Molecular epidemiology of Cercospora leaf spot on resistant and susceptible sugar beet hybrids

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Abstract

Cercospora leaf spot (CLS), caused by *Cercospora beticola*, is a major foliar disease impacting sugar beet production worldwide. The development of new resistant sugar beet hybrids provides a powerful tool to better manage the disease, but it is unclear how these hybrids affect CLS epidemiology. We used a molecular epidemiology approach to dissect natural epidemics of CLS affecting two susceptible and two resistant sugar beet hybrids with different levels of resistance at two field sites. Infected plants were geotagged on a weekly basis. Isolations of *C. beticola* were made from infected leaves and genotyped using six simple sequence repeat loci to identify clones. We determined that CLS epidemics had a later onset in plots planted to resistant hybrids, but once the pathogen established an infection, there was little difference between resistant and susceptible hybrids in the probability of localized spread and dispersal. We found that different clones often infected the same leaf and that clusters of infected plants were often colonized by a mixture of clones. There was little overall difference in genetic diversity between resistant and susceptible hybrids, however genotypic evenness differed at one site. In this site we found one genotype restricted to the resistant cultivars at a high frequency. We established that infections were not randomly distributed across the fields and we found that a single clone could spread over a distance of 100 m during a growing season.

Keywords: *Beta vulgaris*, *Cercospora beticola*, population genetics, cercospora leaf spot, resistant cultivars

Introduction

Sugar beet (*Beta vulgaris* L.) contributes approximately one-fifth of global sucrose production annually (Rangel et al., 2020). The most destructive disease of sugar beet is the foliar disease Cercospora leaf spot (CLS) caused by the fungus *Cercospora beticola*. CLS affects the production of sugar beet in almost all sugar beet growing regions worldwide (Holtschulte, 2000). Under high disease pressure, the yield loss can exceed 40% (Shane and Teng, 1992). The main method of control is multiple applications of fungicides, however this has resulted in the emergence of widespread fungicide resistance in *C. beticola* populations (Bolton et al., 2013). Resistant cultivars are seen as an important means of control in addition to fungicides, with CLS-resistant cultivars providing protection under low to moderate disease pressure (Francis and Luterbacher, 2003; Vogel et al., 2018; Weiland and Koch, 2004; Wolf and Verreet, 2005). A new resistance gene named *BvCR4* has recently been identified and bred into sugar beets (Chen et al., 2023). This new gene has shown a high level of CLS resistance and could be a useful method of control.

As with the evolution of fungicide resistance, pathogens can evolve to overcome host resistance through selection for virulent strains. How quickly pathogens evolve virulence to resistant cultivars is strongly influenced by the genetic architecture of the resistance – single major resistance (R) genes with large effect are usually less durable compared to quantitative resistance encoded by several genes with smaller, additive effects (Brown, 2015). The evolution of virulence is also influenced by the amount of genetic diversity in the selected pathogen population, the deployment strategies used for the resistance genes, and the distribution of virulent pathogen genotypes across a heterogenous host population (Milgroom and Peever, 2003; Stam and McDonald, 2018).

The interaction between pathogen genotypes and host genotypes shapes both the development of epidemics at the field scale and the evolutionary landscape of the entire pathosystem at larger spatial and temporal scales. High genetic diversity within a pathogen population can promote the evolution of virulence (McDonald and Linde, 2002a), but the type and degree of host resistance can influence the distribution of genetic diversity in the corresponding pathogen population. When a new resistant cultivar carrying a major R gene is first deployed in the field, the corresponding pathogen population experiences a selection

bottleneck, because only genotypes that carry the compatible virulence factors can infect the resistant cultivar. This selective bottleneck will likely result in a reduction in genetic diversity in the pathogen population. If the resistance of the cultivar is quantitative, selection is likely to have less effect on the overall genetic diversity, and genetic variation at the selected loci can be maintained (Cowger and Brown, 2019). How quickly the pathogen population and the corresponding genetic diversity can recover following this selection bottleneck depends upon the life history and evolutionary potential of the pathogen - in particular the frequency of sexual reproduction and the efficiency of dispersal of virulent genotypes (Barrett et al., 2008; McDonald and Linde, 2002b). The subsequent population recovery and dispersal are also likely to promote rapid evolution. The rate of sexual reproduction in *C. beticola* is unknown as no sexual stage (teleomorph) has yet been described, but the presence of both mating types at the *MAT* locus, together with high genotypic diversity suggests that it undergoes regular sexual cycles (Bolton et al., 2012; Groenewald et al., 2006, 2007b; Vaghefi et al., 2017a). The rapid evolution of fungicide resistance in CLS indicates that *C. beticola* has a high evolutionary potential (Bolton et al., 2013; Pethybridge et al., 2020; Trkulja et al., 2017), suggesting that *C. beticola* populations could also adapt quickly to deployment of resistant host genotypes.

The epidemiology of CLS has been well studied (Imbusch et al., 2021; Rossi et al., 2000; Tedford et al., 2018; Wolf and Verreet, 2005). *C. beticola* is polycyclic, with the primary inoculum coming from four known sources, including overwintering structures called pseudostromata which can persist on plant debris for two years (Khan et al., 2008), windborne conidia blowing in from outside newly planted fields, conidia produced on alternative hosts growing in or nearby new fields, and infested seeds (Spanner et al., 2022). In as little as 7 days these first infections can reproduce asexually to form conidia that establish secondary infections (Rangel et al., 2020). These conidia can be dispersed to adjacent plants by rainsplash, wind and insects (Khan et al., 2008; Vereijssen et al., 2007; Weiland and Koch, 2004), with the longest recorded dispersal distance exceeding 45 m (Imbusch et al., 2021). On resistant hosts, the latent period did not change, but the spore yield was reduced (Rossi et al., 2000). This finding illustrates that resistant hosts may influence CLS epidemiology, which may in turn affect the emergence and distribution of virulent genotypes.

