Population genomics provide insights into the global genetic structure of *Colletotrichum graminicola,* the causal agent of maize anthracnose

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Abstract

Background: *Colletotrichum graminicola*, the causal agent of maize anthracnose, is an important crop disease worldwide. Understanding the genetic diversity and mechanisms underlying genetic variation in pathogen populations is crucial to the development of effective control strategies. The genus *Colletotrichum* is largely recognized as asexual, but several species have been reported to have a sexual cycle. Here, we employed a population genomics approach to investigate the genetic diversity and reproductive biology of *C. graminicola* isolates infecting maize. We sequenced 108 isolates of *C. graminicola* collected in 14 countries using restriction site-associated DNA sequencing (RAD-Seq) and whole-genome sequencing (WGS). **Results:** Clustering analyses based on single-nucleotide polymorphisms showed populational differentiation at a global scale, with three genetic groups delimited by continental origin, compatible with short-dispersal of the pathogen, and geographic subdivision. Distinct levels of genetic diversity were observed between these clades, suggesting different evolutionary histories. Intra and inter-continental migration was predicted between Europe and South America, likely associated with the movement of contaminated germplasm. Low

clonality and evidence of genetic recombination were detected from the analysis of linkage disequilibrium and the pairwise homoplasy index (PHI) test for clonality. We show evidence that even if rare (possibly due to losses of sex and meiosis-associated genes) *C. graminicola* can undergo sexual recombination based on lab assays and genomic analyses. **Conclusions:** Our results support hypotheses of intra and intercontinental pathogen migration and genetic recombination with great impact on *C. graminicola* population structure.

Keywords: Fungal plant pathogen, population genetics, phylogeography, phytopathology, recombination, migration

Introduction

Crop diseases are one of the biggest concerns in global agriculture responsible for major losses in food production [1,2]. Fungi are primary causal agents of plant diseases and can cause catastrophic damage [3,4]. Disease outbreaks caused by fungal plant pathogens during recent decades have increased and are recognized as emergent threats to food security worldwide [5–8]. Human activity tends to intensify fungal disease dispersal by promoting pathogen movement far from their native location [9,10]. The modification of natural environments caused by modern agriculture provides new opportunities for natural selection [10]. The employ of crops with a limited genetic diversity increases the risks of global disease spread since pathogen genotypes can rapidly spread through genetically uniform host populations [11,12]. Additionally, ongoing climate changes can shift plant-pathogen distributions on a global scale since temperature changes may affect diseases development,

73 hence acting in the spread and survival of pathogens and their establishment in hitherto
74 unsuitable regions [8,13,14].

75 Maize (*Zea mays*) is the second most extensively cultivated cereal crop globally, with
76 a harvested area of almost 200 million hectares [15]. Together with rice and wheat, maize
77 supplies nearly half daily calories in human food [14, 16]. Although many diseases negatively
78 impact maize production, anthracnose is one of the most important diseases, it is so difficult to
79 control that requires to use of integrated practices [17–20]. Anthracnose disease is caused by
80 *Colletotrichum graminicola*, an ascomycete fungus that can infect most plant tissues, though
81 the stalk rot and seedling blight can bring more significant economic damage [21–23].
82 Taxonomically, *C. graminicola* belongs to the Graminicola species complex, a well-defined
83 monophyletic clade encompassing 16 *Colletotrichum* species pathogenic to the Poaceae family
84 [23–25].

85 Prior to 1960, maize anthracnose was a minor leaf spot disease in the USA, and the
86 causal agent *C. graminicola* was primarily studied for its taxonomic significance [22,26].
87 However, this view changed during the following decades when devastating outbreaks caused
88 unexpected losses across north-central and eastern regions of the USA [19,22]. Afterwards, the
89 disease became increasingly common, and in some seasons extremely destructive throughout
90 the eastern USA [27–29]. The occurrence of these outbreaks are thought to be associated with
91 the use of hybrids particularly susceptible to anthracnose, more favorable weather conditions,
92 and the emergence of more aggressive strains of the pathogen with rapid spread through the
93 maize belt [30–32]. Nowadays, maize anthracnose is a well-established endemic disease in the
94 USA, and better managed mainly through the use of resistant cultivars, but it still can cause
95 important damage in some regions of the country [33–37]. Given the history of the disease, it

may have the potential to become much more significant in the future.

Outside of the USA, *C. graminicola* is globally distributed and is of major importance in the Americas, and other regions of the world [38]. In North America, Canada reports variable disease incidence, but anthracnose is still the cause of significant concern [36,39,40]. In addition to temperate areas, maize anthracnose is also reported in tropical and subtropical regions, including Brazil and Argentina, where warm and humid conditions contribute to augment the disease [41–48]. Since the first report of anthracnose in Europe, (Italy in 1852) [49], several countries have reported the occurrence of the disease, including Germany, France, and ex Czechoslovakia [50–52], and more recently Croatia, Portugal, Switzerland, Bosnia and Herzegovina [53–57]. Recently, maize anthracnose also was reported in Asia [58], indicating that the pathogen is increasing its geographic range.

Pathogens widely disseminated over continents indicate an intense dispersal of their propagules, either from aerial dispersal, or via human-mediated movement of infected plant material, and/or seeds [11,59–61]. The nature of fungal propagules, i.e., their small and numerous spores, favor long-distance dispersal resulting in the spread of pathogens across and between continents [11]). Although many pathogens are cosmopolitan in distribution, their populations often can differ in some aspects, for instance, virulence, resistance to chemical controls, and genetic diversity [62]. The migration of plant pathogens can have epidemiological and evolutionary implications since the movement of individuals from one population to another may result in gene flow when migrants contribute their genes to the gene pool of another population [63]. *Colletotrichum graminicola* is seed-transmitted, also known as a seed-born pathogen [64–66], therefore, contaminated seeds serve as inoculum sources, and promote pathogen movement. Furthermore, though the transport of seeds and infected plant material,

119 fungi can naturally be spread via the dispersal of both sexual and asexual spores [11,59,67]. In
120 addition to the dispersal process, the reproductive biology of a species is crucial to
121 understanding the evolutionary forces that shape the genetic diversity of pathogen populations
122 over time and space [68]. Asexual reproduction predominates in the most of plant-pathogenic
123 fungi, but many species undergo sexual cycles, and many others employ both reproductive
124 strategies [63]. Sexual reproduction produces genetic recombination by the independent
125 assortment of chromosomes, increasing genotype diversity and generating a random
126 association between alleles at different loci [69,70]. Yet, alternative mechanisms, such as
127 parasexual reproduction, and cryptic sexual cycle can also create genetic recombination in
128 fungal species [71,72]. While sexual reproduction may be costly (in terms of fitness) and
129 disrupt favorable gene combinations, experimental evidence has suggested that sex in fungi
130 may increase the rate of adaptation to new environments [73,74]. Recombination can create
131 particular genotypes through the combination of more fit alleles, which can accelerate the
132 evolution of resistance to multiple fungicides and overtake R genes of resistant cultivars
133 [69,75,76].

