distinct from other eccDNAs**

333 MicroDNAs have previously been studied as a distinct set of molecules within the eccDNA category.

334 Besides being small (less than 400bp), microDNAs are found to be enriched in genic regions, exons,

335 5'UTRs and CpG islands [14,41]. We examined if microDNAs in *M. oryzae* showed these characteristics

336 by analyzing eccDNA forming regions less than 400 bp in length with less than 10% LTR retrotransposon

337 sequence across different organisms. Enrichment of microDNAs in CpG islands was the most consistent

338 result across all organisms we analyzed, though this enrichment was not found in *M. oryzae* (Additional

339 File 1: Fig. S16). Similarly, we found no enrichment of microDNAs in 5'UTRs in *M. oryzae*. We did

340 however find a small enrichment of microDNAs in genic regions in *M. oryzae* as in many of the other

341 sequenced organisms (Additional File 1: Fig. S16 and S17). In general, our analysis suggested that the

342 previously described characteristics of microDNAs are not common across all organisms and sample

343 types.

344

345 MicroDNAs also displayed distinct features from the remaining subset of non-LTR eccDNAs which we

346 called large eccDNAs. Among other differences, we found that, unlike microDNAs, large eccDNAs tended

347 to be enriched in intergenic regions (Additional File 1: Fig. S17 and S18). Additionally, eccDNAs are often

348 associated with active transcription [1,9], and we found a slight but significant correlation between

349 expression and junction split reads for large eccDNAs but not for microDNAs (Additional File 1: Fig. S19).

350

351 In yeast, eccDNA amplification is thought to often occur with the help of autonomously replicating

352 sequences (ARSs) which contain ARS consensus sequences (ACs) [5,19,42]. In *M. oryzae*, we found that

353 ACSs were enriched in large eccDNAs (permutation test, mean of expected: 5320.14 regions, observed:
354 6950 regions, $p < 0.01$, $n = 100$ replicates) but depleted in microDNAs (permutation test, mean of
355 expected: 818.09 regions, observed: 714 regions, $p < 0.01$, $n = 100$ replicates). However, for both large
356 eccDNAs and microDNAs, presence of an ACS in the eccDNA forming region did not result in an
357 increased number of junction split reads (Additional File 1: Fig. S20). Finally, microDNAs have been
358 found to be associated with chromatin marks and increased GC content [14,41]. However, we did not
359 find any of these enrichments in microDNAs or large eccDNAs in *M. oryzae* (Additional File 1: Fig. S21).

360

361 **Many genes are found encompassed by eccDNA forming regions**

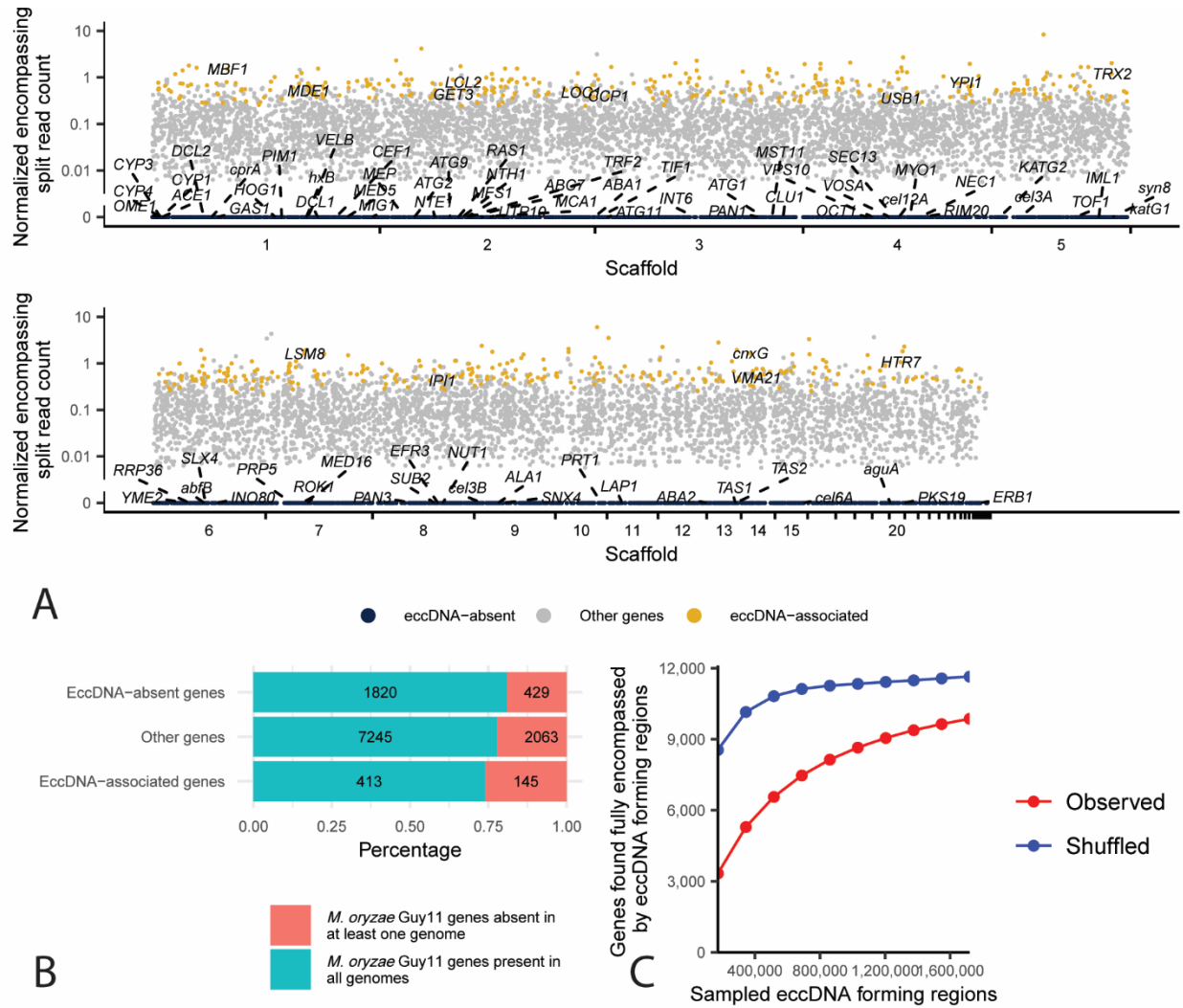
362 Many eccDNAs contain genes and these eccDNAs can provide genotypic and phenotypic plasticity in
363 other organisms. In *M. oryzae* we found that, out of the 12,115 genes in Guy11, 9,866 were fully
364 contained by an eccDNA forming region in at least one sample (for an example, see Fig. 2B). These genes
365 included *TRF1* (MGG_04843) and *PTP2* (MGG_00912) which have been shown to be involved in
366 fungicide resistance in *M. oryzae* [43,44]. EccDNA forming regions containing these two genes were
367 validated using outward PCR (Additional File 1: Fig. S2). However, not all genes were observed in
368 eccDNA forming regions at the same frequency and their presence on eccDNAs was heterogenous
369 across samples. To further understand what types of genes are enriched in eccDNA forming regions, we
370 focused on a robust set of eccDNA-associated genes. To identify these genes, we first counted the
371 number of times each gene was found fully contained by a junction split read in each sample. We
372 referred to this count as the number of “encompassing split reads” for each gene. We then normalized
373 this count to the number of junction split reads in each sample and averaged it across technical
374 replicates for each biological replicate. Finally, we sorted the genes by their prevalence in each biological
375 replicate and chose genes that were found in the top third of genes for this count in all three biological

376 replicates. In total, using these metrics, we identified 558 eccDNA-associated genes shared across all
377 biological replicates (Additional File 1: Fig. S22 and Additional File 21).

378

379 To identify biological processes enriched in eccDNA-associated genes, we performed gene ontology (GO)
380 enrichment analysis. We found that eccDNA-associated genes were enriched for GO terms related to
381 vesicle transport, mitosis, and the cytoskeleton among other terms (Fig. 6A, Additional File 1: Fig. S23
382 and Additional Files 22-24). We also explored whether eccDNA-associated genes showed differences in
383 gene expression or other genomic features from other genes. However, we found no difference
384 between eccDNA-associated genes and other genes in gene expression, GC content, or histone marks,
385 aside from a significant difference in H3K36me3 (Additional File 1: Fig. S24 and S25).

386



387

388 **Fig. 5.** EccDNA forming regions contain most *M. oryzae* genes, but not all, and many are associated with presence-absence

389 variation. **A.** Manhattan plot showing the number of encompassing split reads per million junction split reads averaged across

390 biological replicates for each gene in the *M. oryzae* Guy11 genome. Each dot represents one gene. EccDNA-associated genes

391 with known gene names are labeled according to their normalized encompassing split read count and position in the genome.

392 EccDNA-absent genes with known gene names are labeled with lines pointing to their location in the genome. **B.** Stacked bar

393 plot showing the percentage of eccDNA-absent genes, other genes, and eccDNA-associated genes in the *M. oryzae* Guy11

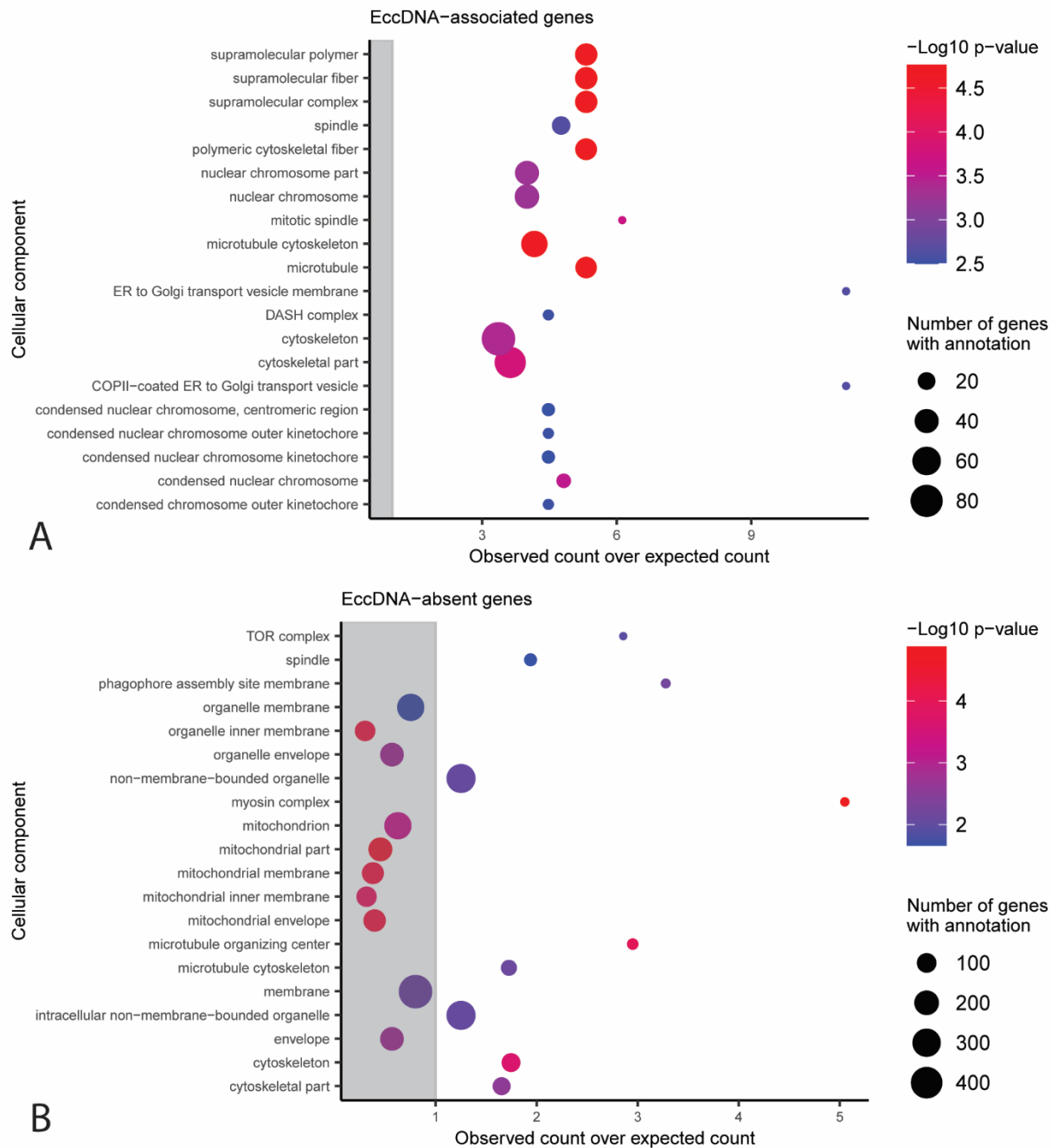
394 genome that had an ortholog in all other 162 *M. oryzae* genomes analyzed or not. Numbers indicate the number of genes in

395 each category. **C.** Rarefaction analysis of the observed number of genes found fully encompassed by eccDNA forming regions at

396 different subsamples of all found eccDNA forming regions, compared to the same number of randomly selected genomic

397 regions.