97

98 Previous work established that *C. beticola* has high genetic diversity and a high evolutionary
99 potential, and that host resistance may influence aspects of CLS epidemiology, but few
100 studies have investigated the evolution of virulence in *C. beticola* populations or used
101 neutral genetic markers to understand how CLS epidemics develop in fields during a growing
102 season. Molecular epidemiology bridges the gaps between disease epidemiology and
103 pathogen population genetics. By including genetic markers into epidemiology studies, new
104 research questions can be addressed around the origins of epidemics, pathogen
105 movements, dispersal pathways and the spatio-temporal aspects of disease progression
106 (Archie et al., 2009; Desbiez et al., 2002; Xhaard et al., 2012).
107

108 Here we analyzed the molecular epidemiology of natural CLS epidemics occurring in
109 resistant and susceptible hybrids of sugar beet to better understand the evolutionary
110 potential of this pathogen on newly developed hybrids. The main objects were 1) to
111 determine the time of onset of CLS epidemics on different hybrids; 2) to measure the local
112 dispersal pattern for both primary and secondary inoculum of CLS; 3) to assess the genetic
113 diversity of *C. beticola* populations during epidemics across different spatial and temporal
114 scales, including: on the same leaves; on neighboring plants; and across hybrids within and
115 between fields; 4) to determine the degree of clonality on different host populations and
116 identify the most likely sources of primary and secondary inoculum in natural field
117 infections.
118

119 **Methods**

120 **Field trials in Switzerland**

121 Two trial sites were located in Switzerland: Rudolfingen, Trüllikon 47°38'18.6"N, 8°40'46.1"E
122 and Hendschiken, Othmarsingen 47°23'40.4"N, 8°13'41.1"E. At both sites, four sugar beet
123 hybrids were planted in March 2020 into single fields that had not been planted with sugar
124 beets in the previous three years. The four hybrids differed in their resistance to CLS,
125 including two highly resistant hybrids with the *BvCR4* gene – Hybrid_R2 and Hybrid_R3, and
126 two susceptible hybrids lacking *BvCR4*– Hybrid_S2 and Hybrid_S3 (Table 1). In Hendschiken,
127 12 rows of each hybrid were planted in each plot in a field covering 3600 m^2. In Rudolfingen,
128 18 rows of each hybrid were planted in each plot in a field covering 1950 m^2. Rows were

129 separated by approximately 45 cm in both locations (Figure S1). Sugar beets were harvested
130 in early November. No fungicides were applied at either field site and all CLS infection was
131 natural.
132
133 **Temporal sampling strategy**
134 Our sampling was designed to identify the naturally infected plants in each plot and
135 determine how natural infection spreads among plants. We systematically searched each
136 plot for infected plants once per week from July 21st to September 24th 2020. Individual
137 sugar beet plants served as sampling units and one leaf with CLS lesions per plant was
138 collected. The sampling design is shown in Figure S1. All sampled plants were marked with
139 flags to prevent resampling. In the early stages of the epidemic, we found single newly
140 infected plants surrounded by healthy plants, which we referred to as solitary infections. As
141 the epidemic progressed, the disease often spread from a single infected plant to
142 neighboring plants to form a cluster of infected plants, hereafter called a hotspot. For each
143 solitary infection, we recorded the GPS location (Garmin) and gave it a two-letter code (e.g.
144 AA) and the number 1 to indicate it was the first infected plant at that GPS position (e.g.
145 AA1). If the infected plant was adjacent to a previously identified and flag-labeled plant, it
146 was considered part of the same hotspot. Infected plants in the same hotspots were given
147 the same alphabetic code and a number to indicate the sampled plants were the 2nd, 3rd,
148 4th or 5th plant found in the hotspot (e.g. AA4 was the fourth plant sampled in the AA
149 hotspot). One leaf with CLS lesions was taken from each plant, with a preference for young
150 and otherwise healthy leaves with more than one clear CLS lesion. The excised leaves were
151 kept flat and cool until they were transported back to the laboratory for *C. beticola* isolation.
152 We sampled a maximum of five plants from each hotspot. We stopped sampling a hybrid
153 plot when it was no longer possible to find new solitary infected plants because hotspots
154 had coalesced. Our sampling design allowed us to track CLS epidemic progression and track
155 hotspot formation within and between plots planted to resistant and susceptible hybrids.
156
157 **Leaf storage**
158 In the field, the leaves were stored flat and cool until being transported back to the lab. To
159 store leaves flat, each leaf was placed on a labeled piece of paper and covered with a dry
160 paper towel. Multiple leaves were stacked between stiff cardboard sheets and secured with

161 strong rubber bands. The stacked leaves were then stored in a cool box with ice blocks to
162 maintain a low temperature during transport. Upon arrival at the laboratory, the leaves
163 were air dried at room temperature in a drying cupboard. Dried leaves were stacked and
164 placed in plastic bags with anhydrous silica gel and stored at 4°C until used for pathogen
165 isolation.
166
167 **Pathogen isolation and DNA extraction**
168 To obtain pure strains of *C. beticola*, spores were collected from the dried leaves. Three or
169 four pieces of leaf tissue with visible lesions were cut from each dried leaf. The tissue
170 samples were surface sterilized by immersing them in a 2% sodium hypochlorite solution for
171 3 minutes, followed by two rinses in sterile de-ionized water. The leaf pieces were placed on
172 dry paper towels to remove excess water, then were put into glass petri dishes on an
173 elevated sterile net placed on a piece of moist filter paper. The petri dishes were sealed with
174 parafilm and incubated at 24°C for 48-72 hours to induce sporulation. The induced lesions
175 were checked for the typical black pseudostromata and hyaline conidia under a dissecting
176 microscope (Olympus Schweiz AG). The spores were picked from sporulating lesions using
177 hand-made fine glass needles and transferred to fresh potato dextrose agar (PDA, Difco).
178 The plates were cultured under constant darkness at 24°C for 7-10 days. After a
179 characteristic *C. beticola* colony had formed, a 5-mm agar plug was excised from the edge of
180 each colony and transferred to a new PDA plate for 14 days to form a pure culture. Any
181 contaminated plates were discarded. For long-term storage of each pure culture, 4-6 agar
182 plugs were cut from the edges of a 2-week-old pure culture and put into cryotubes in
183 duplicate - with and without anhydrous silica gel, and stored at 4°C. For most leaves,
184 between 1-3 colonies were isolated from different lesions, but for some leaves, more than 3
185 strains were isolated to investigate pathogen diversity within the same leaf. In total, 1251 *C.*
186 *beticola* isolations were made. For practical reasons, not all isolates were transferred into
187 long-term storage. Instead, we aimed for a single isolation per leaf and preference was
188 given to isolates from hotpots, which were expected to be more virulent and capable of
189 dispersing to nearby healthy plants.
190
191 To obtain fresh mycelia for DNA extraction, we transferred hyphae from plates to liquid
192 culture by scraping small pieces of hyphae from the edge of each pure culture and placing