134 The genetic control underlying sexual development in the genus *Colletotrichum* is not
135 well known and differs from the typical bipolar mating system of ascomycetes [77–79]. All
136 species studied so far contain just one of the two mating types alleles typical of ascomycetes
137 (*MAT1-1/2*) [23], which would seemingly limit their ability to mate. However, for at least some
138 *Colletotrichum* species, including *C. graminicola* sexual reproduction has been demonstrated.
139 Although the teleomorph of *C. graminicola* has been characterized under laboratory conditions
140 [78,79], the asexual state is the one commonly found in the field conditions, which plays a
141 significant role in the lifecycle. This species is reported to be a homothallic species [78], i.e.,

142 possesses both sets of genes and thus can complete the sexual cycle independently [80].
143 However, some strains are self-sterile and cross-fertile [79,81], which means that some strains
144 are capable of reproducing sexually when cultured alone, while other isolates require a mating
145 partner to produce sexual offspring.

146 Genetic recombination is reported as an important factor in the ecology and evolution
147 of many *Colletotrichum* species [82–87]. However, only a few population studies have
148 characterized the genetic diversity of grass-infecting *Colletotrichum* species, such as *C.*
149 *sublineola* and, *C. cereale* [85–89], and little is known about *C. graminicola* population
150 diversity. Studies using single marker genes and limited sample size showed high genetic
151 variability on *C. graminicola* isolates [87,90]. Therefore, in this study, we investigated the
152 global population structure of *C. graminicola* to get insights into the reproductive biology of
153 this species. Our population genomic analyses using single-nucleotide polymorphisms (SNPs)
154 revealed population subdivision into three genetic clades with isolates grouped according to
155 their country of origin, with distinct levels of genetic diversity between them, suggesting
156 different evolutionary histories. We found intra and intercontinental migration, mainly between
157 Europe and South America, likely associated with the movement of contaminated seeds. We
158 discussed the possible evolutionary origins of these genetic groups and the impact of
159 recombination on the genetic structure as well the potential of maize anthracnose becoming a
160 much more significant disease in the future.

161

162 **Materials And Methods**

163

164 **Sampling and isolation of *C. graminicola***

165 Fresh collections of *C. graminicola* samples were obtained from symptomatic maize
166 stems provided by an international network of maize researchers and producers, allowed
167 sampling from nine different countries where anthracnose disease is prevalent (Fig.1). Eleven
168 *C. graminicola* isolates originally collected from five countries (Germany, Japan, Netherlands,
169 Nigeria, and Zimbabwe) [91,92] were obtained from public culture collections (Supplementary
170 Table S1) and also included in the collection. Symptomatic stems were dissected, surface
171 disinfected, and plated in culture medium to fungal isolation, as previously described [21,57].
172 Fungal structures (hyphal tips, acervuli) emerging from maize tissue were transferred to potato
173 dextrose agar (PDA) medium allowed to grow for 7 days at 23°C then single-spore purified.
174 Monosporic cultures were stored at -80°C from suspension conidia in 7.5% skim milk (Merck,
175 Darmstadt Germany) mixed on silica gel (S/0730/53 Fisher) [93]. Isolates were verified as *C.*
176 *graminicola* by inspection of colony morphology, spore size and shape, and sequencing of the
177 internal transcribed spacer (*ITS*) region of rDNA using the primers ITS4 and ITS5 [94],
178 following PCR amplification conditions as described by Rech et al. [85]. The amplified ITS
179 fragments were purified using NucleoSpin gel and PCR Clean-up kits (Macherey Nagel,
180 Düren, Germany) and sequenced using Sanger technology by University of Salamanca (Spain).
181 Specie identity was confirmed by BLAST searches performed against in GenBank (NCBI). All
182 materials used in this study were used in accordance with the rights and obligations of the
183 recipient as described in the International Treaty on Plant Genetic Resources for Food and
184 Agriculture adopted by the FAO on November 3, 2001.

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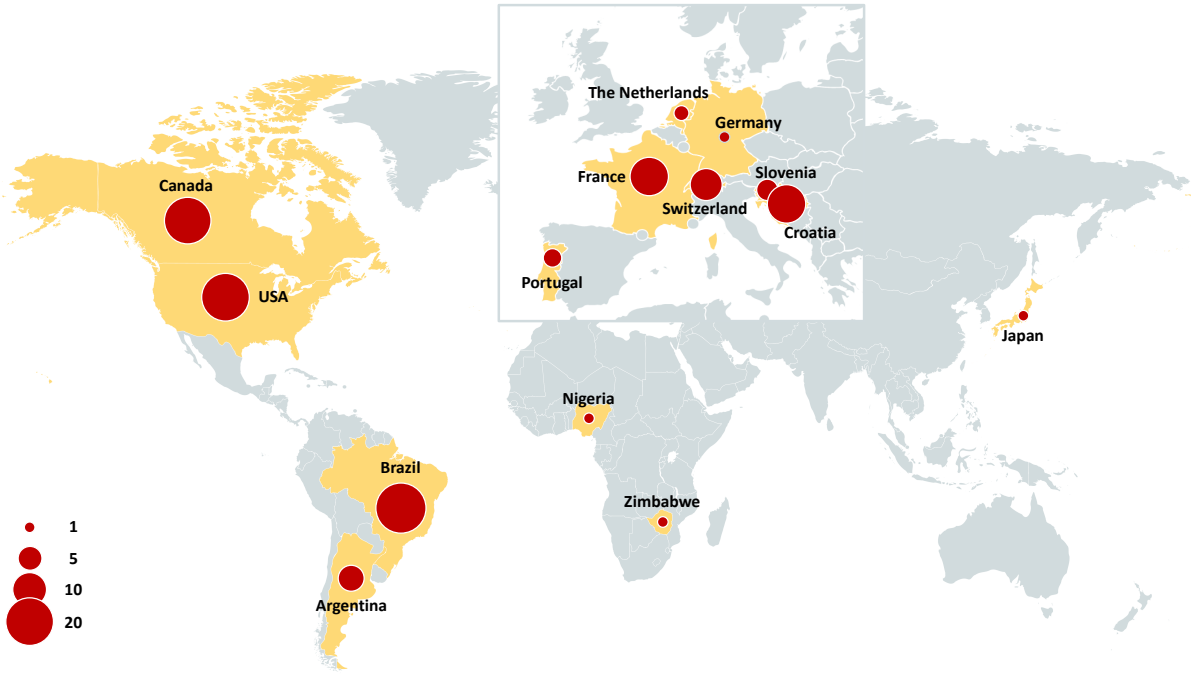


187 **Figure1.** Worldwide *Colletotrichum graminicola* sampling.

188

189 **DNA extraction, library preparation, and sequencing**

190 Mycelia from monosporic cultures were incubated on an orbital shaker in potato
191 dextrose broth (PDB) (Difco, Becton, Dickinson and Company, Franklin Lakes, USA) for three
192 days at 25°C and 150 rpm under continuous light [54]. Genomic DNA was extracted using
193 DNeasy Plant Mini Kits (Qiagen Inc., Valencia, California, USA) and quantified using a Qubit
194 3.0 fluorimeter (Invitrogen, Waltham, USA). DNA for *C. graminicola* isolates used for whole
195 genome sequencing (WGS) was extracted using the cetyltrimethylammonium-bromide
196 (CTAB) protocol adapted from Baek and Kernerley [95].