398



399
400 **Fig. 6.** GO terms associated with eccDNA-associated and eccDNA-absent genes in *M. oryzae*. Functional categories in the
401 cellular component Gene Ontology with an observed number of **A.** eccDNA-associated genes or **B.** eccDNA-absent genes that is
402 significantly different from the expected number with correction for gene length bias. The y-axis shows the different functional
403 categories, and the x-axis represents the observed number of genes divided by the expected number of genes in this group.
404 Dots outside of the grey rectangle represent functional categories that are observed more often than expected. The size of dots

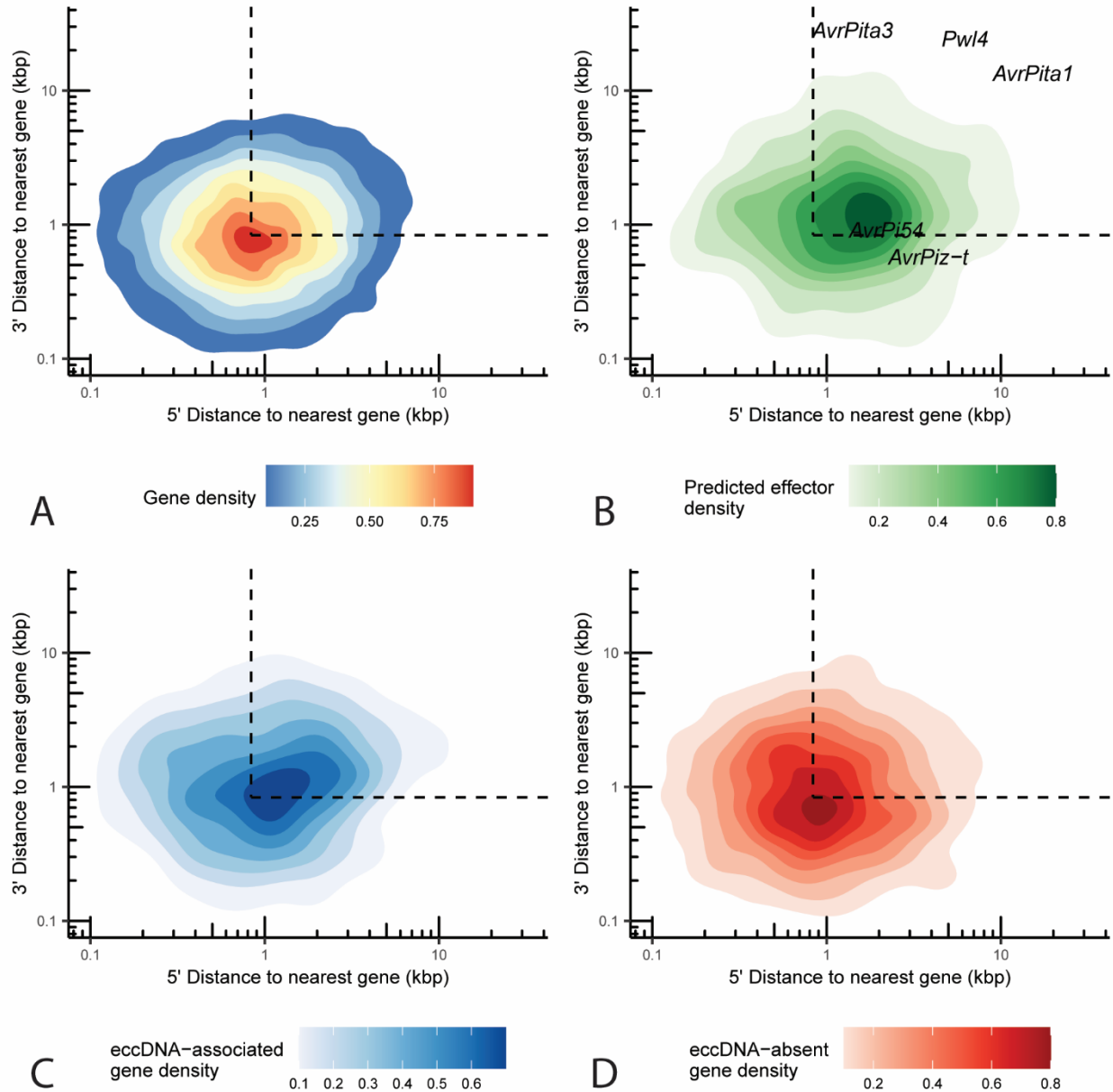
405 indicates the total number of genes in the *M. oryzae* genome that belong to each functional category. Only the 20 categories
406 with the largest $-\log_{10}$ p-values according to a Chi-square test are shown.

407

408 **EccDNA-associated genes are closer to gene sparse and repeat dense regions of the genome than**
409 **other genes**

410 Some plant pathogens are described as having “two-speed” genomes with housekeeping genes found
411 close together in repeat-poor regions and environmentally responsive and disease-associated genes
412 found in repeat-dense and gene-poor regions [28]. To determine if eccDNA-associated genes were
413 enriched in either of these genomic contexts, we analyzed if eccDNA-associated genes were more
414 distant from other genes than expected by chance (Fig. 7). We observed a significant difference
415 (permutation test for difference of medians, $p = 0.0117$, $n = 10,000$ replicates) between the median
416 distance to the nearest gene of eccDNA-associated genes (543 base pairs) and other genes (485 base
417 pairs). We also observed a significant difference (permutation test for difference of medians, $p =$
418 0.0282 , $n = 10,000$ replicates) between the median distance to the nearest genomic repeat of eccDNA-
419 associated genes (663 base pairs) and other genes (769 base pairs, Additional File 1: Fig. S26). This
420 difference in proximity was not observed for transposable elements, indicating that transposable
421 elements alone were not responsible for this effect (Additional File 1: Fig. S27). The heterogeneity of
422 eccDNAs and the mechanisms of their formation might be influencing this comparison. However, our
423 data points to a link between genome architecture and eccDNA formation.

424



425

426 **Fig. 7.** EccDNA-associated genes are often found in gene sparse regions of the *M. oryzae* genome. Two-dimensional density

427 plot representing the 5' and 3' distance to the nearest gene in the *M. oryzae* Guy11 genome in kilobase pairs for each **A.** gene,

428 **B.** predicted effector, **C.** eccDNA-associated genes, and **D.** eccDNA-absent genes. Known effectors are shown as text in **B.**

429 Dashed lines represent median 5' and 3' distance to nearest gene.

430

431 **EccDNA-associated genes are more prone to presence-absence variation than other genes**

432 There is evidence of eccDNAs generating structural variation in other organisms [15,16]. We therefore
433 tested whether eccDNA formation is associated with genes prone to presence-absence variation in 162
434 rice-infecting *M. oryzae* isolates (Additional File 25). As expected from previous studies [45,46], our
435 analysis indicated that predicted effectors were more likely to experience presence-absence variation
436 (Additional File 1: Fig. S28; X-squared = 146.33, df = 1, p-value < 2.2e-16). We also found that eccDNA-
437 associated genes were more likely to be prone to presence-absence variation (Fig. 5B; X-squared =
438 16.262, df = 2, p-value = 2.95e-04). This result suggested that eccDNA formation and structural variation
439 occur in similar regions of the genome but did not show whether they are directly linked. To see if a
440 more direct link existed, we surveyed the genomes of the *M. oryzae* isolates for small deletions that
441 completely or partially overlapped genes but did not disrupt neighboring genes. We were able to
442 identify 257 such events (Additional File 26). However, none of these deletions matched our eccDNA
443 forming regions and only 8 of them came within 50 bp. Our rarefaction analyses revealed that there is
444 likely to be a much greater diversity of eccDNAs than what we were able to capture at the sequencing
445 depth of this study, whether we considered samples individually or as a whole (Additional File 1: Fig. S4
446 and S29). Therefore, eccDNA formation that could have contributed to structural variation might have
447 been missed due to either under sequencing or they could have been missing in the conditions tested in
448 this study.

449
450 Similarly, we were interested in identifying any potential DNA translocations that may have occurred
451 through an eccDNA intermediate. While we were able to successfully construct a bioinformatics pipeline
452 that identified one previously described eccDNA-mediated translocation in wine yeast [16] (Additional
453 File 1: Fig. S30), we were unable to identify any such examples in any of the *M. oryzae* genomes we
454 analyzed despite including isolates infecting a variety of hosts in this analysis (306 genomes in total,
455 Additional File 27).

456

457 Finally, since mini-chromosomes have been hypothesized as playing important roles in fungal plant
458 pathogen evolution, we also sought to determine whether genes that were previously found on *M.*
459 *oryzae* mini-chromosomes were associated with eccDNA formation but found no such effect (Additional
460 File 1: Fig. S31).

461

462 **Many eccDNA-absent genes are myosin-complex related**

463 Since most *M. oryzae* genes appeared in eccDNA forming regions in at least one sample, we were
464 particularly interested in the 2,249 genes that never appeared fully encompassed by an eccDNA forming
465 region in any of our technical or biological replicates, which we called eccDNA-absent (Additional File
466 21). We first verified that eccDNA-absent genes were not caused by insufficient sequencing coverage
467 using rarefaction analysis. This analysis differed significantly from our previous ones (Additional File 1:
468 Fig. S4 and S29). Here, we counted the number of genes found in eccDNA forming regions at various
469 subsamples of eccDNA forming regions. This analysis revealed that our observations of eccDNA-absent
470 genes were unlikely to be caused by the under sequencing we described previously as the number of
471 genes found fully encompassed by eccDNA forming regions appeared to plateau at larger subsamples of
472 eccDNA forming regions (Fig. 5C). Additionally, a permutation analysis showed that, given the high
473 coverage of our data, we only expected to find 468 genes in this category by chance, which is far fewer
474 than the 2,249 genes we observed (Fig. 5C).