193 them into 150 mL flasks containing 50 mL potato dextrose broth (PDB, Difco). The liquid
194 cultures were incubated in the dark at 24°C on a shaker at 120 rpm for 10 days. The fungal
195 tissue was harvested, washed twice with sterilized water, and the supernatant was
196 discarded after centrifuging (Allegra X-12R, Beckman Coulter) at 3750 rpm for 10 mins. The
197 pellet was then transferred to 2 mL microcentrifuge tubes and lyophilized overnight. The
198 lyophilized pellets were ground into a fine powder using ~15 glass beads in a grinding
199 machine (Qiagen MM300 Retsch TissueLyser). A total of 560 DNA samples of isolates coming
200 from separate leaves were extracted using an automated DNA extraction robot, the
201 Kingfisher Flex (Thermo Fisher Scientific), using the recommended reagents and a protocol
202 for fungal samples (LGC Genomics GmbH). The resulting DNA samples were quantified using
203 a Nanodrop spectrophotometer (Thermo Fisher Scientific). We performed a polymerase
204 chain reaction (PCR) using species-specific primers (Knight and Pethybridge, 2020) to
205 confirm the samples were *C. beticola*. Only samples with a clear band at around 200 bp
206 (~85%) were retained.
207
208 **Simple sequence repeats analysis**
209 The six SSRs were amplified in 2 multiplexes (multiplex1: SSRCb1, SSRCb3, SSRCb21;
210 multiplex2: SSRCb22, SSRCb25, SSRCb27) (Groenewald et al., 2007a; Vaghefi et al., 2017b).
211 The SSR primers were blasted against the reference genome to ensure they were not
212 physically linked (Table S1). The PCR amplifications were performed using Qiagen Type-it
213 kits (Qiagen) in a total volume of 11 µL. The reaction mixture contained 20 ng of template
214 DNA, 0.4 µM of each forward and reverse primer, 4 µL Type-it mix (with buffer and $MgCl_2$), 1
215 µL Q-solution (1 U Taq polymerase), and 2 µl deionized water. The initial denaturation was
216 carried out at 95°C for 5 mins, followed by 30 cycles of denaturation at 95°C for 30 s,
217 annealing at 58°C or 56°C for 90 s, elongation at 72°C for 30 s and final extension at 60°C for
218 30 mins. The PCR amplicons were analyzed on an ABI 3730xl DNA Analyzer (Applied
219 Biosystems) at the Genetic Diversity Centre (GDC) of ETH Zürich. Multiple samples were
220 repeated at different stages to create technical replicates in order to estimate SSR
221 genotyping error rates and ensure consistent allele calling.
222
223 SSR allele size was scored using the R package *Fragman* (Covarrubias-Pazaran et al., 2016).
224 To reduce the genotyping error, allele fragment sizes of each SSR marker were visualized

225 and binned using the R package MsatAllele v 1.05 (Alberto, 2009). Fragment stutter patterns
226 varied between the SSRs. Visual inspection of the allele calling and consistency between
227 repeats was used to assess the accuracy of the MsatAllele automatic binning. Automatic
228 binning was accurate for three SSRs (SSRCb27, SSRCb3, SSRCb21) but manual adjustment of
229 the bin sizes was required for the rest. The consistency of allele calling was evaluated using
230 isolates that were replicated (n=51). In total we obtained SSR genotypes for 475 isolates.
231 Genotype calling was repeated for 20% of isolates chosen at random. Samples with multiple
232 allele calls were assumed to be a mixture of strains and were excluded from the dataset. All
233 individuals with missing SSR data were also removed, leaving 340 fully genotyped isolates
234 (158 from Hendschiken and 182 from Rudolfingen) for downstream analyses.
235
236 **Statistical analysis**
237 Statistical analysis was performed in R version 4.3.1 (R Core Team, 2023) and R Studio
238 version 2023.6.0.421 (Posit team, 2023). To investigate the localized spread between plants
239 to form a hotspot, we used a generalized linear model with a binomial distribution. To
240 compare across hybrids, we needed to control for the fact that CLS continued to spread
241 after we stopped sampling in some hybrid plots, meaning that any new infection cases that
242 were identified at the end of the sampling period were not followed to determine if they
243 spread to neighboring plants. Our solution was to right-censor the data by excluding the
244 new infection cases observed 1 and 2 weeks before the final sampling date in each hybrid
245 plot. This is a common practice when sampling ends before an event has been observed.
246
247 As the sampling period varied for each hybrid, the right-censor date also varied (Table 2).
248 The rate at which a hotspot developed was analyzed using a linear model. The pairwise
249 distances between geotagged infection sites were calculated using the R package *geosphere*
250 v1.5-18 (Hijmans, R. J., 2022). Whether the distributions of geotagged plants differed
251 between hybrids or between the beginning and the end of the season were assessed using
252 Kolmogorov-Smirnov (K-S) tests in the R package R4PDE (Del Ponte, 2023). Multi-locus
253 genotypes (MLGs) were identified and analyzed with the R package *poppr* v.2.9.3 (Kamvar et
254 al., 2015, 2014). We performed MLG analysis separately for each hybrid in each site to
255 compare the epidemics between them.
256

257 We performed clone-correction for each site separately as our earlier analyses indicated it
258 was unlikely to observe the same MLG between two sites (whole genome sequence data of
259 256 isolates found only one case of the same clone present at the two sites (Chen et al.,
260 2023)). The number of alleles, number of unique MLGs, the expected number of MLGs
261 controlling for differences in sample size (eMLG) and Nei's gene diversity, Simpson's
262 diversity and evenness were calculated using the R package *poppr* v.2.9.3 for each hybrid
263 and site. Bootstrapped confidence intervals were calculated for genetic diversity statistics
264 using function diversity_ci in *poppr*. Clonal fraction was calculated as (N–number of
265 MLGs)/N, where N equals the number of isolates.
266
267 **Results**
268 **Onset and distribution of CLS disease**
269 The first symptoms of *C. beticola* were observed in the susceptible hybrids, for Hybrid_S2 on
270 July 21st and one week later in Hybrid_S3. Three weeks later, the first infections appeared
271 on the resistant hybrids (Table 2). The appearance of the first *C. beticola* infections on
272 resistant hybrids with *BvCR4* varied between fields. In Hendschiken, the disease appeared
273 on Hybrid_R2 before Hybrid_R3, while in Rudolfingen, the first symptoms were observed
274 later in Hybrid_R2. GPS coordinates were used to map the locations of infected plants. The
275 spatial distribution of all infection cases is shown in Figure 1.
276
277 The accumulation of geotagged infection cases is shown in Figure 2. The sampling period
278 varied for each hybrid because the dates of disease onset differed (Table 2) and because we
279 stopped sampling when the hotspots coalesced and solitary infections could no longer be
280 clearly distinguished. Thus, we stopped sampling first in Hybrid_S2, then Hybrid_S3 and
281 then Hybrid_R2. Sampling continued until our final visit (September 24) in Hybrid_R3 as
282 hotspots had not coalesced. Infections continued to increase in all hybrids after we stopped
283 sampling. It should be noted that due to some initial sampling problems, we missed the
284 start of the epidemic in Hybrid_S3. Hence caution must be taken when comparing the
285 temporal spread in Hybrid_S3 with other hybrids. By the end of the season, 454 plants were
286 sampled (one leaf per plant) from 196 geotagged infections in Hendschiken, and 503 plants
287 were sampled from 209 geotagged infections in Rudolfingen.
288

289 **Localized spread of CLS in each hybrid**

290 Across all hybrids, about 80% of infection cases spread to at least one neighboring plant,
291 resulting in the formation of hotspots (Table 3). There was no significant difference between
292 fields or hybrids in the probability that CLS would spread from one solitary infected plant to
293 neighboring plants to form a hotspot of two or more infected plants, or to form a large
294 hotspot with at least five infected plants (hotspot: Field $F_{(1,209)}$ =1.23, p=0.26; Hybrid $F_{(3,206)}$
295 =4.84, p=0.1, Large hotspot Field $F_{(1,209)}$ =2.69, p=0.10; Hybrid $F_{(3,206)}$ =4.90, p=0.17). NB: the
296 numbers in Table 3 differ slightly compared to the total cases and hotspots (Figure 2) due to
297 the right-censoring of datasets.