197 New DNA sequence datasets were generated from 101 *C. graminicola* isolates using
198 either RAD-seq of WGS; sequences for an additional seven isolates were also included in the
199 analyses [92] (Supplementary Table S1, S2). A total of 88 isolates were genotyped with RAD-
200 seq by Floragenex Inc. (Beaverton, Oregon, USA). Briefly, RAD-seq libraries with sample-

201 specific barcode sequences were constructed from DNA digested with restriction enzyme PstI,
202 then single-end sequenced (1 x 100 bp) on one lane using TrueSeq PE150 kit (Illumina, Inc.,
203 San Diego, CA) on an Illumina HiSeq 2000 instrument. WGS data was obtained from eight *C.*
204 *graminicola* isolates (CBS252.59, F-64330-2, F-64330-7, F-40330-1, SW-8046-2, CRO-I-35,
205 CR-10370-50, SW-8046-2) from libraries prepared using the Nextera XT DNA kit (Illumina,
206 Inc, San Diego, CA, and sequenced on Illumina HiSeq4000 (2x150 bp). WGS data was
207 obtained from six *C. graminicola* isolates (ARG-2700-2, CA-CHAT-1, CA-N8H, CRO-I-41,
208 P-7565-072-1, SW-8046-1) from paired-end libraries prepared using the Illumina TruSeq DNA
209 Nano kit and sequenced on an Illumina MiSeq (2x300 bp). Sequence quality was checked on
210 FASTQC v0.11.7 (Babraham Bioinformatics) and low-quality reads and adaptors were trimmed
211 with TRIMMOMATIC v0.3.8 [96]. *De novo* assemblies were performed with SPADES v3.11.1 [97].
212 Low coverage scaffolds were identified and removed; scaffolds from the mitochondrial
213 genome and rRNA clusters were identified and masked. The assembly completeness was
214 assessed based on the sordariomyceta_odb9 lineage dataset using BUSCO v.3 [98].
215

216 **Read mapping and SNP calling**

217 The RAD-Seq reads were inspected with FASTQC v0.11.7 (Babraham Bioinformatics,
218 Cambridge, UK) to confirm quality and were demultiplexed and quality filtered with the
219 process_radtags program in STACKS v.2.10 [99]. The demultiplexed reads were trimmed using
220 TRIMMOMATIC v.0.3.8 [96] using the parameters ('LEADING:15 TRAILING:15
221 SLIDINGWINDOW:4:15 MINLEN:36') and aligned to the *C. graminicola* reference genome
222 M1.001 (NCBI accession number SAMN02953757) using BWA v.0.7.8 [100]. Genetic variants
223 were identified with FREEBAYES V.0.9.21 [102]. The WGS reads were quality trimmed with

224 TRIMMOMATIC v.0.3.8 and aligned to the reference genome with SPEEDSEQ V.0.1.2 [101], which
225 uses BWA-MEM for read mapping and FREEBAYES for variant identification. The vcf files were
226 merged and filtered using VCFTOOLS [103] to remove nonbiallelic SNPs do not present in all
227 isolates, sites with quality scores lower than 30, and minor allele frequency (MAF) lower than
228 3%.
229
230 **Population structure and genetic diversity**
231 We defined a population as a group of individuals of the same species that occupy that
232 share the same area and colonize the same type of host simultaneously, then sharing a common
233 evolutionary history [63]. Analysis of molecular variance (AMOVA) was performed with the
234 hypothesis of no genetic differentiation using the package POPPR v2.8.6 [106]. Weir and
235 Cockerham's genetic differentiation index (F_{ST}) was calculated with the R package HIERFSTAT
236 using the WC84 method [107]. We used three clustering methods to examine the C.
237 *graminicola* population structure. First, we used a principal component analysis (PCA), with *a*
238 *priori* geographical knowledge from sampling (by continent). This analysis was conducted in
239 the R environment with the package ADEGENET v2.0.1 [108] using the DUDI.PCA function.
240 Second, we performed a discriminate analysis of principal components (DAPC) with R
241 package ADEGENET v2.0.1 [108]. The FIND.CLUSTER function was used to identify the most
242 likely grouping in the data based on the Bayesian information criterion (BIC) calculated for 1
243 to 10 clusters. Third, to confirm the pattern of population subdivision inferred by the PCA and
244 DAPC we used the fast maximum-likelihood genetic clustering method SNAPCLUST [109]. The
245 function SNAPCLUST.CHOOSEK was applied as a prior step to determine the optimal number of
246 clusters (*K*), based on the Bayesian information criterion (BIC). Additionally, we implemented

247 a neighbor network using the software SPLITSTREE V4 [110] to visualize phylogenetic signals,
248 indicated by the presence of reticulations in the network, from a distance matrix with default
249 parameters.

250 The number of multilocus genotypes (MLGs) was calculated using the R package POPPR
251 v2.8.6 [106]. We used the MGL.FILTER function to determine the true number of MLGs from a
252 genetic distance obtained by DISS.DIST function using the threshold determined in the
253 cutoff_predictor tool. To visualize relationships among genotypes a minimum spanning
254 network (MSN) was generated using the *msn* function in the R package POPPR. Genetic
255 diversity was estimated by comparison of the number of SNP variations by counting the
256 number of segregating sites between individuals, using the FIND.VARIATIONS tool in GENEIOUS
257 [111]. Tajima's D was calculated in nonoverlapping 10 kb windows using POPGENOME [112].
258

259 **Linkage disequilibrium**

260 The coefficient of linkage disequilibrium (r^2) [113] was calculated using VCFTOOLS
261 [103]. The rate of decay in linkage disequilibrium with physical distance was calculated for all
262 pairs of SNPs less than 100 kb apart using the option --hap-r2 and excluding sites with minor
263 allele frequencies below 5%. We also used the pairwise homoplasy index (PHI)
264 test implemented in SPLITSTREE V4 [110] to test the null hypothesis of clonality.
265

266 **Investigation of putative sex-related genes**

267 To identify orthologues of sex-related genes in the *C. graminicola* genomes, BLASTp
268 searches (e-value cutoff 1e-3) were performed using for the query a set of 96 gene with a role
269 in sexual reproduction in fungal model systems [114,115]. All nucleotide sequences were

270 checked manually to confirm no interruption in the translation frame. For this analysis, we used
271 the 12 *C. graminicola* assemblies generated in this study (see Supplementary Table S2 for the
272 summary assemblies statistics), plus seven *C. graminicola* genome assemblies used by Rech et
273 al. [92] and genome assemblies from nine other fungal species: *Colletotrichum fiorinae* ([116];
274 Genome accession: JARH00000000.1), *Colletotrichum fructicola* ([117]; Genome accession:
275 ANPB00000000.1), *Colletotrichum gloeosporioides* ([118]; Genome accession:
276 AMYD00000000.1), *Colletotrichum higginsianum* ([119]; Genome accession:
277 LTAN00000000.1), *Colletotrichum nymphaeae* ([120]; Genome accession:
278 JEMN00000000.1), *Colletotrichum salicis* ([120]; Genome accession: JFFI00000000.1),
279 *Colletotrichum simmondsii* ([120]; Genome accession: JFBX00000000.1), *Colletotrichum*
280 *sublineola* ([121]; Genome accession: JMSE00000000.1), *Colletotrichum orbiculare* ([117];
281 Genome accession: AMCV00000000.1), *Verticillium dahliae* ([122]; Genome accession:
282 ABJE00000000.1),
283
284 **Pathogenicity assays**
285 Pathogenicity characterization was performed by *in vivo* leaf blight assays using a
286 derivative of the susceptible maize inbred line Mo940 [123]. Plants were cultured in a
287 greenhouse for two weeks (V3 developmental stage), and inoculation via *C. graminicola* spore
288 suspensions were performed according to Vargas et al. [124]. Virulence was quantified by
289 measuring the area of necrotic lesions on leaves 4 days after inoculation. Symptomatic
290 leaves were excised from the plants, scanned with a flat-bed scanner, and quantified using the
291 imaging processing software Paint.NET v.4.2.16 (dotPDN LCC) [54], with three biological
292 replicates per isolate. For the comparison of differences in virulence among isolates, Analysis