475

476 We next explored whether gene expression or other genomic features could explain the observed
477 eccDNA-absent genes. However, we found no strong differences between eccDNA-absent genes and
478 other genes in gene expression, GC content, or histone marks (Additional File 1: Fig. S24 and S25).

479 EccDNA-absent genes also did not differ from other genes in terms of their distance to the nearest gene,
480 repeat or transposable element (Fig. 7, Additional File 1: Fig. S26 and S27).

481

482 Finally, we performed GO enrichment analysis on these genes and found, amongst many other enriched
483 terms, that genes related to cytoskeletal proteins, and especially the myosin complex, were enriched
484 within eccDNA-absent genes (Fig. 6B, Additional File 1: Fig. S32, and Additional Files 28-30). While genes
485 related to the cytoskeleton were also enriched among eccDNA-associated genes, these were related to
486 mitosis and microtubule polymerization, rather than the myosin complex (Fig. 6A, Additional File 1: Fig
487 S23). This result is of particular interest given that the actin gene has been used in a previous study [19]
488 as well as this one, as a marker for linear DNA due to its negative fitness effect at high copy numbers in
489 yeast [33]. As expected, the *M. oryzae* actin gene (MGG_03982) was one of the eccDNA-absent genes,
490 meaning it was never found in an eccDNA forming region in its entirety in any of our samples.

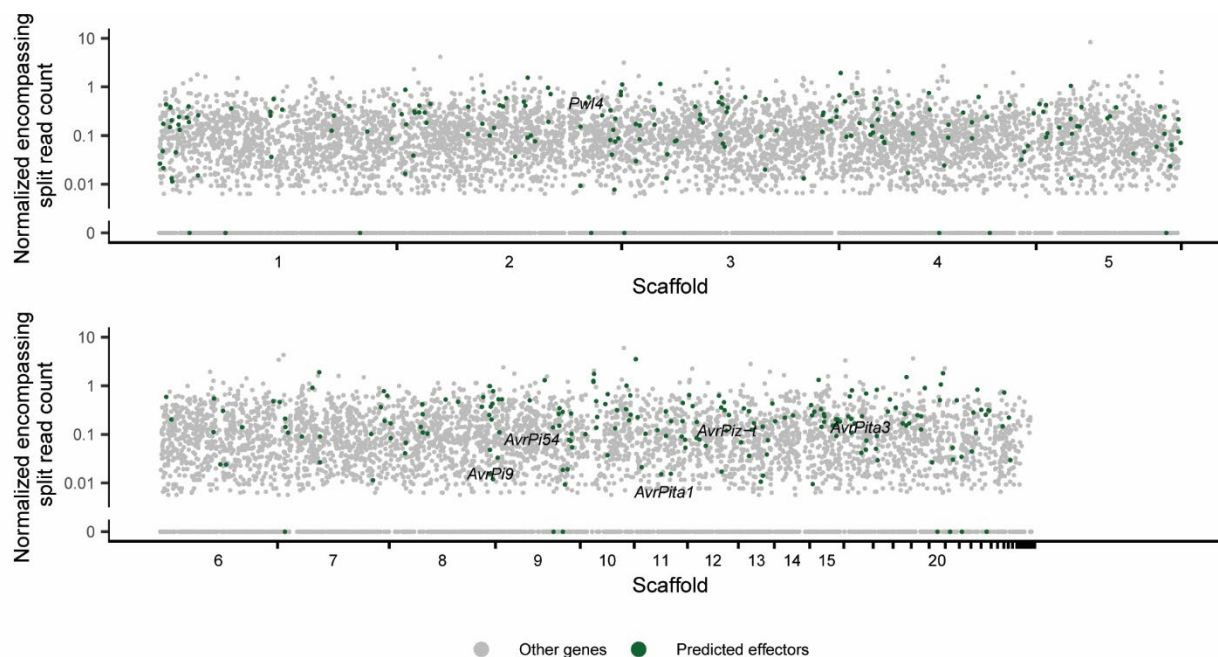
491 Furthermore, in agreement with our GO enrichment results, *MYO1* was also one of these eccDNA-
492 absent genes. To validate our bioinformatics analysis, we tested whether we could amplify the full
493 sequences of these genes from our eccDNA samples using PCR. In agreement with our findings, we were
494 only able to amplify these sequences from our genomic DNA sample (Fig. S33). These results suggested
495 that eccDNA formation is not random in *M. oryzae* and that certain groups of genes may be protected
496 from eccDNA formation or maintenance of these eccDNAs in the cell.

497

498 **Effectors are enriched in eccDNA forming regions compared to other genes**

499 Finally, we wanted to identify whether eccDNA forming regions contained disease-causing effectors. We
500 found that many known *M. oryzae* effectors were encompassed by eccDNA forming regions in at least
501 one sample. This included *AvrPita3*, *AvrPita1*, *AvrPi9*, *AvrPi54*, *AvrPiz-t*, and *Pwl4* (Fig. 2 and 8 and
502 Additional File 21). We validated eccDNA forming regions containing these effectors using outward PCR

503 (Additional File 1: Fig. S2). Additionally, we found that many predicted effectors were found in eccDNA
504 forming regions (Fig. 8 and Additional File 21). We also found that many of these putative effectors were
505 associated with larger numbers of encompassing split reads and found this difference to be statistically
506 significant (Additional File 1: Fig S34; permutation test for difference in medians, $p < 0.0001$, $n = 10,000$
507 replicates). Effectors are often small genes and, given the often-small size of eccDNA forming regions in
508 our data, which may have been caused by the bias of RCA towards small molecules [1,47] (Additional
509 File 1: Fig. S35), we felt that our analysis could be affected by this bias. To address this issue, we
510 repeated our permutation test, comparing predicted effectors to a set of non-effectors of similar lengths
511 and again found a significant difference in number of encompassing split reads (permutation test for
512 difference in medians with correction for gene length distribution, $p = 0.0206$, $n = 10,000$ replicates).
513 This result suggests that effectors are more likely to be found on eccDNAs than other genes in *M. oryzae*
514 and that this effect is not simply due to their size. Additionally, a small proportion of effectors are found
515 among our eccDNA-absent genes (Fig. 8). These candidates might be more evolutionarily stable and
516 therefore useful as targets for disease resistance.
517



518

519 **Fig. 8.** Effectors are enriched in eccDNAs in *M. oryzae*. Manhattan plot showing the number of encompassing split reads per
520 million reads averaged across biological replicates for each gene in the *M. oryzae* Guy11 genome. Each dot represents one
521 gene. Predicted effectors are shown in green and known effectors are shown as text.

522

523 Discussion

524 EccDNAs have been shown to be a source of significant phenotypic [5,6,9,11,12] and genotypic [15,48]
525 plasticity that can help organisms adapt to stress. While eccDNAs have been extensively studied in
526 human cancer [1], very few studies have attempted to study the circularome of other organisms, and
527 even fewer have generated high quality whole circularome sequencing data. To expand our
528 understanding of eccDNAs across the tree of life, we studied the circularome of the fungal plant
529 pathogen *M. oryzae* and, through this analysis, developed many tools to analyze whole circularome
530 sequencing data, which can often be difficult to interpret. These include a new pipeline to identify
531 eccDNA forming regions and frameworks for comparing this data across organisms, identifying
532 mechanisms of eccDNA formation of LTR retrotransposons, identifying gene sets enriched or depleted in

533 eccDNAs, and identifying structural variants that may have been caused by eccDNAs. Our analysis also
534 revealed that the circularome of *M. oryzae* contains a wide diversity of eccDNA forming regions that
535 appeared to exceed those of other previously characterized organisms. This wide diversity likely
536 contributed to under sequencing of our samples and a small overlap in exact eccDNA forming regions
537 across samples. However, our analysis throughout this study showed that our samples clustered tightly
538 together with regards to various features of the circularome, indicating that while exact eccDNA forming
539 breakpoints were mostly not shared across samples, eccDNA formation hotspots were. We also found
540 that eccDNA forming regions in *M. oryzae* were more commonly made up of LTR retrotransposons than
541 other organisms. Though the results of our comparative analysis need to be verified using standardized
542 protocols, these differences highlight the need to further characterize the circularome of other
543 eukaryotes to obtain a better understanding of how they differ. Additionally, it is important to note that
544 the data analyzed in this study only represent snapshots of the circularomes of the organisms described
545 and could vary greatly across developmental stages and environmental stresses that were not included
546 in these analyses. Further studies of eccDNAs across these different conditions are necessary to
547 definitively describe and compare these molecules across organisms.

548

549 We analyzed the types of genes that were found on eccDNAs in *M. oryzae* and found that eccDNA-
550 associated genes were often prone to presence-absence variation, hinting at a link between eccDNAs
551 and genomic plasticity. However, we could not find direct evidence of gene deletions occurring through
552 an eccDNA intermediate in *M. oryzae*. Similarly, we could not find any evidence of eccDNA-mediated
553 translocations. These results could be due to our sequencing coverage and our bioinformatics pipelines
554 not showing the full diversity of eccDNAs in *M. oryzae*. For example, our pipeline was unable to detect
555 eccDNAs formed from HR between perfect repeats. Additionally, our scripts were able to identify an
556 eccDNA-mediated translocation in wine yeasts but were limited to non-repetitive regions of the genome

557 and may have missed some of these events in those regions in *M. oryzae*. Finally, it is possible that
558 eccDNA-mediated translocations occur on a larger time scale than what we were able to sample within
559 the *M. oryzae* species. However, it is likely that experimental approaches, including inducing the
560 formation of specific eccDNAs, are necessary to determine whether these events lead to chromosomal
561 deletions or rearrangements. On a genome-wide scale, single cell sequencing of the circularome as well
562 as genomic DNA could also lead to a more precise view of eccDNA formation and structural variation as
563 they occur in the cell during vegetative growth. These techniques will likely also need to be paired with
564 amplification-free eccDNA sequencing protocols as well as high coverage, long read sequencing to fully
565 resolve the structure of eccDNA molecules. Additionally, we found that eccDNA-associated genes
566 presented characteristics associated with the gene-sparse, repeat-rich, and “fast” part of the plant
567 pathogen genome where rapid adaptation to stress occurs [28]. The fact that eccDNA-associated genes
568 were closer to repeats than other genes, but not transposons specifically, indicated that this effect was
569 not simply caused by eccDNA formation by LTR retrotransposons. We also found that predicted
570 effectors were enriched in eccDNA forming regions. These results show that eccDNA formation occurs in
571 the same genomic contexts as rapid genome evolution in *M. oryzae* and could also point to eccDNAs
572 directly playing a role in the plasticity of important genes like effectors.