298

299 To assess the rate of hotspot development in each hybrid, we calculated the rate of spread
300 as the number of infected plants divided by the number of days between sampling periods
301 for plants sampled in each hotspot. There was no difference in the rate of spread between
302 sites (Field $F_{(1,115)}$ =2.87, p=0.09), there was a significant effect of hybrid (Hybrid $F_{(3,115)}$ =6.5,
303 p<0.001) and no interaction (Field*Hybrid $F_{(3,115)}$ =2.17, p=0.09). In Hendschiken, the rate of
304 spread was slower in Hybrid_R3 and faster in Hybrid_R2 compared to Hybrid_S2
305 (Hybrid_R2: t=3.95, p=0.002, Hybrid_R3: t=-2.73, p=0.008). In Rudolfingen, there was no
306 difference in the rate of spread, although Hybrid_R3 tended to be slower (t=-1.82, p=0.07).

307

308 **Spatial distribution of infections**

309 We compared the distribution of geotagged infections at the beginning and the end of the
310 epidemic and across hybrids within each site. The first two sampling dates in each hybrid
311 plot were designated as the beginning of the epidemic and were used to investigate the
312 spatial distribution of the first CLS infections. The initial distributions of hotspots and
313 infection sites for most hybrids were randomly distributed (Table S2, p >0.10) with two
314 exceptions: Hybrid_S2 in Hendschiken and Hybrid_R2 in Rudolfingen. During the epidemic,
315 the minimum distance between infection sites decreased or remained constant, while the
316 maximum distance increased throughout each epidemic (Table 4). In some cases, the GPS
317 was not recorded for an infection site, resulting in smaller numbers in Table 4 compared to
318 Figure 2. The final distributions of hotspots and infection sites for most hybrids were
319 aggregated (non-random) (Table S2, p <0.10) with two exceptions: Hybrid_R2 in
320 Hendschiken and Hybrid_S2 in Rudolfingen, where the infection sites were randomly

321 distributed. Although the number of infection cases increased over the season, there was
322 little difference in the spatial distribution of geotagged infections at the beginning and at
323 the end of the season (Table S3, $p > 0.10$, Figure S2).
324
325 We compared the distribution of geotagged infection sites in each hybrid and found that
326 Hendschiken Hybrid_R3 was often different from the other plots (Table S4, $p < 0.10$). In
327 Rudolfingen, there were more differences; Hybrid_R2 was different from both susceptible
328 hybrids, Hybrid_R3 was also different from Hybrid_S3, but was similar to Hybrid_S2, while
329 Hybrid_S2 and Hybrid_S3 had different distributions.
330
331 **Genetic and genotypic diversity at different hierarchical levels**
332 The six SSR loci used in our study were polymorphic, resulting in 2 to 9 alleles per locus.
333 Allelic diversity after clone correction ranged from 0.23 to 0.83 (Table S5) and there was
334 some variation in the number of alleles for each SSR between hybrids (Table S6). There were
335 some alleles unique to each field; for example, the 183 bp allele at SSRCb22 was found only
336 in Hendschiken and the 373 bp allele at SSRCb27 was only detected in Rudolfingen.
337
338 We investigated genetic diversity at different hierarchical levels, including within and
339 between hybrids and fields, and within leaves and hotspots. In Hendschiken, 158 isolates
340 were distributed among 82 unique MLGs. In Rudolfingen, 182 isolates resulted in 77 unique
341 MLGs (Table 5). The genetic diversity ranged from 0.40 to 0.57 across different hybrid plots,
342 and the genotypic diversity ranged from 0.73 to 0.95 (Table 5). There was little difference in
343 Nei's gene diversity between sites or hybrids. The clonal fraction differed significantly among
344 the hybrids in Rudolfingen ($\chi^2 = 11.15$, df=3, p=0.01), while it was similar among hybrids at
345 Hendschiken ($\chi^2 = 3.38$, df=3, p=0.33). In Rudolfingen, the clonal fraction was lower in
346 Hybrid_S3 and higher in Hybrid_R2. The frequency of MLGS differed in each site (Figure 3).
347 In Rudolfingen, there was greater skew in MLG frequencies; R_MLG77 appeared in 46
348 isolates (20 in Hybrid_R2 and 26 in Hybrid_R3) and was restricted to the resistant hybrids
349 with *BvCR4*. In Hendschiken, differences between the hybrids were less pronounced, with
350 the most frequent MLG (H_MLG75) appearing 13 times in the resistant hybrids with *BvCR4*
351 (Hybrid_R2 and Hybrid_R3) (3 and 10 times respectively). The evenness was significantly

352 higher in susceptible hybrids in Rudolfingen, and no difference was found in Hendschiken
353 (Figure 4).
354
355 **Genotypic diversity within a leaf and hotspot**
356 To investigate genotypic diversity within individual leaves, multiple isolations were made
357 from the same leaf for a total of 48 leaves (Table 6a). It was common to find more than one
358 genotype per leaf, with 23 out of the 48 (0.48) tested leaves having more than one genotype
359 on the same leaf. The maximum number of MLGs from one leaf was two, irrespective of the
360 number of isolations (2-4). Although there was not enough data to make statistical
361 comparisons between hybrids or sites, we commonly observed the coexistence of multiple
362 genotypes in resistant hybrids. Specifically, in Hendschiken 6 out of 14 (0.39) tested leaves
363 from resistant hybrids had multiple strains and 10 out of 16 (0.63) resistant leaves had
364 multiple strains in Rudolfingen. We also considered the genotypic diversity within a hotspot
365 (n= 48) (Table 6b). Just over half of the hotspots had more than one MLG. In resistant
366 hybrids, 8 out of 13 (0.61) tested hotspots had multiple strains in Hendschiken and 11 out of
367 19 (0.57) hotspots had multiple strains in Rudolfingen.
368
369 **Genotypic diversity at the start of each epidemic**
370 Genotypic diversity and the spatial distribution of isolates from the first two sampling dates
371 in each plot were used to infer the most likely sources of primary inoculum in each
372 epidemic. In Hybrid_S2 (July 21st and 27th) in Rudolfingen we genotyped 13 isolates coming
373 from seven hotspots and identified nine MLGs (clonal fraction (CF) = 0.31). In Hendschiken
374 we genotyped 14 isolates from six hotspots and identified seven MLGs (CF = 0.50, but five
375 isolates from the same hotspot had the same MLG). At the beginning of the epidemic, no
376 MLGs were shared between hotspots, indicating that different pathogen strains established
377 each hotspot. Only four out of the seven initial hotspots in Rudolfingen were geotagged,
378 with pairwise distances ranging from 18.4 to 64.7 meters. Three out of the six initial
379 hotspots in Hendschiken were geotagged, with pairwise distances ranging from 25.0 to 68.7
380 meters. For the first two sampling dates in Hybrid_S3, only one isolate was genotyped from
381 each site, and thus the distribution of MLGs cannot be compared.
382