293 of Variance (ANOVA) with Bonferroni and Sidak corrections for pairwise comparisons was
294 performed. Seventeen isolates were assessed, divided into batches because we could not
295 manage the whole set simultaneously in the laboratory. Given the possibility of a batch effect,
296 one of the strains (M1.001) was analyzed in all the batches to estimate the possible systematic
297 differences among them. Once all the isolates were analyzed, we compared the isolate repeated
298 in the different batches using ANOVA and obtained highly significant differences ($p < 0.0001$),
299 indicating a clear batch effect. Therefore, to eliminate the systematic differences among
300 batches, we standardized each strain using the mean and the standard deviation of the control
301 of its batch.
302

303 ## Results

304 **Population genetic analyses**

305 A total of 5811 biallelic variants were detected among 108 *C. graminicola* isolates
306 collected from 14 countries, which were used for population genetic analyses (Fig.1;
307 Supplementary Table S1). Analysis of SNP density showed an average of 1.4 SNPs per 10 kb
308 across contigs. Information about reads and coverage per individual are listed in the
309 supplementary table S1.

310 Clustering methods revealed population subdivision, supporting the existence of three
311 genetic groups, with isolates grouped by their continent of origin corresponding to the isolates
312 from South America, Europe, and North America, henceforth referred to as Brazilian (BR),
313 European (EU), and North American (NAM) clades (Fig. 2). The PCA and DAPC grouped
314 individuals according to their geographic origin (Fig. 2a, Supplementary Fig. S1).
315 Nevertheless, the isolates LAR318, CBS113173, and MAFF511343, from Zimbabwe, Nigeria,

316 and Japan respectively, obtained from culture collections did not group with the main three
317 genetic clusters. We do not discard the possibility of their geographic origins may not be
318 correctly reported in the collections. The SNAPCLUST function based on the BIC criterion
319 supported the clustering pattern by showing the optimum value of K = 3
320 clusters (Supplementary Fig. S2). The neighbor-net network generated by SPLITSTREE
321 identified three groups in the data (Fig. 3). Clusters in the network were connected by extensive
322 reticulated branches (looping) and long branches among samples, indicating a large
323 evolutionary distance separating individuals. AMOVA performed between *C. graminicola*
324 clusters showed that 65.5% of the total genetic variance was within clusters and 34.5% was
325 among them. Pairwise F_{ST} values between clusters were significant and revealed strong
326 differentiation, ranging from 0.26 to 0.42 (Supplementary Table S3).
327 Among the 108 isolates, we detected 76 MLGs unique and 15 MLGs shared between
328 at least two isolates. Most MLGs included isolates from the EU clade (13 MLGs in the total)
329 and shared between isolates from the same clade, and country (Supplementary Table S4).
330 However, one MLG encompassed isolates from France and Switzerland (F-40300-3 and SW-
331 8046-3), and another with isolates from Argentina and Croatia (ARG-2700-2 and CRO-I-35).
332 Two isolates from culture collections (M1.001 and LAR318) from USA and Nigeria were also
333 shared. Such genotypes shared were almost identical genetically, with few SNPs between
334 samples (Supplementary Table S4). The minimum spanning network (MSN) showed a similar
335 relationship among the aforementioned genotypes. The European cluster displayed an inner
336 position with MLGs closer genetically, while Brazilian and North American clusters were
337 placed on the edges of the network, likely due to their separate evolutionary history.
338

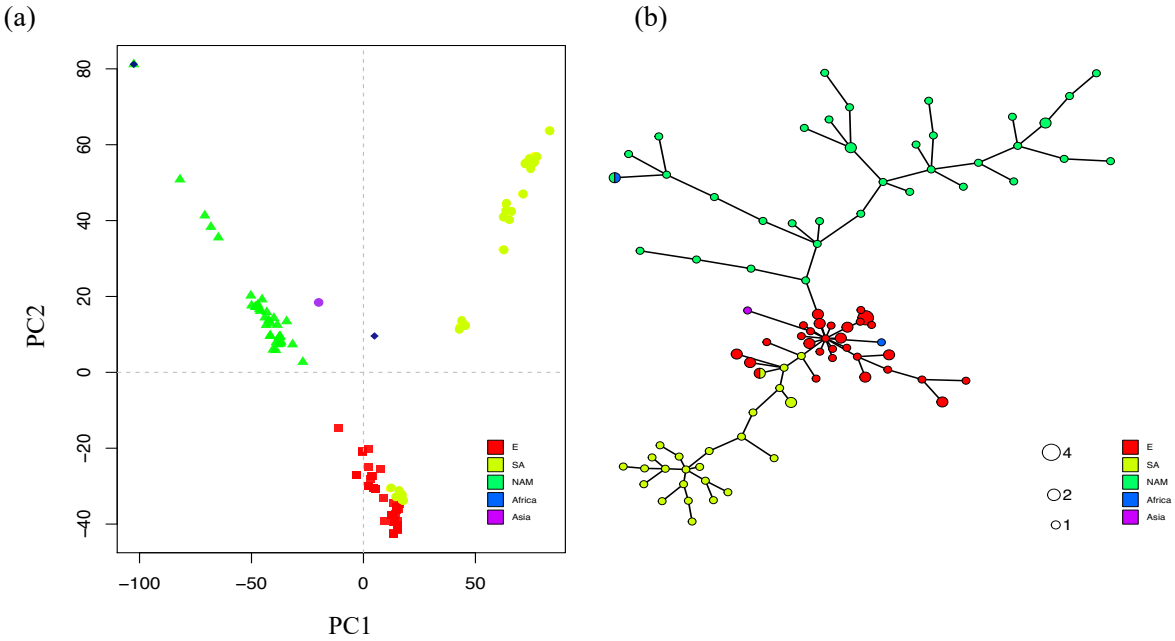


Figure 2. Population subdivision of *Colletotrichum graminicola*. (a) Principal components analysis (PCA). Isolates are colored according to their continent of origin. (b) Minimum spanning network (MSN) showing the multilocus genotypes (MLGs) based on Nei's genetic distance. The distance between nodes represents the genetic distance between MLGs

To estimate genetic diversity, we computed the number of segregating sites within genetic groups. Genetic diversity was twice as high in NAM compared to BR and EU clades (Table 1). Based on all the SNPs from the full dataset, NAM showed almost all sites segregating when considering all isolates belonging to this clade. In contrast, isolates from the EU clade showed fewer segregating SNPs and consequently lower genetic diversity. Tajima's D values were high and positive in the three lineages; a positive value is consistent with balancing selection or population contraction.

Table 1. Single nucleotide polymorphism (SNP) variation within *Colletotrichum graminicola* clusters and Tajima's neutrality statistic averaged over 10 kb nonoverlapping windows.