573

574 We also identified a set of eccDNA-absent genes, which were never found fully encompassed by eccDNA
575 forming regions under our experimental conditions. This observation was not explained by incomplete
576 sequencing. Histone marks, expression and proximity to repetitive DNA did not appear to set these
577 genes apart either. Though it is possible that other factors contribute to this phenomenon and directly
578 prevent eccDNA formation in these regions, our data indicates that eccDNA formation in *M. oryzae* is
579 not a random process and hints at selective pressure acting against cells that accumulate high copy

580 numbers of these genes through eccDNA formation. This idea is supported by the absence of genes
581 related to the myosin complex, which are deleterious at high copy numbers in other organisms.
582
583 Selective pressure during growth under stress could favor *M. oryzae* cells containing higher copy
584 numbers of genes important for survival under these conditions as has been extensively shown in other
585 organisms [5,6,9,11,12]. For example, we identified two genes associated with fungicide resistance in
586 our eccDNA forming regions, which, if amplified, could lead to drug resistance, as previously observed
587 [6,11,12]. Further experimentation and characterization of the *M. oryzae* circularome under stress is
588 necessary to investigate if this eccDNA-mediated phenotypic plasticity is present in the plant pathogen.
589 These experiments could also be used to assess how LTR retrotransposon activity changes in response to
590 stress in *M. oryzae* and how the mechanisms of eccDNA formation that we described might be affected.
591 We attempted to perform such experiments by sequencing *O. sativa* tissue infected by *M. oryzae* but
592 found that *O. sativa* eccDNAs crowded out the circularome sequencing signal and prevented meaningful
593 analysis, highlighting the need for a dedicated enrichment or single cell sequencing protocol.
594 Additionally, analyzing the biological significance of the amplification of specific genes on eccDNAs,
595 especially across treatments, may prove challenging and will require further tool development. For
596 example, the same genes may be on eccDNAs of varying sizes and composition across samples. Multiple
597 genes could also be on each eccDNA, further complicating the analysis. The complexity of eccDNAs
598 combined with the limitations of current eccDNA sequencing techniques severely limits the analysis of
599 circularome sequencing data, which is why we chose to focus our analysis on hotspots of eccDNA
600 formation and groups of genes rather than individual genes. In the future, high coverage, long read
601 sequencing of eccDNAs collected without amplification will likely be necessary to perform more
602 thorough analyses of eccDNAs, and this type of study is likely to become the gold standard for the field
603 once cost is no longer prohibitive.

604

605 **Conclusion**

606 This study commences the characterization of the *M. oryzae* circularome and highlights its potential for
607 generating phenotypic and genotypic plasticity. If eccDNAs were to facilitate these phenomena, they
608 could become potential drug targets to prevent the rapid adaptation of the blast pathogen to
609 environmental stress, fungicides, and resistant crop varieties. Furthermore, regions and genes prone to
610 forming eccDNAs could be excluded as drug targets or as targets for engineered resistance in crops. On
611 the other hand, we found 1,820 genes including several predicted effectors in the *M. oryzae* genome
612 that were conserved in all other rice infecting isolates that we analyzed and that were in the eccDNA-
613 absent group. These genes could be high potential targets for fungicide design or engineered resistance.
614 Our study also describes the great diversity of eccDNAs and the enrichment of LTR retrotransposons in
615 the *M. oryzae* circularome. These observations, in addition to the potential consequences of eccDNA
616 formation, highlights the need to study these molecules in more organisms, including other fungal plant
617 pathogens.

618

619 **Methods**

620 ***M. oryzae* growth and DNA extraction**

621 *M. oryzae* Guy11 was grown on Difco oatmeal agar plates for 21 days under constant light in a Percival
622 Scientific Incubator Model CU-36L4 equipped with half fluorescent lights and half black lights. 1 cm² of
623 mycelium was scraped from the colony edge and used to start 3 liquid cultures (biological replicates) in
624 15 ml complete medium [49] (CM) in petri dishes. Liquid cultures were incubated without shaking for 3
625 days in the same growth chamber.

626

627 Total DNA extraction was performed according to a protocol from the Prof. Natalia Requena group at
628 the Karlsruhe Institute of Technology. Briefly, mycelium grown in liquid culture was washed 3 times with
629 water and then ground in liquid nitrogen. Ground mycelium was incubated in extraction buffer (0.1M
630 Tris-HCl pH 7.5, 0.05 M EDTA, 1% SDS, 0.5 M NaCl) at 65°C for 30 minutes. 5M potassium acetate was
631 then added to the samples which were then incubated on ice for 30 minutes. The supernatant was then
632 washed with isopropanol and ethanol. Finally, the DNA pellet was resuspended in water and treated
633 with RNase A (Thermo Scientific).

634

635 ***O. sativa* growth and DNA extraction**

636 *O. sativa* samples were originally intended to serve as control samples to be compared to tissue infected
637 by *M. oryzae* and therefore the methods below reflect this original intent. However, circularome
638 sequencing data obtained from infected tissue was not included in this study as it included very little
639 sequencing data that mapped to the *M. oryzae* Guy11 genome.

640

641 *O. sativa* cv. Nipponbare seeds were surface sterilized in 70% ethanol for 1 minute and 10% bleach for
642 10 minutes with thorough rinsing in sterile water after each before being placed on wet filter paper in a
643 petri dish. The petri dish was wrapped in foil and placed at 4°C for 2 days to germinate. Germinated
644 seedlings were planted in potting mix made up of 50% Turface and 50% Super Soil. Seedlings were
645 grown for three weeks in a greenhouse under standard conditions. For three samples, the first true leaf
646 was cut from one rice plant, its tip was removed, and it was then cut into two equal segments,
647 approximately 10mm in length. This pair of segments was then placed on their abaxial surface on wet
648 filter paper in a petri dish. Five hole-punches of filter paper soaked in 0.25% gelatin and 0.05% Tween-20
649 were then placed on each segment. The petri dishes were then placed in an airtight container with wet
650 paper towels and placed on a windowsill for 7 days. Hole-punches were removed and non-chlorotic

651 tissue in contact with hole-punches was ground in liquid nitrogen. DNA extraction was then performed
652 using the Qiagen Plant DNeasy mini kit.

653

654 **Circular DNA enrichment**

655 Total DNA obtained from DNA extractions (biological replicates) were then split into three samples
656 (technical replicates) before circular DNA enrichment. This enrichment was performed according to a
657 protocol from Lanciano *et al.* with a few modifications [18]. 5 µg of extracted DNA was used as input for
658 circular DNA enrichment in *M. oryzae* and 750 ng of extracted DNA were used for *O. sativa*. To purify the
659 samples and begin removing large linear DNA fragments, the samples were treated using a Zymo
660 Research DNA Clean and Concentrator kit and standard protocols. Linear DNA digestion was then
661 performed using Epicentre PlasmidSafe DNase and incubated at 37°C for 24 hours. DNase, ATP, and
662 reaction buffer were then added to the samples every 24 hours while the incubation continued. In total,
663 the reaction was allowed to proceed for 96 hours. Remaining DNA was then precipitated overnight at
664 4°C by adding 0.1 volume 3M sodium acetate, 2.5 volumes ethanol and 1 µl glycogen (20 mg/ml). Rolling
665 circle amplification was then performed using the Illustra TempliPhi 100 Amplification Kit (GE
666 Healthcare). Precipitated DNA was resuspended directly in 20 µl of the Illustra TempliPhi sample buffer
667 and the amplification reaction was allowed to proceed for 24 hours at 30°C.

668

669 **Verification of circular DNA enrichment**

670 In a separate experiment, 5 samples of *M. oryzae* mycelium were grown up in liquid culture and total
671 DNA was extracted. Circular DNA enrichment was performed as before with some exceptions and
672 without technical replicates. First, linear DNA digestion was only performed for 72 hours for 3 samples.
673 Second, aliquots of the incubating samples were taken at 0 hours, 24 hours, 48 hours and 72 hours for
674 these 3 samples, and 0 hours, 48 hours, 72 hours and 96 hours for the last 2 samples. qPCR was then

675 used to verify linear DNA depletion in each sample using an Applied Biosystems QuantStudio 5
676 instrument and the QuantStudio Design and Analysis desktop software. Primers were used to amplify a
677 portion of the *M. oryzae* actin gene (MGG_03982) along with Lightcycler 480 Sybr Green I master mix
678 (Additional File 2: Table S3). Data from four qPCR technical replicates was obtained. Remaining linear
679 DNA fraction in each sample at each timepoint was then calculated using the $2^{-\Delta\Delta Ct}$ method.

680

681 **Illumina library preparation and sequencing**

682 Library preparation was performed by the QB3-Berkeley Functional Genomics Laboratory at UC
683 Berkeley. DNA was fragmented with an S220 Focused-Ultrasonicator (Covaris), and libraries prepared
684 using the KAPA Hyper Prep kit for DNA (Roche KK8504). Truncated universal stub adapters were ligated
685 to DNA fragments, which were then extended via PCR using unique dual indexing primers into full length
686 Illumina adapters. Library quality was checked on an Agilent Fragment Analyzer. Libraries were then
687 transferred to the QB3-Berkeley Vincent J. Coates Genomics Sequencing Laboratory, also at UC Berkeley.
688 Library molarity was measured via quantitative PCR with the KAPA Library Quantification Kit (Roche
689 KK4824) on a BioRad CFX Connect thermal cycler. Libraries were then pooled by molarity and sequenced
690 on an Illumina NovaSeq 6000 S4 flowcell for 2 x 150 cycles, targeting at least 10Gb per sample. FastQ
691 files were generated and demultiplexed using Illumina bcl2fastq2 version 2.20 and default settings, on a
692 server running CentOS Linux 7. One technical replicate did not pass quality control before library
693 preparation and was omitted.

694

695 **PacBio library preparation and sequencing**

696 Using a Covaris S220 Focused-Ultrasonicator, 2 ug of each DNA sample was sheared to an approximate
697 fragment size of 5000 bp and purified using AMPure XP beads (Beckman Coulter). Library preparation
698 was performed using the NEBNext Ultra DNA Library Prep Kit (kit number E7370L, New England Biolabs)

699 and 8 cycles of PCR. Barcode sequences and barcodes assigned to each sample are described in
700 Additional files 31 and 32. Libraries were then quality controlled using a Bioanalyzer high sensitivity DNA
701 chip and the Agilent 2100 Bioanalyzer system. One technical replicate did not pass quality control before
702 library preparation and was omitted. The samples were then submitted to Novogene (Tianjin, China) for
703 PacBio sequencing which was performed on the PacBio Sequel platform using a 600-minute sequencing
704 strategy and three SMRT cells.

705

706 **Inferring eccDNA forming regions from short read sequencing data**

707 Illumina sequencing signal was analyzed using a custom pipeline inspired by previously published
708 methods [13]. Illumina reads were first trimmed of Illumina TruSeq adapters using CutAdapt [50] version
709 2.4 with the nextseq-trim=20 option. Trimmed reads were then mapped to the *M. oryzae* Guy11
710 genome [37] and the 70-15 mitochondrial sequence [51] obtained from the Broad Institute
711 ([https://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-](https://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/magnaporthe-comparative-genomics-proj)
712 [initiative/magnaporthe-comparative-genomics-proj](https://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/magnaporthe-comparative-genomics-proj)) using BWA-MEM [52] version 0.7.17-r1188 and the
713 `-q` and `-a` options. Reads mapping to mitochondrial sequences were excluded. Uniquely mapped reads
714 were then mined for split reads that mapped in the same orientation, had at least 20 bp of alignment on
715 either side of the split, mapped to only two places in the genome, and where the start of the read
716 mapped downstream from the end. This last filter sets these split reads apart from split reads that
717 would indicate a deletion in the genome. Split reads for which one side of the split read mapped more
718 than 50kbp away from the other or to a different scaffold than the other were excluded. Opposite facing
719 read pairs were also obtained from uniquely mapped reads. Candidate eccDNA forming regions were
720 then inferred by combining these two structural read variants. A split read that contained an opposite
721 facing read pair that mapped no more than a combined 500 bp from the borders of the region contained
722 within the two halves of the split read was considered a candidate eccDNA and a junction split read. The

723 length distribution of these candidate eccDNA forming regions (Additional File 1: Fig. S35A) was then
724 used to probabilistically infer candidate eccDNA forming regions from multi-mapping reads (Additional
725 File 1: Fig. S35B). For each multi-mapping split read, a list of potential combinations of alignments that
726 satisfied the previously described criteria for split reads was generated and one of these combinations
727 was chosen at random, weighted by their length according to the generated length distribution. The
728 chosen combinations were then used to infer additional candidate eccDNA forming regions by
729 combining these with opposite facing read pairs as before, except this time obtained from unique and
730 multi-mapping reads.