For the resistant Hybrid_R2: In Rudolfingen, on the first sampling date (August 25th) 15 isolates from 11 hotspots were genotyped. These contained six MLGs (CF = 0.6) with pairwise distances ranging from 1.3 to 49.2 m. At the beginning of this epidemic the R_MLG77 genotype was found 10 times in eight hotspots that were separated by pairwise distances ranging from 1.3 to 19.6 m. In Hendschiken, during the first sampling dates in Hybrid_R2 (August 11th and 20th), five isolates were sampled from four hotspots composed of four MLGs (CF = 0.2), with the pairwise distances ranging from 4.2 to 61.4 m. No MLGs were shared between hotspots.

For the resistant Hybrid_R3: In Rudolfingen, on the first two sampling dates (August 20th and 25th), four isolates from four hotspots were genotyped and each had a unique MLG (CF = 0), with pairwise distances ranging from 5.7 to 46.0 m. The frequent MLG R_MLG77 appeared in one of these hotspots. In Hendschiken, 15 isolates sampled from eight hotspots belonged to nine MLGs (CF = 0.4) (four isolates from the same hotspot had the same MLG), with pairwise distances between hotspots ranging from 6.2 to 58.3 m. One MLG was shared between two hotspots that were 7.8 m apart.

Distribution of isolates with the same MLGs over time

The primary CLS infections in each plot gave rise to asexual conidia that provided secondary inoculum. The spatial distribution of the resulting clones with identical MLGs provides insights into the dispersal of secondary inoculum during a growing season. Common MLGs, observed ≥ 5 times in each field (eight in Rudolfingen and five in Hendschiken), were used to calculate the dispersal distance of the same genotype (Table 7). The minimum pairwise distance of 0 m was not included in the table. It was common to find the same MLG separated by distances exceeding 10 meters. The maximum distance between isolates sharing the same MLG was 108.5 m in Rudolfingen and 68.7 m in Hendschiken.

Discussion

The major objective of this study was to compare CLS epidemics on sugar beet hybrids exhibiting different levels of resistance. We combined detailed epidemiological surveys with geo-referenced sampling and SSR genetic data to understand the fine scale temporal and spatial distribution of *C. beticola* genotypes on different resistant hybrids grown under the

415 same environmental conditions during a single season. The dynamics of different pathogen
416 populations were assessed on their respective hybrids during each epidemic. Resistant
417 hybrids showed delayed epidemics, however, the host genotype did not affect the likelihood
418 of spread. There were no differences in genetic diversity between pathogen populations on
419 different hosts, however we found high frequencies of some MLGs on resistant hosts. The
420 distribution of hotspots tended to be aggregated and was similar at the beginning and end
421 of each epidemic in each hybrid, with a few exceptions. We found that a single genotype
422 could disperse over a hundred meters during an epidemic, indicating the possibility of long-
423 distance dispersal of individual clones during a growing season.
424
425 **Onset of the disease varied according to host genotype**
426 CLS emerged at least 3 weeks later in the hybrids with *BvCR4* (Hybrid_R2 and Hybrid_R3)
427 compared to the hybrids without *BvCR4* (Hybrid_S3 and Hybrid_S4). The delayed
428 appearance of CLS in resistant hybrids at both sites suggests that the resistant hybrids
429 suppressed the establishment of CLS early in the season, agreeing with observations from
430 earlier field trials conducted by KWS (personal communication). Similar delays (2 to 4
431 weeks) in the onset of CLS on resistant sugar beet hybrids were also reported in previous
432 studies (Weiland and Koch, 2004; Weltmeier et al., 2011; Wolf and Verreet, 2005). It is
433 possible that we did not always detect the start of an epidemic due to the size of the plots
434 and our sampling frequency. For example, for Hybrid_R2 in Rudolfingen and Hybrid_S2 in
435 Hendschiken, we collected leaves from 43 and 22 plants respectively on the first day of
436 sampling (Table 2), suggesting that the epidemic had already started in these plots in the
437 days between our sampling visits. For the six remaining hybrid plots, the number of plants
438 collected on the first sampling date was < 10, suggesting that we caught the beginning of
439 those epidemics. If we consider only the six plots with less than 10 infected plants at the
440 onset of the epidemic, the average distance between infected plants was 23.0 m in
441 Hendschiken and 30.8 m in Rudolfingen. If we add the other two plots, the average distance
442 between infected plants was 23.9 m in Hendschiken and 25.7 m in Rudolfingen. Our
443 interpretation in both cases is that the primary inoculum was airborne. Given the high
444 genetic diversity found on these first infected plants and the random distributions of
445 primary infections across the fields in both locations (Table S2), we hypothesize that the
446 primary inoculum was mainly airborne ascospores.

447

448 **Localized spread was not strongly affected by host genotype**

449 There was no effect of host genotype on the probability that a single infected plant would
450 develop into a hotspot, with about 80% of infection cases spreading to neighboring plants
451 (Table 3), but the speed of localized spread (involving 2-5 plants) varied between hybrids
452 and fields. In Rudolfingen, there was no difference among hybrids in how quickly CLS spread
453 to neighboring plants, but in Hendschiken, the spread was slower in Hybrid_R3 and faster in
454 Hybrid_R2 compared to the susceptible Hybrid_S2. This field-dependent pattern may be
455 attributed to differences in the distribution of the hybrid plots, in particular the
456 susceptibility of the hybrid planted in a neighboring plot. Previous studies suggested that
457 the level of host resistance did not influence the latent period, however, susceptible hybrids
458 produced more conidia per unit of necrotic leaf area (Oerke et al., 2019). Thus, plots planted
459 next to susceptible hybrids may experience higher local disease pressure. In Hendschiken,
460 Hybrid_R2 was planted next to the susceptible Hybrid_S3, while in Rudolfingen, Hybrid_R2
461 was planted next to the resistant Hybrid_R3, which might have acted as a buffer zone that
462 reduced the influx of conidia onto Hybrid_R2 (Figure S1). In addition to differences in local
463 spore production, differences in local microclimates may also have affected epidemic
464 development at each field site (Imbusch et al., 2021; Rossi et al., 2000; Vereijssen et al.,
465 2006). It also is possible that our weekly sampling interval was not frequent enough to
466 accurately monitor localized spread, as some hotspots spread from one to five plants
467 between two successive time points.