Cluster	SNPs within clusters	Tajima's D*
BR	2484	0.62 (0.88)
EU	2077	0.25 (1.03)
NAM	5264	1.42 (0.99)

*Standard deviation in parentheses.

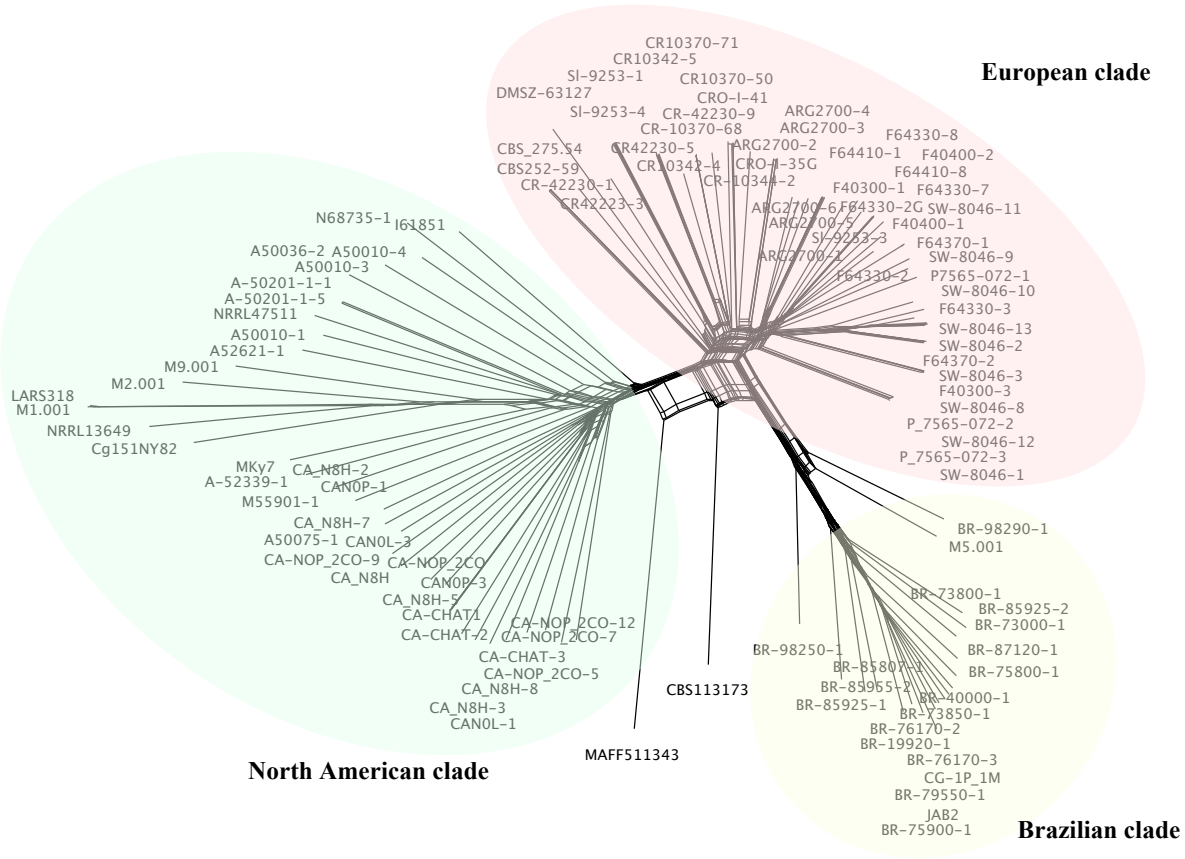
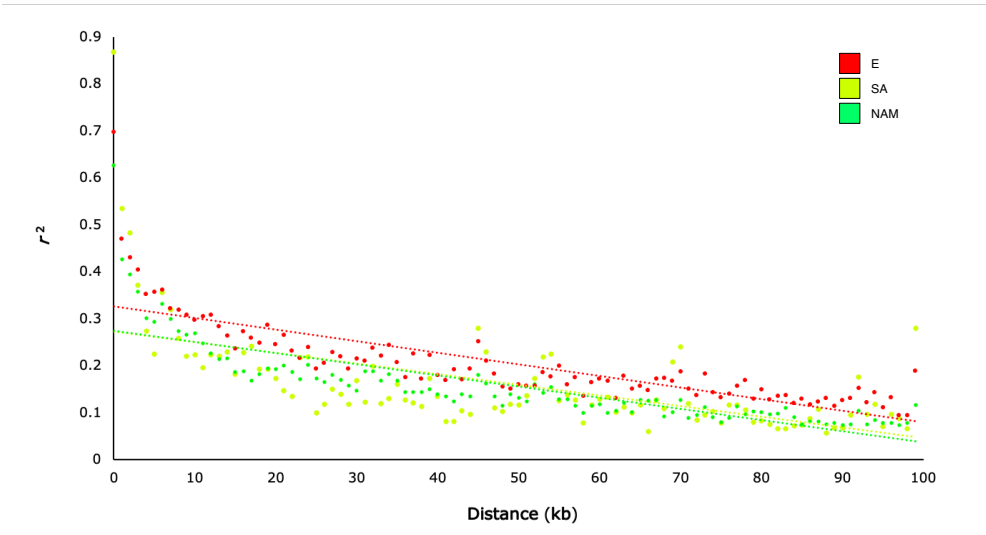


Figure 3. Neighbor-Net network showing relationships between *Colletotrichum graminicola* isolates.

363 **Footprints of recombination**

364 We assessed the genomic impact of recombination by analyzing patterns of linkage
365 disequilibrium. For this, we computed LD on the genetic clusters separately to avoid combining
366 evolutionarily distinct populations, which can lead to an overestimate of the linkage
367 equilibrium [69]. For all genetic clusters, the LD decayed quickly with physical distance,
368 reaching 50% of its maximum value in less than 1kb, suggesting a random association of alleles
369 at different loci, likely caused by recombination events. We also used the pairwise homoplasy
370 index (PHI) test implemented in SPLITSTREE to test the null hypothesis of no recombination,
371 which rejected the null hypothesis for all three genetic populations ($p < 0.001$).

372 To test the hypothesis that *C. graminicola* sex and meiosis-associated genes are being
373 maintained, we explore the presence of 96 genes previously identified as having roles in sexual
374 reproduction in Ascomycetes (Supplementary Table S5). We observed the presence of 94 genes
375 out of the 96 sex-related genes in three *C. graminicola* genomes (M1.001, LARS318 and
376 CBS113173), while all the other strains were missing three or more genes. A summary of the
377 15 genes showing the highest variability in presence/absence in the *C. graminicola* genomes is
378 shown in Table 2.



379

380 **Figure 4.** Linkage disequilibrium (LD) decay plots of three genetic populations of
381 *Colletotrichum graminicola* (European, Brazilian, and North America). LD was calculated for
382 all pairs of SNPs less than 100 kb distance apart.
383
384 **Table 2.** *Colletotrichum graminicola* orthologs of *Fusarium graminearum, Neurospora*
385 *crassa, Podospora anserina, Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe* sex-
386 related genes. Fifteen genes showing the highest variability in terms of present/absence in the
387 *C. graminicola* genomes. Genes absent are marked with an x.