731

732 Each candidate eccDNA forming region was then validated by verifying that the region had over 95%
733 read coverage and at least two junction split reads with the exact same coordinates. Candidate eccDNA
734 forming regions that did not pass these criteria were considered low quality and were not included in
735 the analysis.

736

737 **Inferring eccDNA forming regions from long read sequencing data**

738 Circular consensus sequences (CCS) were first called from PacBio data using ccs version 3.4.1
739 (<https://ccs.how/>). Demultiplexing was then performed using lima version 1.9.0 (<https://lima.how/>) and
740 sequences of barcodes used for library preparation (Additional Files 31 and 32). CCSs were then mapped
741 to the *M. oryzae* Guy11 genome using minimap2 [53] version 2.18-r1015. Only uniquely mapped reads
742 were kept for analysis. We then identified eccDNA forming regions by looking for split reads that either
743 mapped to the same orientation to the same exact region multiple times or pairs of split alignments that
744 were less than 50 kb apart, mapped in the same orientation and oriented properly so that they were
745 indicative of a circular junction rather than a deletion.

746

747 **Outward PCR validation of eccDNA forming regions and PCR validation of eccDNA-absent genes**

748 Outward facing primers were designed to 8 eccDNA forming regions of interest to validate their
749 presence in our eccDNA sequencing samples. Primers were designed to amplify the junction of each
750 eccDNA but not result in a product of the same size when used on genomic DNA (Additional File 2: Table
751 S3). Primer3 [54] was used for primer design. The oligonucleotides were then synthesized by Integrated
752 DNA technologies. PCR was performed using New England Biolab's Phusion High-Fidelity DNA
753 polymerase on *M. oryzae* Guy11 genomic DNA and rolling circle amplification products for the sample
754 each eccDNA forming region was found in. 5ng DNA of each sample was used per 50 µl PCR reaction as
755 well as 5X Phusion HF buffer, 10 mM dNTPs, 10 µM forward primer, 10 µM reverse primer and 1 unit of
756 Phusion DNA polymerase. PCR conditions were as follows: initial denaturation at 98°C for 30 seconds, 35
757 cycles of denaturation at 98°C for 10 seconds, annealing at 64°C or 65°C for 30 seconds, extension at
758 72°C for 10 seconds, and a final extension at 72°C for 5 minutes. PCR products were run on a 2% agarose
759 gel to check for amplification. Bands of the expected size were extracted from electrophoresis gels using
760 Zymo Research's Zymoclean Gel DNA Recovery Kit. Sanger sequencing was performed by the UC
761 Berkeley DNA Sequencing Facility and Sanger sequences were examined for matches to corresponding
762 eccDNA forming regions using BLASTN [55] version 2.2.9 and manual inspection.

763

764 PCR validation of eccDNA-absent genes was performed using similar methods. Primers were designed to
765 amplify the entire annotated gene region of *MYO1* and the actin gene (MGG_03982) and a small
766 segment of the *MAGGY* LTR retrotransposon from genomic DNA. 2ng DNA of each sample was used per
767 20 µl PCR reaction as well as 5X Phusion HF buffer, 10 mM dNTPs, 10 µM forward primer, 10 µM reverse
768 primer and 0.4 units of Phusion DNA polymerase. PCR conditions were as follows: initial denaturation at
769 98°C for 30 seconds, 25 cycles of denaturation at 98°C for 10 seconds, annealing at 64°C or 65°C for 30

770 seconds, extension at 72°C for 5, 60 or 120 seconds, and a final extension at 72°C for 5 minutes. PCR
771 products were run on a 1% agarose gel to check for amplification.

772

773 **Comparing eccDNA forming regions inferred from Illumina data and eccDNA forming regions inferred**
774 **from PacBio data**

775 EccDNA forming regions called using Illumina data and PacBio data were found to be identical if their
776 start and end coordinates were within 10 bp of each other to account for mapping errors. EccDNA
777 forming regions were then called with less stringent requirements to verify if any of the missing eccDNA
778 forming regions were being filtered out somewhere in the pipeline. In this test, all uniquely mapped split
779 reads that had 10 or more bp overlap on either side, were properly oriented, and were less than 50kb
780 apart were considered eccDNA forming regions.

781

782 **Benchmarking eccDNA forming regions called using our pipeline on previously published data**

783 EccDNA forming regions called using our pipeline were compared to eccDNA forming regions previously
784 published for *H. sapiens* [13]. EccDNA forming regions were found to be identical if their start and end
785 coordinates were within 10 bp of each other. EccDNA forming regions described as low quality by the
786 authors were excluded from the published dataset before comparison. High coverage eccDNA forming
787 regions were chosen for comparison if they had more than 10 associated junction split reads. Finally,
788 multi-mapping reads were excluded from the pipeline to identify eccDNA forming regions called using
789 only uniquely mapped reads.

790

791 **Comparing eccDNA sequencing samples to each other**

792 Overlaps in eccDNA forming regions between samples were first calculated based off the exact
793 coordinates of the eccDNA forming regions and Venn diagrams based off these overlaps were generated

794 using the ggVennDiagram R package [56] version 1.2.0. EccDNA forming regions found in all technical
795 replicates taken from each biological replicate were first combined before looking for overlaps between
796 biological replicates. Overlaps were then calculated with various levels of tolerance for the start and end
797 coordinates of the eccDNA forming regions so that regions in one sample that were within 10, 100, or
798 1000 bp from the start and end coordinates of a region in another sample were considered to be found
799 in both samples. Rarefaction analysis for eccDNA forming regions in all samples was performed by
800 sampling mapped eccDNA sequencing reads at random in increasing 10% intervals. For each subsample,
801 eccDNA forming regions were called as previously described and counted. Principal component analysis
802 of read coverage was performed by first calculating junction split read coverage for all 10kbp windows in
803 the genome for each sample. These values were then normalized to the total number of junction split
804 reads in each sample. The matrix of normalized junction split read coverage for all samples was then
805 processed using the prcomp function in R version 3.6.1 with the scale = TRUE option, and the first 6
806 principal components were plotted using the ggbiplot R package [57] version 0.55.

807

808 **Gene and effector annotation**

809 The *M. oryzae* Guy11 genome along with 162 other rice-infecting *M. oryzae* genomes (Additional File 25)
810 were annotated using the FunGAP [58] version 1.1.0 annotation pipeline. For all genomes, RNAseq data
811 (SRR8842990) obtained from GEO accession GSE129291 was used along with the proteomes of *M.*
812 *oryzae* 70-15, P131 and MZ5-1-6 taken from GenBank (accessions GCA_000002495.2,
813 GCA_000292605.1, and GCA_004346965.1, respectively). The 'sordariomycetes_odb10' option was used
814 for the busco_dataset option and the 'magnaporthe_grisea' option was used for the augustus_species
815 option. For repeat masking, a transposable element library generated by combining the RepBase [59]
816 fngrep version 25.10 with a *de novo* repeat library generated by RepeatModeler [60] version 2.0.1 run
817 on the *M. oryzae* Guy11 genome with the LTRStruct option was used for all genomes. Genes in *M.*

818 *oryzae* Guy11 were assigned names according to the gene names listed on UniProtKB for *M. oryzae* 70-
819 15 accessed in October 2021. To make this assignment, *M. oryzae* Guy11 proteins were aligned to the
820 *M. oryzae* 70-15 proteome using BLASTP [55] version 2.7.1+ and hits with greater than 80% sequence
821 identity and that spanned more than 80% of the length of both the *M. oryzae* Guy11 protein and the *M.*
822 *oryzae* 70-15 protein were assigned names.

823

824 Effectors were predicted among *M. oryzae* Guy11 genes by first selecting genes with signal peptides
825 which were predicted using SignalP [61] version 5.0b Darwin x86_64. Genes with predicted
826 transmembrane domains from TMHMM [62] version 2.0c were then excluded. Finally, EffectorP [63]
827 version 2.0 was used to predict effectors from this secreted gene set. Previously well-characterized
828 effectors were identified using previously published protein sequences [64] and DIAMOND [65] version
829 2.0.9.147.

830

831

832 **High quality LTR-retrotransposon annotations in *M. oryzae***

833 High quality, full length, consensus sequences for known *Gypsy* elements in *M. oryzae* (*MAGGY*,
834 *GYMAG1*, *GYMAG2*, *PYRET*, *MGRL3*) and one *Copia* element (*Copia1*) were generated using the
835 WICKERsoft [66] suite of tools. Reference sequences from other genomes for each element were
836 obtained from the RepBase [59] fngrep version 25.10 library. The *M. oryzae* Guy11 genome was then
837 scanned for the presence of these sequences using BLASTN [55] version 2.2.9 and then filtered to hits
838 with 90% sequence identity and that contained 90% of the sequence length. Hits for each reference
839 sequence were then extended to include 500 base pairs of genomic sequence upstream and
840 downstream of the hit. A multiple sequence alignment of hits for each reference sequence was then
841 generated using ClustalW [67] version 1.83 and boundaries were visually inspected and trimmed.

842 Consensus sequences for each element were then generated from these multiple sequence alignments.
843 These consensus sequences were split into LTR and internal regions by self-alignment using the BLASTN
844 [55] webserver in August 2020 to identify LTRs. These consensus sequences are available in Additional
845 File 33. Finally, the locations of these elements in *M. oryzae* Guy11 genome were annotated with
846 RepeatMasker [68] version 4.1.1 with the -cutoff 250, -nolow, -no_is, and -norna options to identify
847 their locations in the *M. oryzae* Guy11 genome. For read coverage plots as well as histone and GC
848 content plots, full length LTR retrotransposon copies were required. These were identified by using the
849 original full length consensus sequences with RepeatMasker as before and then filtering to hits greater
850 than 3000 bp in length and greater than 90% sequence identity.

851

852 **Comparative analysis of eccDNA forming regions**

853 Analysis of eccDNA forming regions in organisms other than *M. oryzae* were performed as described
854 above for Illumina sequencing data using previously published genome, gene annotation and
855 transposable element annotation files (Additional File 34). However, unlike the other data used in this
856 study, the sequencing data in the *S. cerevisiae* dataset was single-end and therefore opposite facing
857 read pairs could not be used to infer eccDNA forming regions. Instead, only eccDNA forming regions
858 with three overlapping junction split reads were used for analysis. For all organisms, reads mapping to
859 unplaced scaffolds and organellar genomes were removed after mapping as described above for the *M.*
860 *oryzae* mitochondrial genome. These scaffolds were also removed from genome size, number of coding
861 base pairs, and number of LTR retrotransposon base pairs calculations for comparative analysis. To
862 calculate the percent of the genome that was covered in each sample, the genomecov command of the
863 BEDtools [69] suite versions 2.28.0 was used with the -d option along with the coordinates of eccDNA
864 forming regions for each sample. Any base pair with a coverage value greater than zero was counted as
865 being a portion of the genome in an eccDNA forming region.

866

867 **Characterization of eccDNA formation by LTR retrotransposons**

868 To generate the Manhattan plot, junction split reads were filtered by selecting regions that were made
869 up of 90% LTR retrotransposon sequences. Junction split read coverage was then calculated for each 100
870 bp window in the genome. Coverage values were then normalized to the total number of LTR eccDNA
871 junction split reads per sample. These coverage values were then averaged across technical replicates
872 for each biological replicates, and then averaged across biological replicates. Finally, only 100 bp bins
873 that overlapped at least 50 bp with an LTR retrotransposon were plotted in Fig 3A. For Additional File 1:
874 Fig. S10, only bins with coverage greater than 0 were plotted.