468

469 To summarize, our findings suggest that the host genotype strongly influenced the
470 emergence of CLS but had little impact on the probability that CLS would spread and form
471 hotspots. This is in line with previous work that demonstrated that the latency of spore
472 production was not influenced by host resistance (Rossi et al., 2000). If *C. beticola* strains
473 succeeded in overcoming the resistance of a host and became established on a resistant
474 plant, their ability to spread to neighboring plants did not differ from the speed of dispersal
475 found on susceptible plants.

476

477 **Infection sites were not randomly distributed in fields**

478 At the end of the season, CLS infection sites were mostly aggregated (not randomly
479 distributed) with one exception (Table S2). The distributions of infections changed little
480 between the onset and the end of the epidemic, except for Hybrid_S2 and Hybrid_R2 in
481 Rudolfingen (Table S3). The minimum distance between infection cases was approximately
482 one meter, while the maximum distance reached 116.3 meters (Table 4). In line with earlier
483 findings, we observed higher levels of CLS in Hybrid_S2, Hybrid_S3 and Hybrid_R2 compared
484 to Hybrid_R3 at the end of the season in both sites. Lesions coalesced and caused wilting
485 and death of entire leaves, leading to new growth from the roots in hybrids S2, S3 and R2.
486 Hybrid_R3 did not exhibit obvious lesion coalescence, leaf death, or regrowth despite having
487 many plants with CLS. This may reflect the shorter time interval between disease onset and
488 final sampling in Hybrid_R3. Studies have shown that later infection results in lower yield
489 losses for resistant varieties (Kaiser et al., 2010), and that the delayed CLS onset observed in
490 resistant hybrids was positively correlated with sugar yield (Wolf and Verreet, 2009).
491
492 **Genotypic variation and spatial distribution of genotypes**
493 Earlier studies illustrated how pathogen populations with higher genetic diversity can have a
494 higher evolutionary potential to overcome R genes (McDonald and Linde, 2002b; Stam and
495 McDonald, 2018). In our study, the average Nei's gene diversity based on six SSR markers
496 ranged from 0.40 to 0.57 (Table 5). It is important to note that our estimates of genotypic
497 diversity were limited because only six SSR loci were used to identify putative clones. To
498 estimate how accurately the six SSRs identified clones, we compared the clones identified
499 using the SSR results with clones identified using whole genome sequences for 113 isolates
500 where we obtained both types of data (Chen et al., 2023). The six SSR loci correctly
501 identified clones or unique genotypes in 84% of cases (47 out of 58 in Rudolfingen and 48
502 out of 55 in Hendschiken). The incorrect assignments were a mix of wrongly identifying two
503 isolates as sharing the same genotype (underestimating genotypic diversity) and failing to
504 identify two isolates as being clones (overestimating genotypic diversity) at roughly equal
505 proportions (9.7% and 6.2% respectively). While this illustrates the potential errors of our
506 SSR genotypes, we expect that this error rate will be randomly distributed across hybrids, so
507 comparisons of relative genotypic diversity between hybrids remain valid.
508

509 The high genotypic diversity found in each population supports the hypothesis that *C.*
510 *beticola* populations undergo regular cycles of sexual recombination, as proposed in earlier
511 studies (Birla et al., 2012; Groenewald et al., 2007b, 2006). We detected high genotypic
512 diversity at small spatial scales, with 23 out of 48 tested leaves infected by more than one
513 MLG and 27 out of 48 tested hotspots containing more than one MLG. Other studies also
514 observed multiple SSR MLGs on a single leaf in table beet and Swiss chard (Vaghefi et al.,
515 2017c) and using other genetic markers (TAPD and DAMD) on sugar beet (Moretti et al.,
516 2006). The presence of multiple MLGs on a single leaf suggests that co-infection is common
517 and that multiple strains may contribute to the spread of disease within a CLS hotspot.
518
519 We hypothesized that virulent isolates would be more prevalent on *BvCR4* hybrids due to
520 their ability to overcome this resistance gene. If only a small fraction of isolates in the
521 primary inoculum are virulent and able to produce asexual secondary inoculum on the
522 resistant hybrids, we would expect these virulent clones to increase in frequency on
523 resistant hybrids during the epidemic, resulting in lower genotypic diversity and higher
524 clonal fractions on resistant hybrids compared to susceptible hybrids. Consistent with this
525 expectation, we observed a skewed frequency distribution of MLGs between hybrids with
526 and without *BvCR4* at both sites. In Rudolfingen, the clonal fraction was higher in the
527 resistant hybrids and evenness was strongly skewed (Figure 3): one MLG (R_MLG77)
528 appeared in 46 isolates exclusively in resistant Hybrid_R2 and Hybrid_R3. In Hendschiken,
529 the differences in frequency were less striking, but one MLG (H_MLG75) appeared in 13
530 isolates solely found on the *BvCR4* hybrids. A subset of the isolates collected from resistant
531 hybrids were tested for virulence against hybrids with and without *BvCR4* under field
532 conditions in a later study. Under experimentally elevated disease pressure that study
533 confirmed that the highest frequency MLGs were virulent strains that could overcome
534 *BvCR4* (Chen et al., 2023). It also found that only half of the isolates collected from hybrids
535 with *BvCR4* were virulent against this gene. The lack of evidence for evolutionary
536 bottlenecks in our study may be attributed to the frequent coexistence of virulent and
537 avirulent strains within the same hybrid. Most of our temporal sampling was during the
538 epidemic growth, thus we did not capture the end-of-season dynamics which might show
539 stronger bottlenecks following clone competition. Transect or random sampling of the

540 pathogen populations at the end of the season may provide a better test of the
541 effectiveness of resistant hybrids as selection sieves.
542
543 **Source of inoculum and spatial distribution of MLGs**
544 One of our aims in this study was to identify the potential sources of primary inoculum for
545 CLS epidemics. We hypothesized that a CLS epidemic can be initiated by wind-dispersed
546 ascospores which are capable of long-distance dispersal over hundreds of meters or by
547 conidia on infected leaves from earlier crops that are moved by wind or watersplash. If the
548 primary inoculum in a plot originates from windborne ascospores coming from outside the
549 field, we would expect to find genetically different isolates sparsely and randomly
550 distributed across individual plants at the onset of an epidemic. Alternatively, if the primary
551 inoculum arises from locally produced asexual spores transmitted through watersplash from
552 crop debris or human activity (e.g. infested seeds or machinery), we would expect to find
553 the same genotype occurring simultaneously in different hotspots that might be aggregated
554 in the field (for the case of infested machinery). In plots planted with Hybrid_S2, at both
555 sites we observed unique genotypes in each hotspot, suggesting that unique genotypes led
556 to the establishment of each hotspot and that epidemics were initiated by genetically
557 diverse primary inoculum coming from outside the fields. Similar patterns were observed in
558 Hybrid_R3 at both sites and in Hybrid_R2 in Hendschiken, with no or few isolates shared
559 between hotspots. But a different pattern was found for the resistant Hybrid_R2 in
560 Rudolfingen. In this epidemic, the genotypic diversity of the first infections was lower, and
561 the dominant genotype R_MLG77 appeared simultaneously 10 times in 8 hotspots
562 distributed widely across the field. This suggests that asexual spores originating from a
563 neighboring unsampled border planted to Hybrid_R2 may have disproportionally affected
564 the genotypic diversity found in the sampled Hybrid_R2 plot. We postulate that the
565 R_MLG77 strain emerged from standing genetic variation and attained a relatively high
566 frequency in the border plots before being transmitted into the sampled Hybrid_R2 plot
567 (Figure S1). With the exception of Hybrid_R2 in Rudolfingen, we found that genetically
568 distinct primary inoculum was distributed widely across most plots (Table S2), as expected
569 for wind-dispersed ascospores originating from outside of the field.
570