Gene[1]	**Gene annotation / putative function**	M1.001	DMSZ63127	LARS318	CBS113173	NRRL47511	NRRL13649	CRO-I-35	F40300-1	CR10370-50	F64330-7	SW8046-2	CBS252.59	F64330-2	SW8046-1	P7565-07212	CA-CHAT-1	CRO-I-41	CA-N8H	ARG-2700-2
GLRG_06331T0	Meiotic recombination protein REC4								x	x	x	x		x	x			x	x	
GLRG_03069T0	Splicing factor 3B subunit 4							x	x	x	x	x	x	x						
GLRG_06080T0	Strand exchange protein RAD55p						x	x	x	x	x	x	x	x	x	x		x		x
GLRG_04547T0	Cell cycle checkpoint protein RAD17													x						
GLRG_06289T0	Histone H2A.Z							x		x			x							
GLRG_10150T0	Casein kinase I							x	x	x		x	x	x			x			
GLRG_02257T0	Nucleotide excision repair protein RAD23									x										
GLRG_02375T0	DNA topoisomerase							x	x	x	x	x	x	x						
GLRG_02369T0	Ku80 protein							x	x	x	x			x						
GLRG_02331T0	Chromosome segregation protein SUDA							x												
GLRG_00702T0	WD repeat-containing protein slp1					x														
GLRG_00302T0	Histone-lysine N-methyltransferase									x										
GLRG_03812T0	Peptide pheromone maturation								x											
GLRG_00900T0	Histone deacetylase HOS2		x																	
GLRG_09043T0	G protein-coupled receptor gpcr1								x	x	x		x							

388 [1]Genes from *C. graminicola* locus tag from reference genome strain M1.001 (O'Connell et al. 2012).
389
390

391 **Pathogenicity**

392 Seventeen isolates representative of the subclades identified in the phylogeny inference
393 and showing diversity of colony morphology and pigmentation were chosen for pathogenic
394 characterization (Supplementary Fig. S3). Considering the continental origin, the following
395 isolates were chosen, America: M1.001, A-52339-1, ARG-2700-5, BR-85925-1, CA-NOP-
396 2CO, CA-CHAT-1, and JAB2; Africa: CBS113173, LARS318; Europe: CBS252.59, CR-
397 42230-1, CRO-I-41, F-64330-2, P-7565-072-3, SI-9253-1, and SW-8046-2; Asia:
398 MAFF511343. All isolates, except two - CBS252.59 and MAFF511343, were pathogenic on
399 the maize cultivar evaluated producing necrotic lesions at the inoculation points by 4 days post
400 inoculation (dpi) (Fig. 5). Isolates CBS252.59 and MAFF511343 also failed to produce
401 necrotic lesions at 6 dpi (not shown). A high variation in virulence among the isolates was
402 found compared to the strain M1.001. We compared all the strains to the virulence of M1.001
403 using a classical analysis of variance $p < 0.0001$). Isolates ARG-2700-5, P-7565-072-3, SI-
404 9253-1, CA-CHAT-1, and CR-42230-1 had significantly higher virulence levels, while isolate
405 JAB2 was significantly less virulent. Population structure was not correlated with virulence.
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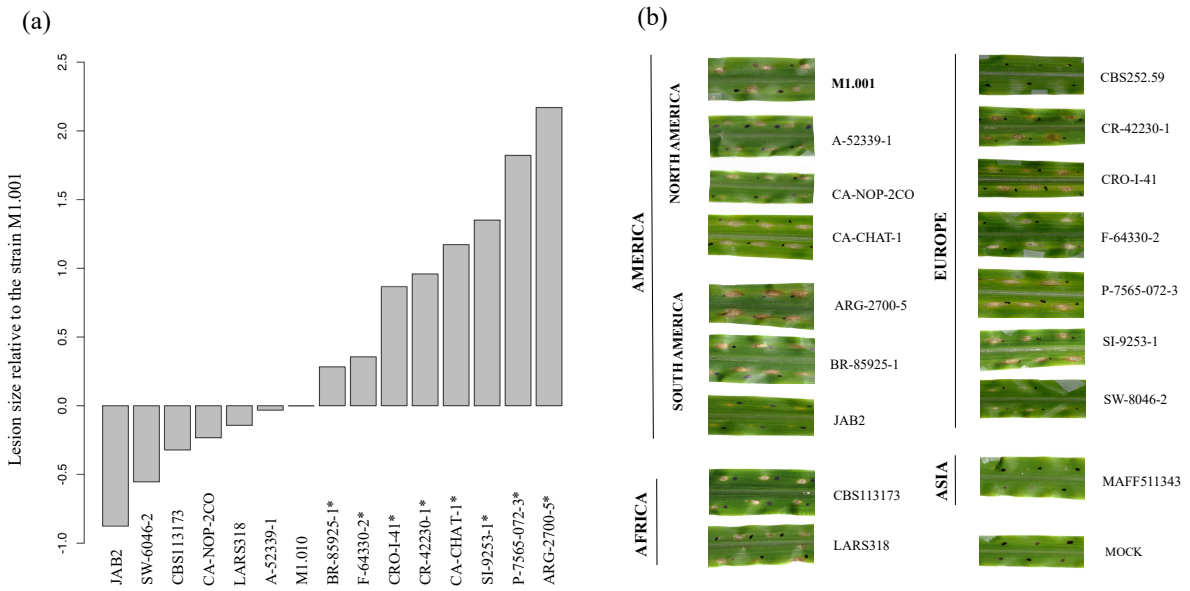

Figure 5. Pathogenic characterization of *Colletotrichum graminicola* isolates. (a) Graphic representation of pairwise comparisons of 14 isolates in order of virulence (relative to strain M1.001 used as control strain) in ascending order. The isolates CBS252.59 and MAFF511343 were non-pathogenic to the maize cultivar evaluated and were not included in the figure. Isolates marked with an asterisk are significantly different from isolate M1.001. (b) Necrotic lesions on maize leaves at 4 days after inoculation with spore suspensions of the isolates.

Discussion

We employed a population genomics approach to understand the genetic diversity of *C. graminicola* isolates infecting maize sampled from fourteen countries. Using SNP markers generated through RAD-seq and WGS, we investigated the distribution of genetic variation considering our samples as a global population. We found populational differentiation at a worldwide scale, with three genetic groups delimited by continental origin, compatible with

428 short dispersal of the pathogen, and geographic subdivision. However, we observed migration
429 intra and intercontinental migration, likely associated with the movement of contaminated
430 seeds. The genetic clusters, corresponding to Brazilian, European, and North American clades,
431 showed distinct levels of genetic diversity that could reflect different evolutionary histories.
432 Evidence of genetic recombination was detected from analysis of linkage disequilibrium and
433 the PHI test for clonality. Investigation of sex-related genes revealed gene losses in most
434 isolates. We show evidence that even if rare (possibly due to losses of sex and meiosis-
435 associated genes) *C. graminicola* can undergo sexual recombination based on lab assays and
436 genomic analyses. We present the possible evolutionary origins of these genetic groups and the
437 impact of recombination on genetic structure and the potential of maize anthracnose becoming
438 a much more significant disease in the future.