875

876 To simulate expected read coverage for different types of LTR eccDNAs, the *Copia1* consensus sequence
877 was taken as a reference, though the *MAGGY* consensus sequence yielded identical results. Simulated
878 DNA sequences were then generated for each type of LTR eccDNA. The expected 2-LTR circular
879 sequence generated by NHEJ (scenario 1, Fig. 4A) was simply made up of two LTR sequences and the
880 internal sequence, and the expected 1-LTR circle sequence generated by HR (scenario 3, Fig. 4C) was
881 made up of one LTR sequence and the internal sequence. These sequences were shuffled 1000 times to
882 generate 1000 sequences starting at various points of the expected circularized sequence. For the 1-LTR
883 circle sequence generated by autointegration (scenario 2, Fig. 4B), the random autointegration events
884 were simulated by choosing a random length segment of the internal sequence starting with its start or
885 end, adding the LTR sequence to this sequence, and randomly shuffling the sequence to simulate a
886 circular sequence. This process was repeated 1000 times to generate 1000 sequences. Finally, for each
887 scenario, Illumina reads were simulated to reach 2000x coverage for each of the simulated sequences
888 using ART Illumina [70] version 4.5.8 and the following parameters: 150 bp read length, 450 bp mean
889 insert size, 50 bp insert size standard deviation, HiSeqX TruSeq. Reads were mapped to the simulated

890 sequences using BWA-MEM [52] version 0.7.17-r1188 with default settings and coverage for each base
891 pair was calculated.

892

893 To generate observed coverage for each element, sequencing read coverage across the genome was
894 calculated for all 10 base pair windows in the *M. oryzae* Guy11 genome for each sample. Coverage
895 values were then normalized to the total number of mapped sequencing reads in each sample. These
896 coverage values were then averaged across technical replicates for each biological replicates, and then
897 averaged across biological replicates. Finally, profile plot data was generated for full length, high
898 confidence sequences for each LTR retrotransposon using computeMatrix scale-regions and plotProfile
899 of the DeepTools [71] suite of tools version 3.5.1 using full length, high confidence LTR retrotransposon
900 sequences. Profile plots were also generated using previously published whole genome sequencing data
901 by averaging sequencing coverage across all three samples [32,37,38].

902

903 **Identification of split reads associated with eccDNA formation from LTR retrotransposons**

904 Split reads were first identified as any read that mapped to only two places in the genome with at least
905 20 base pairs of alignment on either side. LTR-LTR split reads were then selected from these split reads
906 for each LTR retrotransposon if both sides of the split read had any overlap with any copy of that
907 retrotransposon's LTR in the genome. LTR-internal split reads were selected if one side of the split read
908 had any overlap with any copy of the retrotransposon's LTR in the genome and the other side had any
909 overlap with any copy of the retrotransposon's internal region in the genome. Read coverage, LTR-LTR
910 split read coverage, and LTR-internal coverage was then calculated for each annotation of each LTR
911 retrotransposon. Coverage values were then normalized to the total number of mapped sequencing
912 reads in each sample. These coverage values were then averaged across technical replicates for each
913 biological replicates, and then averaged across biological replicates.

914

915 **Comparison of microDNAs and large eccDNAs across organisms**

916 Genome, gene annotation, and transposable element annotation files for each organism used for this
917 analysis were as previously described (Additional File 34). Again, organellar genomes as well as unplaced
918 contigs were filtered out of these files before analysis. Introns and UTRs were added to gene annotation
919 files that were missing these elements using the 'agat_convert_sp_gff2gtf.pl' and
920 'agat_sp_add_introns.pl' commands from the AGAT toolkit version 0.6.2
921 (<https://github.com/NBISweden/AGAT>). CpGplot of EMBOSS [72] version 6.6.0.0 was used to annotate
922 CpG islands in each genome. Upstream and downstream regions were defined as being 2000 base pairs
923 upstream from the transcription start site and downstream from the transcription end site, respectively.
924 Genic regions were defined as being made up of all sequences between transcription start and end sites
925 and intergenic regions were the opposite. Junction split reads were counted as being from a specific
926 region if they overlapped to any extent within that region.

927

928 The observed percentage of junction split reads overlapping with each region type was calculated for
929 each sample for each organism and an average of these percentages was calculated. The junction split
930 reads of each sample were then shuffled across the genome 10 times, excluding LTR retrotransposon
931 locations, and an expected percentage for each region was calculated, averaged across all permutations,
932 then averaged across all samples for each organism. Finally, the \log_2 of the fold enrichment was
933 calculated by taking the \log_2 of the observed average percentage over the expected average percentage.

934

935 **Correlation of expression and eccDNA formation**

936 Previously published RNAseq data from *M. oryzae* Guy11 grown in liquid culture in rich medium was
937 obtained [73] (Additional File 35). The data was mapped to the *M. oryzae* Guy11 genome using STAR

938 [74] version 2.7.1a with the quantMode GeneCounts option. Read counts per gene were then divided by
939 library size and multiplied by the length of each gene in order to obtain reads per kilobase million
940 (RPKMs). RPKMs per gene were then averaged across all samples.

941
942 Junction split read counts per gene used to analyze the correlation of expression and eccDNA formation
943 were generated for each gene by counting the number of junction split reads that intersect the gene to
944 any extent. Counts per gene were first assessed for each sample and normalized to the number of
945 junction split reads in that sample. Normalized counts were then averaged across technical replicates for
946 each biological replicate. Average counts per biological replicate were then averaged to obtain the final
947 result.

948
949 To compare gene content and eccDNA formation, the *M. oryzae* genome was divided into 100kbp bins
950 and the number of genes per bin was then calculated. Junction split reads per bin were calculated for
951 each sample using the same method. Junction split read per bin values were then normalized to the
952 total number of junction split reads in each sample. These values were then averaged across technical
953 replicates for each biological replicate, and then averaged across biological replicates.

954

955 **ARS consensus sequence enrichment analysis**

956 The published ACS sequence profile [42] was used to identify ACSs in eccDNA forming regions using the
957 FIMO [75] software version 4.12.0. Only hits scoring greater than 17 were kept. In order to test for
958 enrichment of these sequences, an expected distribution of ACS sequences was generated by randomly
959 shuffling eccDNA forming regions across the *M. oryzae* Guy11 genome, excluding regions containing LTR
960 retrotransposons. The observed number of ACS sequences in eccDNA forming regions was then
961 compared to the expected distribution to generate a p-value.

962

963 **Histone mark and GC content profile plots**

964 Previously published ChIPSeq data for H3K27me3, H3K27ac, H3K36me3 and loading controls was
965 obtained [73]. Sequencing reads for each technical replicate were combined before reads for each
966 treatment for each biological replicate were mapped to the *M. oryzae* Guy11 genome using BWA-MEM
967 [52] version 0.7.17-r1188 with default settings. The bamCompare command from the DeepTools [71]
968 suite of tools version 3.5.1 with the scaleFactorsMethod readCount option was then used to compare
969 the signal from each treatment to the loading control for each biological replicate. computeMatrix scale-
970 regions was then used in conjunction with the plotProfile command to generate processed data for
971 profile plots. After verifying that all biological replicates resulted in similar profile plots, only the first
972 biological replicate was chosen for presentation.

973

974 To generate tracks used for profile plots, a few different strategies were used. GC content profile plots
975 were generated by calculating GC percentage for 50 base pair windows throughout the genome. Profile
976 plot data was then generated using computeMatrix scale-regions and plotProfile commands as before.
977 Methylated and acetylated genes were determined using the methylation and acetylation peaks
978 published by Zhang *et al.* [73]. Marked genes were called when at least 50% of the gene overlapped with
979 a peak. Large eccDNAs, microDNAs, and LTR-eccDNAs from all *M. oryzae* Guy11 samples were combined
980 into a single list which was filtered for duplicates and used for the corresponding tracks in the profile
981 plots. The genome baseline track was generated by combining all of these eccDNA forming regions and
982 shuffling them randomly across the genome. Finally, the full length, high quality LTR-retrotransposon
983 annotations described above were used for LTR retrotransposon tracks. The same approach was used
984 for generating profile plots to compare histone marks and GC content for eccDNA-associated and
985 eccDNA-absent genes.

986

987 **Identification of eccDNA-associated and eccDNA-absent genes**

988 Encompassing split read counts per gene for determining eccDNA-associated and eccDNA-absent genes
989 were generated for each gene by counting the junction split reads that fully encompass the gene using
990 the intersect command of the BEDTools [69] suite version 2.28.0 with the -f 1 option. This count was
991 normalized to the total number of junction split reads in each sample, then averaged across technical
992 replicates for each biological replicate. Genes with a count of zero were removed from each biological
993 replicate before being sorted by this count. Genes in the top third for this count were compared
994 between biological reps using the ggVennDiagram R package [56] version 1.2.0. This count was averaged
995 across biological replicates to obtain the encompassing split read count per gene for visualizations in Fig.
996 5 and Fig. 8 and for comparison between predicted effectors and other genes (Additional File 1: Fig.
997 S34).

998

999 **GO enrichment analysis**

1000 GO terms were first assigned to annotated *M. oryzae* Guy11 genes using the PANNZER2 [76] webserver
1001 on August 17th, 2020. Annotated GO terms were then filtered to annotations with a positive predictive
1002 value greater than 0.6. The topGO [77] R package version 2.36.0 was then used to parse assigned GO
1003 terms and reduce the gene list to a list of feasible genes for analysis. Either eccDNA-associated or
1004 eccDNA-absent were assigned as significant genes, and the number of these genes belonging to each GO
1005 term was used as the observed value for the enrichment analysis. A kernel density function was then
1006 generated using the gene lengths of the significant gene set. The same number of genes as the
1007 significant gene set were then sampled at random from the feasible gene set using weighted random
1008 selection with weights obtained from the kernel density function. This random sampling was repeated
1009 100 times and the average of the number of genes belonging to each GO term was used as the expected

1010 value for the enrichment analysis. Finally, the Chi-square statistic was then computed comparing
1011 observed and expected values to test for enrichment or depletion of each GO term.

1012

1013 **Gene presence absence variation**

1014 In order to identify genes prone to presence absence variation in the *M. oryzae* Guy11 genome,
1015 OrthoFinder [78] version 2.5.1 with default settings was used on all of the *M. oryzae* proteomes and the
1016 *Neurospora crassa* proteome obtained from GenBank (accession GCA_000182925.2). Then, for each *M.*
1017 *oryzae* genome, we queried whether each gene annotated in the *M. oryzae* Guy11 genome had an
1018 ortholog identified by OrthoFinder in that genome. Finally, the absence of genes without orthologues
1019 was confirmed using BLASTN [55] version 2.7.1+.

1020

1021 Small, genic deletions were identified using orthologs identified by OrthoFinder [78] version 2.5.1 as
1022 before. For each genome, we looked for genes in the *M. oryzae* Guy11 genome that had no ortholog in
1023 that genome, but that were flanked by two genes with orthologs in that genome. One-to-many, many-
1024 to-many, and many-to-one orthologs were excluded from this analysis. Candidate gene deletions were
1025 validated using alignments performed using the nucmer and mummerplot commands of the MUMmer
1026 [79] suite of tools version 4.0.0rc1 to verify that a DNA deletion truly existed, and that this deletion
1027 overlapped the gene of interest.