571 Our experiment also aimed to better understand the dispersal pattern of secondary
572 inoculum within a season. In Rudolfingen, high frequency MLGs (≥5) were separated by
573 between 49 and 108 meters and in Hendschiken by between 9 and 68 m (Table 7). This is
574 the first study in the CLS pathosystem to measure the dispersal of individual genotypes
575 within a season. The dispersal distances we observe are unlikely to be due solely to water-
576 splash, as water-splash can only disperse *C. beticola* spores short distances (several meters)
577 (Carlson, 1967). Wind dispersal is a more likely mechanism, and although wind was already
578 recognized as an important mechanism, it was not clear before how far wind could disperse
579 *C. beticola* (Khan et al., 2008). Other possible dispersal mechanisms cannot be ruled out. It is
580 possible that insects could also disperse the spores, along with human activity, including
581 machine operations or spores moving on contaminated clothing during our weekly sampling
582 visits.
583

584 **References**

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Table 1. Sugar beet hybrids and their resistance to cercospora leaf spot.

Hybrids	**Cercospora leaf spot resistance level**
Hybrid_S2	Low quantitative resistance
Hybrid_S3	Moderate quantitative resistance
Hybrid_R2	*BvCR4* with low quantitative background resistance
Hybrid_R3	*BvCR4* with moderate quantitative background resistance

Table 2. The first appearance of CLS, the number of plants sampled at the first sampling date (No. plants), the number of days after the first appearance of symptoms on July 21st (onset delay), the total length of the sampling period, and the last sampling date (the right censored date is shown in parenthesis, see Methods) for each hybrid at each field site.

Location	**Hybrid**	**First infection date**	**Onset delay**	**No. plants**	**Sampling period**	**Last sampling date (right censored date)**
Hendschiken	Hybrid_S2	21-07-20	0	22	30	20-08-20 (11-08-20)
	Hybrid_S3	27-07-20	6	1	24	20-08-20 (11-08-20)
	Hybrid_R2	11-08-20	21	6	33	08-09-20 (03-09-20)
	Hybrid_R3	20-08-20	30	3	35	24-09-20 (03-09-20)
Rudolfingen	Hybrid_S2	21-07-20	0	7	30	20-08-20 (11-08-20)
	Hybrid_S3	27-07-20	6	2	24	20-08-20 (11-08-20)
	Hybrid_R2	25-08-20	35	43	14	08-09-20 (03-09-20)
	Hybrid_R3	20-08-20	30	4	35	24-09-20 (08-09-20)

Table 3. The number of infection cases (IC) and the proportion of these that developed into hotspots (≥2 infected plants) and the proportion that developed into large hotspots (≥5 infected plants) for each hybrid. The data has been right censored (see Methods).

Location	Hybrid	No. IC	Number of hotspots (proportion)	Number of large hotspots (proportion)
Hendschiken	Hybrid _S2	37	26 (0.70)	5 (0.14)
	Hybrid _S3	20	17 (0.85)	4 (0.20)
	Hybrid _R2	22	18 (0.82)	7 (0.32)
	Hybrid _R3	30	24 (0.80)	4 (0.13)
Rudolfingen	Hybrid _S2	23	19 (0.83)	9 (0.39)
	Hybrid _S3	18	17 (0.94)	6 (0.33)
	Hybrid _R2	29	26 (0.90)	9 (0.31)
	Hybrid _R3	31	23 (0.74)	5 (0.16)

Table 4. The number of geotagged infection sites and the minimum, mean and maximum pairwise distance (m) between infection sites at the beginning and the end of the sampling period in each hybrid.

Location	Hybrid	Pairwise distance at the start of epidemics				Pairwise distance at the end of epidemics			
		Count	Min	Mean	Max	Count	Min	Mean	Max
Rudolfingen	Hybrid _S2	14	2.2	28.2	73.7	41	1.3	36.7	110.7
	Hybrid _S3	4	3.8	54.7	98.6	31	1.3	47.8	116.3
	Hybrid _R2	15	1.3	19.7	62.0	37	1.3	29.2	108.3
	Hybrid _R3	8	4.2	33.9	94.5	88	0.2	37.2	110.0
Hendschiken	Hybrid _S2	14	1.3	24.7	72.8	37	0.8	26.0	72.8
	Hybrid _S3	8	1.3	20.0	51.1	24	1.3	23.2	64.6
	Hybrid _R2	6	4.2	29.7	57.3	41	1.3	27.2	76.0
	Hybrid _R3	9	3.1	22.5	58.3	66	0.2	21.6	68.5

Table 5. Indices of gene and genotype diversity based on six SSR loci for *Cercospora beticola* populations collected from four sugar beet hybrids in two fields in Switzerland. The table includes the number of strains (N), the number of multilocus genotypes (MLGs), the expected number of MLGs after rarefaction (eMLGs), the clonal fraction (CF=1-(MLGs/N), the Simpson's complement index (λ) and Nei's index of gene diversity before clone correction (He_0) and after clone correction (He_{cc}).

Location	Hybrid	N	MLGs	eMLGs	CF	λ	He_0	He_{cc}
Hendschiken	Hybrid_S2	30	20	15.6	0.33	0.91	0.56	0.60
	Hybrid _S3	22	16	16	0.27	0.92	0.51	0.54
	Hybrid _R2	43	28	17.3	0.35	0.95	0.52	0.54
	Hybrid _R3	63	37	16.2	0.41	0.94	0.56	0.58
	Total/Mean	**158**	**82**	**18.3**	**0.48**	**0.97**	**0.56**	**0.56**
Rudolfingen	Hybrid _S2	49	27	16.7	0.45	0.95	0.51	0.54
	Hybrid _S3	23	19	19	0.17	0.94	0.57	0.60
	Hybrid _R2	40	16	10.7	0.60	0.73	0.40	0.56
	Hybrid _R3	70	35	14.1	0.50	0.85	0.52	0.57
	Total/Mean	**182**	**77**	**16.3**	**0.58**	**0.92**	**0.55**	**0.57**

Table 6. Genotypic diversity within (a) a leaf and (b) a hotspot (HS). The first column indicates a frequency class relating to the number of isolations per leaf/hotspot. There are three classes: 2, 3 or 4 isolations were made per leaf leaf/hotspot. The table includes the number of leaves/hotspots that had only one MLG (all clones) and with more than one MLG. The proportions are provided in parenthesis.