439 Clustering analyses based on single-nucleotide polymorphisms showed strong
440 population differentiation, grouping of the isolates according to their country of origin. The
441 European clade showed less genetic variation, a higher number of repeated MLGs, and lower
442 pairwise differentiation among clusters, suggesting a larger clonal component as compared to
443 the other clades. Interestingly, all genotypes shared were almost identical genetically,
444 suggesting that gene flow can be dependent on the clonal isolates generated by asexual
445 reproduction. Most MLGs were shared between isolates from the same cluster and country,
446 however, one MLG encompassed isolates from France and Switzerland (more than 1000 km
447 apart) and another one with isolates from Argentina and Croatia. In our cluster analyses, the
448 European clade encompassed all isolates from Argentina. Together, these results indicate intra
449 and intercontinental migration of the pathogen. We argue a likely European origin of the
450 isolates from Argentina due to the movement of maize germplasm into South America. It is

451 common for maize breeders from North America and Europe to use South America (including
452 Argentina) fields for advancing breeding programs during the northern hemisphere winter. As
453 Argentina is a maize exporter, breeding programs can represent a possible source of pathogen
454 importation and consequently a source of pathogen introductions into new areas, for instance
455 in Europe or elsewhere. This result is particularly important from the epidemiological point of
456 view, with direct implications on disease management since migration can cause the movement
457 of more virulent and/or fungicide resistant genotypes.

458 The predominant form of dissemination of *C. graminicola* is thought to be by
459 contaminated seeds [64–66]. Fungi can naturally be spread via the dispersal of both sexual and
460 asexual spores, caused mainly by the transport of infected plant material [11,59,67]. Dispersal
461 of asexual conidia of *C. graminicola* is strongly dependent on water splash and blowing
462 raindrops, responsible for short-distance dispersal [22]. Although airborne dispersal is not
463 typically documented, ascospore dispersal is associated with long-distance dissemination since
464 such spores can be ejected up and dispersed by air currents reaching longer distances [125,126].
465 If sexual reproduction occurs, even sporadically in this species, it can contribute to long
466 distance dissemination, leading to a broader pathogen distribution.

467 The North American clade encompassed isolates from the United States, Canada, and
468 one strain from Africa obtained from a culture collection. This cluster is more genetically
469 diverse and seems to have a separate evolutionary history. Although the first report in the
470 literature of a *Colletotrichum* species infecting maize came from Europe (Italy, 1852) we argue
471 that the North American cluster may be the more ancient lineage (or ancestral lineage) since
472 maize anthracnose was described for the first time in 1914 in the United States [127].
473 Considering that Mesoamerica is the maize center of origin, most probably Mexico, [128,129]

474 and the center of origin of pathogenic species usually correspond to the center of origin of their
475 host [130], given the proximity to the USA, this lineage could be the founder of the outbreak
476 during the 1970s and may have served as the source for introductions in other continents. The
477 highest genetic diversity found in this lineage also corroborates this hypothesis, since the center
478 of origin of a pathogen is likely to consist of populations with more genetic variability than
479 recently founded populations [131]. *C. graminicola* may have evolved together with the host
480 in Central America, and due to the expansion of maize production in the USA green belt the
481 pathogen had an opportunity to spread across the vast maize growing areas. Alternatively, our
482 sampling, which consisted of a large range of years (1975 to 2016), might have overestimated
483 the genetic diversity of this group. A more contemporary sampling can reveal the current
484 genetic variation of populations from North America.

485 In South America, isolates from Argentina and Brazil were grouped into separate
486 clades, suggesting independent introductions. In Brazil, the first disease report was in 1965 in
487 the São Paulo state (Campina's city) [132]. In the country, maize is planted in two distinct
488 growing seasons, the first crop planted in the summer and the second crop during the winter.
489 This practice contributes to increasing primary inoculum in the area. A significant reduction in
490 maize yield in tested hybrids was reported by Cota et al. [43] which was associated, among
491 other factors, with a higher pathogen aggressiveness and genetic differentiation in populations
492 during the crop cycle. This could indicate changes in the population size and their genetic
493 composition that may have contributed to a higher disease virulence in the country.

494 We found molecular evidence of genetic recombination for the three *C. graminicola*
495 populations detected in this study. The rapid decay of linkage disequilibrium and extensive
496 reticulations in the neighbor network is consistent with a possible history of recombination.

497 Furthermore, the PHI test for clonality detected recombination within each population (*P* =
498 0.0). These results corroborate a previous study that found evidence of recombination using
499 the PHI test at the chromosome level [92]. Similar findings of low and fast LDecay are reported
500 in other fungal plant pathogens which suggests the occurrence of genetic mechanisms
501 responsible for creating such patterns in genomes [82,133,134]. In addition, the signature of
502 repeat-induced point mutation (RIP), a genome defense mechanism that occurs during sexual
503 reproduction, [135,136] was detected in *C. graminicola* genomes [137,138]. RIP mutations
504 may indicate an ancestral state of sexual reproduction or that meiosis cryptically occurs in
505 nature [71,139].

506 Despite *C. graminicola* being reported as a homothallic species, strains may exhibit
507 homothallism and heterothallism, but this phenomenon is not yet fully understood [22].
508 Investigation of fertility through crosses of the strains M1.001 and M5.001 produced an
509 offspring exclusively heterothallic, which led Vaillancourt et al. [79] to hypothesize that
510 unbalanced heterothallism could be involved in the mating system. Our investigation of sex-
511 related genes revealed gene losses in most isolates and in those species known to be self-fertile
512 such as *C. salicis,* and in those known to be self-sterile and cross-fertile like *C. fiorinae* [120].
513 Interestingly the only two genes missing in all *C. graminicola* genomes analyzed were *ppg1*
514 and *pre2,* which form a pheromone receptor pair. The importance of this signaling pair varies
515 among homothallic ascomycetes. However, none of the deletion mutants obtained in *Sordaria*
516 *macrospora*, *Aspergillus nidulans* and *Gibberella zeae* completely lost the capability to
517 sexually cross [115].

518 Considering this evidence and the high completeness of the assemblies we conclude
519 that at least three of the *C. graminicola* isolates maintained all the genes required to undergo

520 sexual reproduction. This finding also suggests that some lineages have lost the ability for
521 sexual reproduction or that not all sex-related genes reported in other species are required in
522 *Colletotrichum* spp. [22]. Keeping in mind the presence of homothallic and heterothallic strains
523 in this species, the lack of genes required to sexually cross in one of the strains could mean
524 failure in sexual reproduction. In this scenario, sexual reproduction would become rare (but not
525 inconceivable) due to the low chance of two strains having the full set of genes encounter
526 themselves and mating.

527 The reproductive biology of the genus *Colletotrichum* is mixed, and the sexual state of
528 several species is unknown although several species are known to have the capacity for sexual
529 reproduction. The sexual capability of *C. graminicola* is well-documented [78,140]. We
530 demonstrated experimentally the sexual capability of *C. graminicola* isolates mating in lab
531 conditions, as described by Vaillancourt et al. [79] (see Supplementary Fig. S4 for details),
532 although the teleomorph has not yet been found in field conditions. Although the absence of
533 the sexual state in nature could cause less recombination, population genetic studies show that
534 several *Colletotrichum* species known as exclusively asexual, exhibit evidence of genetic
535 recombination [83,141,142]. Some studies have described alternative mechanisms, such as the
536 parasexual cycle as responsible for creating genetic recombination in *Colletotrichum* species
537 [79,84,143–145].