1028

1029 **Identification of eccDNA-mediated translocations**

1030 Identification of translocations with a potential eccDNA intermediate was done by first aligning two
1031 genomes using the nucmer command of the MUMmer [79] suite of tools version 4.0.0rc1 with the
1032 maxmatch option. The nucmer output was then parsed to look for portions of the reference genome
1033 that had an upstream region that aligned to one query scaffold, followed by two separate adjacent

1034 alignments to another query scaffold, followed by a downstream region that aligned to the original
1035 query scaffold. We also required that the two adjacent alignments in the center of the region were to
1036 adjacent regions in the query scaffold but their order was reversed compared to the reference.
1037 Candidate eccDNA-mediated translocations were verified manually by inspecting alignment plots
1038 generated using the mummerplot command. The *S. cerevisiae* EC1118 (GCA_000218975.1) and M22
1039 genomes (GCA_000182075.2) obtained from GenBank were used to verify the ability of our pipeline to
1040 detect these translocation events. The *M. oryzae* Guy11 genome was then compared to 306 *M. oryzae*
1041 genomes (Additional File 27) to look for these events in the *M. oryzae* species. Before alignment,
1042 transposable elements were masked from these *M. oryzae* genomes using RepeatMasker [68] version
1043 4.1.1 with the -cutoff 250, -nolow, -no_is, and -norna options, as well as a transposable elements library
1044 generated by combining the RepBase [59] fngrep version 25.10 with the de novo repeat library
1045 generated by RepeatModeler [60] version 2.0.1 run on the *M. oryzae* Guy11 genome with default
1046 settings aside from the LTRStruct argument.

1047

1048 **Minichromosome genes and eccDNAs**

1049 Scaffolds corresponding to minichromosomes in the *M. oryzae* FR13 (GCA_900474655.3), CD156
1050 (GCA_900474475.3), and US71 (GCA_900474175.3) genomes were extracted according to previously
1051 published data [80]. Exonerate [81] version 2.4.0 was then used with the protein2genome model to
1052 identify genes in the *M. oryzae* Guy11 genome that were found on minichromosomes in these other
1053 isolates. Hits with greater than 70% sequence identity to any minichromosome scaffold were identified
1054 as genes found on minichromosomes. Encompassing split reads were then counted for all genes. This
1055 count was normalized to total number of junction split reads in each sample, then averaged across
1056 technical replicates for each biological replicate, then averaged across biological replicates. Finally,

1057 normalized encompassing split read counts for genes found on minichromosomes were compared to
1058 genes not found on minichromosomes.

1059

1060 **Rarefaction analysis for eccDNA-absent genes and unique eccDNA forming regions**

1061 Rarefaction analysis for genes found fully encompassed by eccDNA forming regions were performed by
1062 first sampling eccDNA forming regions from all samples at random in increasing 10% intervals. For each
1063 subsample, the number of genes found fully encompassed by eccDNA forming regions was determined
1064 as before. Next, eccDNA forming regions were shuffled across the genome and sampled at random in
1065 increasing 10% intervals. Again, the number of genes found fully encompassed by eccDNA forming
1066 regions was determined for each sample. This analysis was performed 100 times with similar results as
1067 those represented in Fig. 5C. A similar approach was used for rarefaction analysis of eccDNA forming
1068 regions but the number of unique microDNAs, large eccDNAs and LTR-eccDNAs were counted at each
1069 subsample instead.

1070

1071 **Data processing and analysis**

1072 Data processing was performed in a RedHat Enterprise Linux environment with GNU bash version
1073 4.2.46(20)-release. GNU coreutils version 8.22, GNU grep version 2.20, GNU sed version 4.2.2, gzip
1074 version 1.5, and GNU awk version 4.0.2 were all used for file processing and handling. Conda version
1075 4.8.2 (<https://docs.conda.io/en/latest/>) was used to facilitate installation of software and packages.
1076 Code parallelization was performed with GNU parallel [82] version 20180322. Previously published data
1077 was downloaded using curl version 7.65.3 (<https://curl.se/>) and sra-tools version 2.10.4
1078 (<https://github.com/ncbi/sra-tools>). Image file processing was performed with the help of ghostscript
1079 version 9.25 (<https://ghostscript.com/>) and imagemagick version 7.0.4-7
1080 (<https://imagemagick.org/index.php>). BED format files were processed using bedtools [69] version

1081 2.28.0 and bedGraphToBigWig version 4
1082 (<https://www.encodeproject.org/software/bedgraphtobigwig/>). SAM and BAM format files were
1083 processed with SAMtools [83] version 1.8 and Picard version 2.9.0
1084 (<https://broadinstitute.github.io/picard/>).
1085 Data processing was also facilitated by custom Python scripts written in Python version 3.7.4 with the
1086 help of the pandas [84] version 0.25.1 and numpy [85] version 1.17.2 modules. The scipy [86] version
1087 1.4.1 and more-intertools version 7.2.0 (<https://more-itertools.readthedocs.io/>) modules were also
1088 used.
1089 Data analysis and statistical analyses were performed in R version 3.6.1. Data handling was processed
1090 using data.table [87] version 1.13.6, tidyr [88] version 1.1.3, reshape2 [89] version 1.4.4, and dplyr [90]
1091 version 1.0.4 packages. Plotting was performed using the ggplot2 [91] version 3.3.5 package, with help
1092 from RColorBrewer [92] version 1.1.2, scales [93] version 1.1.1, cowplot [94] version 1.1.1, ggprepel [95]
1093 version 0.9.1 and ggpubr [96] version 0.4.0 packages. The Gviz [97] version 1.28.3 was used for BAM file
1094 visualization. Tables were made using gt [98] version 0.3.1.

1095

1096 **Declarations**

1097 **Availability of Data and Materials**

1098 The datasets supporting the conclusions of this article are available in the NCBI's Sequence Read Archive
1099 repository. Illumina circularome sequencing data for *M. oryzae* was submitted under BioProject
1100 accession PRJNA768097. PacBio circularome sequencing data for *M. oryzae* was submitted under
1101 BioProject accession PRJNA556909. Illumina circularome sequencing data for *O. sativa* was submitted
1102 under BioProject accession PRJNA768410. Additional datasets supporting the conclusions of this article
1103 are available on Zenodo. Genomes and annotation files used for comparative circularome are available

1104 under the DOI 10.5281/zenodo.5544950. Annotated genes and predicted proteins for rice-infecting *M.*
1105 *oryzae* isolates are also available under the DOI 10.5281/zenodo.5542597. Outputs from OrthoFinder2
1106 run on rice-infecting *M. oryzae* proteomes are also available under the DOI 10.5281/zenodo.5544260.
1107 Finally, all files used for statistical analysis and plotting are available under the DOI 10.5281/zenodo.
1108 7114261.

1109

1110 Code for the pipeline used to call eccDNA forming regions for Illumina sequencing data is available in a
1111 maintained GitHub repository (https://github.com/pierri/ecc_caller). All other code used for raw data
1112 processing, data analysis, and figure generation is available in a GitHub repository
1113 (https://github.com/pierri/moryzae_eccdnas_manuscript_code_final).

1114

1115 **Competing interests**

1116 The authors declare that they have no competing interests.

1117

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1126

1127 **Authors' contributions**

1128 PMJ and KVK conceptualized and designed the study. PMJ collected and analyzed the data. PMJ wrote
1129 the original draft manuscript. PMJ and KVK reviewed and edited the manuscript. Both authors read and
1130 approved the final manuscript.

1131

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1384

1385 **Supplementary information**

1386 **Additional File 1: Supplementary Figures.**

1387 **Fig. S1.** Degradation of linear DNA using exonuclease treatment.

1388 **Fig. S2.** Outward PCR validation of eccDNA forming regions.

1389 **Fig. S3.** Overlap in exact break points of eccDNA forming regions across samples.

1390 **Fig. S4.** Rarefaction analysis of sequencing coverage and eccDNA forming regions across all samples.

1391 **Fig. S5.** Principal components analysis of sequencing coverage between samples.

1392 **Fig. S6.** Overlap in eccDNA forming regions across samples, with increasing tolerance for start and end
1393 coordinates.

1394 **Fig. S7.** Overlap between eccDNA forming regions called using PacBio sequencing data and Illumina
1395 sequencing data.

1396 **Fig. S8.** Comparison between eccDNA forming regions in human samples called in this manuscript and in
1397 the original publication.

1398 **Fig. S9.** Comparison of eccDNA forming regions between *M. oryzae* and other previously studied
1399 organisms.

1400 **Fig. S10.** EccDNA forming regions composed of more than 90% LTR retrotransposon sequence in *M.*
1401 *oryzae*.

1402 **Fig. S11.** Percentage of the *M. oryzae* Guy11 genome made up of each LTR retrotransposon.

- 1403 **Fig. S12.** Correlation between number of LTR-LTR split reads and sequencing reads in eccDNA
1404 sequencing samples for each LTR retrotransposon in *M. oryzae*.
- 1405 **Fig. S13.** Correlation between number of LTR-internal split reads and sequencing reads in eccDNA
1406 sequencing samples for each LTR-retrotransposon in *M. oryzae*.
- 1407 **Fig. S14.** Number of LTR-LTR split reads and LTR-internal split reads in eccDNA sequencing samples for
1408 each LTR retrotransposon in *M. oryzae*.
- 1409 **Fig. S15.** Expected read coverage for LTR retrotransposons in *M. oryzae*.
- 1410 **Fig. S16.** MicroDNA enrichment and depletion in the genomes of various organisms.
- 1411 **Fig. S17.** Enrichment and depletion of microDNAs and large eccDNAs across various genomic regions in
1412 *M. oryzae*.
- 1413 **Fig. S18.** Correlation between gene count and junction split read count across the *M. oryzae* genome.
- 1414 **Fig. S19.** Correlation between junction split read count and expression for *M. oryzae* genes.
- 1415 **Fig. S20.** Comparison of junction split read counts between eccDNA forming regions with and without an
1416 ACS.
- 1417 **Fig. S21.** GC content and chromatin marks of eccDNA forming regions in *M. oryzae*.
- 1418 **Fig. S22.** Overlap between genes enriched on eccDNAs in biological replicates.
- 1419 **Fig. S23.** GO terms associated with eccDNA-associated genes.
- 1420 **Fig. S24.** GC content and chromatin marks of eccDNA-associated and eccDNA-absent genes in *M. oryzae*.
- 1421 **Fig. S25.** Comparison of expression data between eccDNA-associated genes and eccDNA-absent genes in
1422 *M. oryzae*.
- 1423 **Fig. S26.** Proximity of *M. oryzae* genes to repeats.
- 1424 **Fig. S27.** Proximity of *M. oryzae* genes to TEs.
- 1425 **Fig. S28.** Predicted effectors are prone to presence-absence variation in *M. oryzae*.
- 1426 **Fig. S29.** Rarefaction curves for eccDNA forming regions in *M. oryzae*.

1427 **Fig. S30.** Example of an eccDNA-mediated translocation in wine yeasts.

1428 **Fig. S31.** Comparison of encompassing split read counts between genes found on mini-chromosomes in
1429 *M. oryzae* and other genes.

1430 **Fig. S32.** GO terms associated with eccDNA-absent genes.

1431 **Fig. S33.** PCR validation of eccDNA-absent genes.

1432 **Fig. S34.** Effectors are enriched in eccDNAs in *M. oryzae*.

1433 **Fig. S35.** Lengths of eccDNA forming regions in *M. oryzae*.