538 Species with the potential for genetic exchange are more likely to overcome control
539 measures employed since recombination can result in new combinations of alleles that increase
540 diversity and speed up adaptation at the field scale [63,146]. This study gathered evidence that
541 *C. graminicola* populations may have been affected by genetic recombination, but we also
542 showed evidence that even if rare (possibly due to frequent losses of sex and meiosis-associated

543 genes) *C. graminicola* can undergo sexual recombination. Fine-scale investigations at the
544 genome level can be employed to understand the real impact of genetic recombination on
545 genetic diversity and adaptation, for instance, if genetic exchange enhances virulence factors
546 (also called fitness-related traits) in these populations, as suggested for other fungal species
547 [147–151]. That can be particularly important in the agricultural pathosystem given that crop
548 pathogens are constantly facing a homogeneous environment that imposes strong directional
549 selection that leads to the evolution of more virulent and drug-resistant pathogen populations
550 [130,152].

551 We did not find a correlation between virulence and population structure; however, we
552 observed a substantial difference in the spectrum of virulence. Several isolates were more
553 aggressive than the strain M1.001 which was originally collected from North America in 1972
554 during the US. outbreak [153]. This finding is particularly important because this strain
555 represents the genetic diversity existent in the outbreak and strains more aggressive are
556 currently present in nature, which may be a risk to the development of new, even more,
557 destructive outbreaks. The pathogenicity assay was performed in one maize line (Mo940)
558 notably susceptible to anthracnose. The use of distinct maize genotypes can result in different
559 responses to fungal infection, as already reported in previous studies, and it should be
560 considered in further pathogenic characterizations [42,154,155]. Another significant aspect is
561 that resistance to maize anthracnose is inherited quantitatively, i.e., host resistance is controlled
562 by several genes with additive genetic effects [156–158]. This fact may correlate with the range
563 of virulence found in this study. The three genetic clades of *C. graminicola* detected may have
564 distinct evolutionary origins, hence encompassing a diverse combination of virulence genes,
565 which could cause different defense responses from the host. We also highlighted that the large

566 variability in virulence should be considered for the screening of resistant maize varieties.
567 Population studies can support breeding programs by investigations of intraspecific variability
568 of *C. graminicola* populations since the effectiveness of genetic resistance depends on
569 knowledge of the genetic structure of pathogens.
570

571 **Conclusions**

572 Our study represents the first investigation of the population structure of *C. graminicola*
573 infecting maize. The three main clades (North America, Brazil, and Europe) have different
574 levels of genetic diversity suggesting that each has a unique evolutionary history. We also
575 found evidence of recent migration of isolates between Europe and Argentina, possibly due to
576 the importation of infected plant material. Although the sexual state of *C. graminicola* has only
577 been reported in lab conditions, we show strong evidence that genetic recombination, whether
578 by sexual reproduction, or by alternative mechanisms (such as the parasexual cycle) plays an
579 important role in *C. graminicola* population structure. In contrast to the traditional view of *C.*
580 *graminicola* being mainly asexual, we conclude that genetic recombination is frequent. Fine-
581 scale investigations at the genome level can be employed to understand the real impact of
582 recombination and gene flow on the genetic diversity and adaptation of this species, with a
583 direct effect on control measures and breeding programs aiming at resistance. Finally, we warn
584 of the danger that maize anthracnose has the potential to become much more significant in the
585 future, mainly due to highly aggressive isolates currently present in maize fields and the
586 geographic range expansion of the pathogen, in addition to increased susceptibility of
587 agroecosystems accentuated by ongoing climate changes.
588

589 ## Additional Files

590 **Supplementary Fig. S1.** A) Bayesian information criteria (BIC) indicating the most probable
591 number of genetic groups by discriminant analysis of principal components analysis (DAPC).
592 B) Scatterplot from DAPC of *Colletotrichum graminicola* isolates assigned with *a priori*
593 geographical information (by continent).
594
595 **Supplementary Fig. S2.** Bayesian information criteria (BIC) indicating the most probable
596 number of genetic groups using the SNAPCLUST function.
597
598 **Supplementary Fig. S3.** Colony morphology of *Colletotrichum graminicola* showing
599 phenotypic diversity among isolates. The colonies were cultivated on PDA medium, at 23ºC
600 under continuous light for 6 days.
601
602 **Supplementary Fig. S4.** Sexual structures of *Colletotrichum graminicola* produced by
603 crossing from strains M1.001 (CB130836) and M5.001 (CBS 130839). The cross-inoculation
604 experiment was performed under laboratory conditions as described by Vaillancourt and Hanau
605 1991. (a) Agar plugs containing strains growing on autoclaved leaves of maize. (b) Mature
606 perithecia, picture taken with Olympus SZX9 stereomicroscope outfitted with an Olympus
607 DP70 camera. c) Ascus and ascospores. d) Ascospores.
608
609 **Supplementary Table S1.** Isolates of *Colletotrichum graminicola* used in this study.
610

611 **Supplementary Table S2.** Summary statistics of *Colletotrichum graminicola* genomes
612 generated in this study.
613
614 **Supplementary Table S3.** Measure of pairwise genetic differentiation between genetic
615 clusters of *Colletotrichum graminicola.*
616
617 **Supplementary Table S4.** Multilocus genotypes (MLGs) among *Colletotrichum graminicola*
618 isolates.
619
620 **Supplementary Table S5**. *Colletotrichum graminicola* orthologs of *Neurospora crassa,*
621 *Saccharomyces cerevisiae, Podospora anserina, Fusarium graminearum*, and
622 *Schizosaccharomyces pombe* sex-related genes.
623

624 Data Availability

625 The vcf file generated in this study is provided as Additional file 1. The genome assemblies
626 were submitted to GenBank and their accession numbers are in Supplementary Table S2.
627

628 Abbreviations

629 ANOVA: Analysis of Variance; BIC: Bayesian information criterion; BLAST: Basic Local
630 Alignment Search Tool; BR: Brazilian clade; CTAB: cetyltrimethylammonium-bromide; EU:
631 European clade; FST: Genetic differentiation index; GTR: general Time-Reversible model of
632 nucleotide substitution; ITS: internal transcribed spacer; MAF: minor allele frequency; MLGs:
633 multilocus genotypes; NAM: North American clade; MSN: minimum spanning network;

NCBI: National Center for Biotechnology Information; PDA: potato dextrose agar; PDB: potato dextrose broth; PCA: principal component analysis; PHI: pairwise homoplasy index; RAD-Seq: restriction site-associated DNA sequencing; pairwise homoplasy index; SNPs: single-nucleotide polymorphisms; WGS: whole-genome sequencing.

Competing Interests

The authors declare that they have no competing interests.

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Competing Interest

The authors declare that they have no competing interests.

Authors' Contributions

S.A.S. and M.R.T conceived the study and designed the project; S.A.S performed fungal isolation and DNA extraction; M.R.T., F.R., R.B., S.B. J.L.V.V., S.A.S, and F.B.C.F. performed analyses; M.R.T., F.R., R.B, and S.A.S. wrote the manuscript; F.B.C.F. and S.A.S. performed the pathogenic characterization; M.A.A.P., M.M.W. and J.A.C. performed genome sequencing; I.B., W.B., V.O., J.B., A.T., J.S.D., P.D.E., P.R., T.J.Z., J.H., G.M. collected the samples. All authors reviewed the manuscript.

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