1434

1435 **Additional File 2: Supplementary Tables.**

1436 **Table S1.** Number of eccDNA forming regions called using whole genome sequencing data.

1437 **Table S2.** Summary of protocols used to extract eccDNAs in studies analyzed in this manuscript.

1438 **Table S3.** Primers used for qPCR validation of linear DNA degradation and outward PCR validation of
1439 eccDNA forming regions.

1440

1441 **Additional File 3: List of eccDNA forming regions called using Illumina circularome sequencing data for**
1442 ***M. oryzae* in this study.**

1443 The first column describes the sample the eccDNA forming region was called with, the next three
1444 columns represent the genomic coordinates of the eccDNA forming region, and the last column
1445 represents the number of junction split reads used to call the eccDNA forming region.

1446

1447 **Additional File 4: List of eccDNA forming regions called using PacBio circularome sequencing data for**
1448 ***M. oryzae* in this study.**

1449 The first column describes the sample the eccDNA forming region was called with, the next three
1450 columns represent the genomic coordinates of the eccDNA forming region, and the last column
1451 represents the number of junction split reads used to call the eccDNA forming region.

1452

1453 **Additional File 5: List of eccDNA forming regions called using Illumina circularome sequencing data for**
1454 ***H. sapiens* muscle tissue published by Møller *et al.* [13].**

1455 The first column describes the sample the eccDNA was called with, the next three columns represent the
1456 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1457 junction split reads used to call the eccDNA forming region.

1458

1459 **Additional File 6: List of eccDNA forming regions called using Illumina circularome sequencing data for**
1460 ***H. sapiens* leukocytes published by Møller *et al.* [13].**

1461 The first column describes the sample the eccDNA was called with, the next three columns represent the
1462 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1463 junction split reads used to call the eccDNA forming region.

1464

1465 **Additional File 7: List of eccDNA forming regions called using Illumina circularome sequencing data for**
1466 ***O. sativa* in this study.**

1467 The first column describes the sample the eccDNA forming region was called with, the next three
1468 columns represent the genomic coordinates of the eccDNA forming region, and the last column
1469 represents the number of junction split reads used to call the eccDNA forming region.

1470

1471 **Additional File 8: List of eccDNA forming regions called using Illumina circularome sequencing data for**
1472 ***O. sativa* leaf tissue published by Lanciano *et al.* [18].**

1473 The first column describes the sample the eccDNA was called with, the next three columns represent the
1474 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1475 junction split reads used to call the eccDNA forming region.

1476

1477 **Additional File 9: List of eccDNA forming regions called using Illumina circularome sequencing data for**
1478 ***O. sativa* seed tissue published by Lanciano *et al.* [18].**

1479 The first column describes the sample the eccDNA was called with, the next three columns represent the
1480 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1481 junction split reads used to call the eccDNA forming region.

1482

1483 **Additional File 10: List of eccDNA forming regions called using Illumina circularome sequencing data**
1484 **for *O. sativa* callus tissue published by Lanciano *et al.* [18].**

1485 The first column describes the sample the eccDNA was called with, the next three columns represent the
1486 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1487 junction split reads used to call the eccDNA forming region.

1488

1489 **Additional File 11: List of eccDNA forming regions called using Illumina circularome sequencing data**
1490 **for *A. thaliana* WT tissue published by Lanciano *et al.* [18].**

1491 The first column describes the sample the eccDNA was called with, the next three columns represent the
1492 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1493 junction split reads used to call the eccDNA forming region.

1494

1495 **Additional File 12: List of eccDNA forming regions called using Illumina circularome sequencing data**
1496 **for *A. thaliana epi12* mutant tissue published by Lanciano *et al.* [18].**

1497 The first column describes the sample the eccDNA was called with, the next three columns represent the
1498 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1499 junction split reads used to call the eccDNA forming region.

1500

1501 **Additional File 13: List of eccDNA forming regions called using Illumina circularome sequencing data**
1502 **for *A. thaliana* leaf tissue published by Wang *et al.* [17].**

1503 The first column describes the sample the eccDNA was called with, the next three columns represent the
1504 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1505 junction split reads used to call the eccDNA forming region.

1506

1507 **Additional File 14: List of eccDNA forming regions called using Illumina circularome sequencing data**
1508 **for *A. thaliana* root tissue published by Wang *et al.* [17].**

1509 The first column describes the sample the eccDNA was called with, the next three columns represent the
1510 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1511 junction split reads used to call the eccDNA forming region.

1512

1513 **Additional File 15: List of eccDNA forming regions called using Illumina circularome sequencing data**
1514 **for *A. thaliana* stem tissue published by Wang *et al.* [17].**

1515 The first column describes the sample the eccDNA was called with, the next three columns represent the
1516 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1517 junction split reads used to call the eccDNA forming region.

1518

1519 **Additional File 16: List of eccDNA forming regions called using Illumina circularome sequencing data**
1520 **for *A. thaliana* flower tissue published by Wang *et al.* [17].**

1521 The first column describes the sample the eccDNA was called with, the next three columns represent the
1522 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1523 junction split reads used to call the eccDNA forming region.

1524

1525 **Additional File 17: List of eccDNA forming regions called using Illumina circularome sequencing data**
1526 **for *S. cerevisiae* WT cells published by Møller *et al.* [13].**

1527 The first column describes the sample the eccDNA was called with, the next three columns represent the
1528 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1529 junction split reads used to call the eccDNA forming region.

1530

1531 **Additional File 18: List of eccDNA forming regions called using Illumina circularome sequencing data**
1532 **for *S. cerevisiae* GAP1^{circle} cells published by Møller *et al.* [19].**

1533 The first column describes the sample the eccDNA was called with, the next three columns represent the
1534 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1535 junction split reads used to call the eccDNA forming region.

1536

1537 **Additional File 19: List of eccDNA forming regions called using Illumina circularome sequencing data**
1538 **for *S. cerevisiae* cells from the deletion collection published by Møller *et al.* [19].**

1539 The first column describes the sample the eccDNA was called with, the next three columns represent the
1540 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1541 junction split reads used to call the eccDNA forming region.

1542

1543 **Additional File 20: List of eccDNA forming regions called using Illumina circularome sequencing data**
1544 **for *S. cerevisiae* cells from the deletion collection treated with zeocin published by Møller *et al.* [19].**

1545 The first column describes the sample the eccDNA was called with, the next three columns represent the
1546 genomic coordinates of the eccDNA forming region, and the last column represents the number of
1547 junction split reads used to call the eccDNA forming region.

1548

1549 **Additional File 21: List of genes annotated in the *M. oryzae* Guy11 genome along with other**
1550 **information discussed in this study for each gene.**

1551 The first three columns describe the genomic coordinates of the gene, the fourth column is the gene's
1552 ID, the fifth column describes whether the gene was predicted to be an effector, the sixth column lists
1553 its name if it is a known effector, the seventh column lists the name of the protein in the *M. oryzae* 70-
1554 15 proteome, the eighth column describes whether it is an eccDNA-associated or eccDNA-absent gene,
1555 and the last column describes whether this gene was kept in all rice-infecting *M. oryzae* genomes
1556 analyzed.

1557

1558 **Additional File 22: Enriched GO terms in the cellular components ontology for eccDNA-associated**
1559 **genes.**

1560 The first column lists the GO term, the second column lists the number of genes annotated with each
1561 term, the third column lists the number of genes observed in the eccDNA-associated category, the
1562 fourth column list the number of genes expected in that category, the fifth column shows is a
1563 description of the go term, the sixth column lists the Chi-square value for that GO term, and the final
1564 column lists the ratio of the observed number of genes in the eccDNA-associated category divided by
1565 the expected number of genes in that category.

1566

1567 **Additional File 23: Enriched GO terms in the molecular function ontology for eccDNA-associated**
1568 **genes.**

1569 The first column lists the GO term, the second column lists the number of genes annotated with each
1570 term, the third column lists the number of genes observed in the eccDNA-associated category, the
1571 fourth column list the number of genes expected in that category, the fifth column shows is a
1572 description of the go term, the sixth column lists the Chi-square value for that GO term, and the final
1573 column lists the ratio of the observed number of genes in the eccDNA-associated category divided by
1574 the expected number of genes in that category.

1575

1576 **Additional File 24: Enriched GO terms in the biological pathway ontology for eccDNA-associated**
1577 **genes.**

1578 The first column lists the GO term, the second column lists the number of genes annotated with each
1579 term, the third column lists the number of genes observed in the eccDNA-associated category, the
1580 fourth column list the number of genes expected in that category, the fifth column shows is a
1581 description of the go term, the sixth column lists the Chi-square value for that GO term, and the final
1582 column lists the ratio of the observed number of genes in the eccDNA-associated category divided by
1583 the expected number of genes in that category.

1584

1585 **Additional File 25: List of GenBank accessions for the genomes of rice-infecting *M. oryzae* isolates**
1586 **used in this study for gene annotation.**

1587

1588 **Additional File 26: List of small, genic deletions identified in the *M. oryzae* Guy11 genome.**

1589 The first three columns describe genomic coordinates of the deletion, the fourth column is the missing
1590 gene's ID, and the last column is the name of the genome where the deletion is present.

1591

1592 **Additional File 27: List of GenBank accessions for the genomes of *M. oryzae* used in this study to**
1593 **search for eccDNA-mediated translocations.**

1594

1595 **Additional File 28: Enriched GO terms in the cellular components ontology for eccDNA-absent genes.**

1596 The first column lists the GO term, the second column lists the number of genes annotated with each
1597 term, the third column lists the number of genes observed in the eccDNA-absent category, the fourth
1598 column list the number of genes expected in that category, the fifth column shows is a description of the
1599 go term, the sixth column lists the Chi-square value for that GO term, and the final column lists the ratio
1600 of the observed number of genes in the eccDNA-associated category divided by the expected number of
1601 genes in that category.

1602

1603 **Additional File 29: Enriched GO terms in the molecular function ontology for eccDNA-absent genes.**

1604 The first column lists the GO term, the second column lists the number of genes annotated with each
1605 term, the third column lists the number of genes observed in the eccDNA-absent category, the fourth
1606 column list the number of genes expected in that category, the fifth column shows is a description of the
1607 go term, the sixth column lists the Chi-square value for that GO term, and the final column lists the ratio
1608 of the observed number of genes in the eccDNA-associated category divided by the expected number of
1609 genes in that category.

1610

1611 **Additional File 30: Enriched GO terms in the biological pathway ontology for eccDNA-absent genes.**

1612 The first column lists the GO term, the second column lists the number of genes annotated with each
1613 term, the third column lists the number of genes observed in the eccDNA-absent category, the fourth
1614 column list the number of genes expected in that category, the fifth column shows is a description of the
1615 go term, the sixth column lists the Chi-square value for that GO term, and the final column lists the ratio

1616 of the observed number of genes in the eccDNA-associated category divided by the expected number of
1617 genes in that category.

1618

1619 **Additional File 31: List showing names of barcodes used for each PacBio sequencing sample.**

1620

1621 **Additional File 32: Sequences of barcodes used for library preparation of PacBio sequencing samples**
1622 **in FASTA format.**

1623

1624 **Additional File 33: Consensus sequences of LTR retrotransposons in the *M. oryzae* Guy11 genome in**
1625 **FASTA format.**

1626

1627 **Additional File 34: Genome, gene annotation, and transposable element annotation files used for**
1628 **comparative circularome analysis.**

1629

1630 **Additional File 35: List of SRA accessions for RNAseq data used in this study.**