(a)

Number of isolations per leaf	**Number of leaves**	**Single MLGs Number (proportion)**	**Multiple MLGs Number (proportion)**
2	41	21 (0.51)	20 (0.49)
3	6	3 (0.50)	3 (0.50)
4	1	1 (1.00)	0
Total	48	25 (0.52)	23 (0.48)

(b)

Number of isolations per HS	**Number of HS**	**Single MLGs Number (proportion)**	**Multiple MLGs Number (proportion)**
2	34	17 (0.50)	17 (0.50)
3	11	3 (0.27)	8 (0.73)
4	3	1 (0.33)	2 (0.67)
Total	48	21 (0.43)	27 (0.57)

Table 7. The median, mean and the maximum pairwise distance (m) between the most frequent MLGs that were found at least five times in a field site.

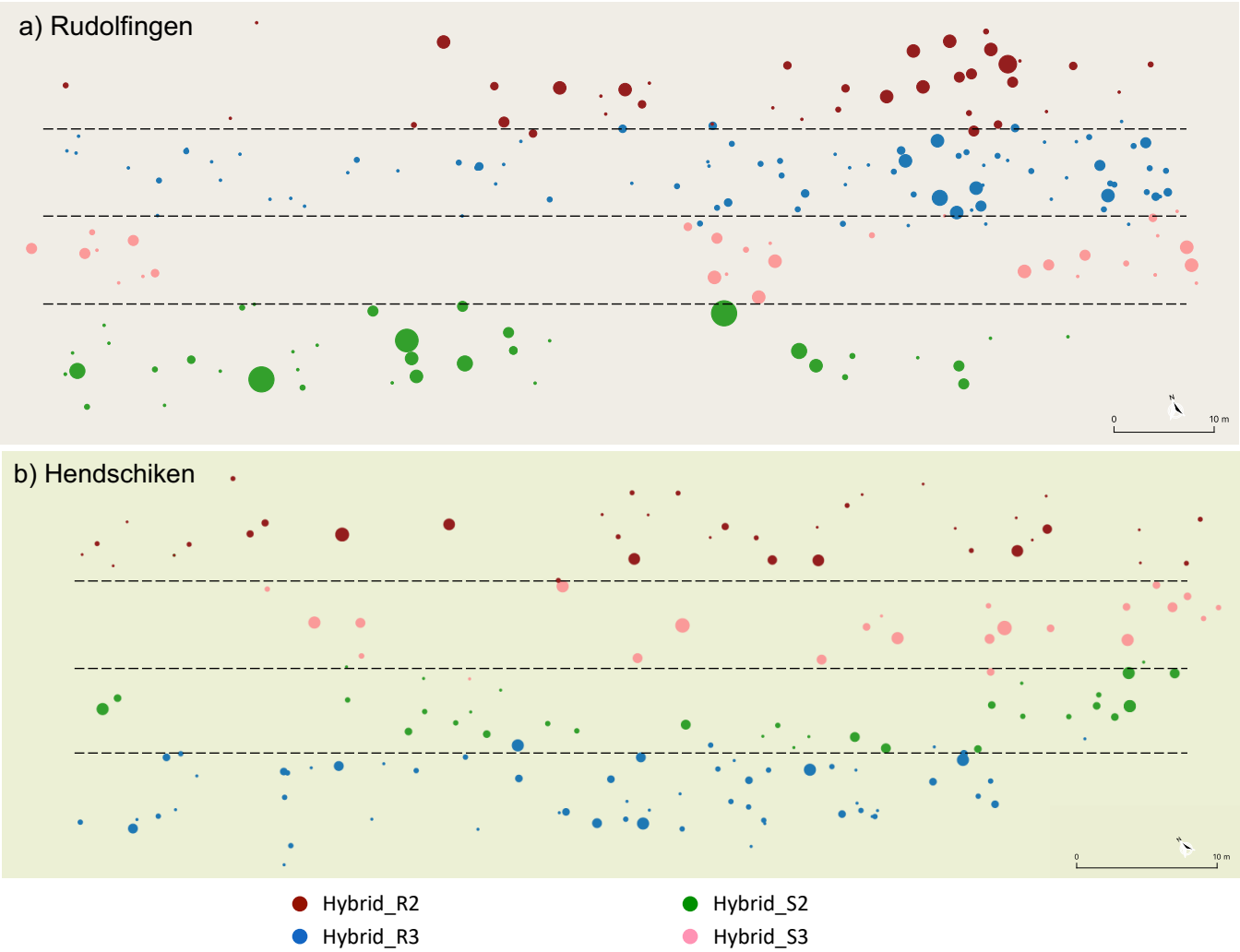
MLG	Count	Median	Mean	Maximum
R_MLG77	46	16.6	22.8	108.5
R_MLG56	7	43.6	38.9	100.5
R_MLG13	6	7.5	16.3	49.8
R_MLG42	6	58.4	48.0	107.8
R_MLG15	5	51.9	43.5	105.0
R_MLG20	5	14.9	28.5	74.0
R_MLG46	5	31.9	23.2	64.4
R_MLG55	5	19.2	30.0	77.1
H_MLG75	13	4.2	7.8	20.5
H_MLG51	11	15.7	18.9	46.7
H_MLG9	9	3.7	4.2	9.4
H_MLG77	8	15.0	13.5	36.7
H_MLG82	8	29.3	27.1	68.7

Figure 1. Spatial distribution of CLS in Rudolfingen (a) and Hendschiken (b). Each point represents a geotagged infection case. The size of the point indicates the number of sampled plants within the hotspot. The point color indicates the plant hybrid: green: Hybrid_S2; pink: Hybrid_S3; brown: Hybrid_R2; blue: Hybrid_R3. Plant hybrid was determined by counting the planted rows and using the different hybrid morphology. The dashed lines approximate the separation of the different hybrids, but in the actual field these lines were not perfectly straight and there was no physical separation. Thus, it can appear that one plot contains more than one hybrid (points with different colors), but this is an artifact of plotting and scaling the figure.

Figure 2. Accumulation of geotagged infection cases over time in Rudolfingen (a) and Hendschiken (b) in each hybrid. Sampling terminated at different dates in each hybrid due to rapid disease progression that resulted in complete infection of the entire experimental plot (see methods and Table 2). Sampling termination prior to end of field trial indicated by red circles (○).

Figure 3. Frequencies of multilocus genotypes (MLGs) appearing in different hybrids in Rudolfingen (a) and Hendschiken (b). Each bar refers to one MLG, the height of the bar indicates the frequency of the MLG in each hybrid.

Figure 4. Evenness of MLGs in each hybrid in Rudolfingen (a) and Hendschiken (b). Points are the values of evenness and the error bars indicate the bootstrapped confidence intervals.



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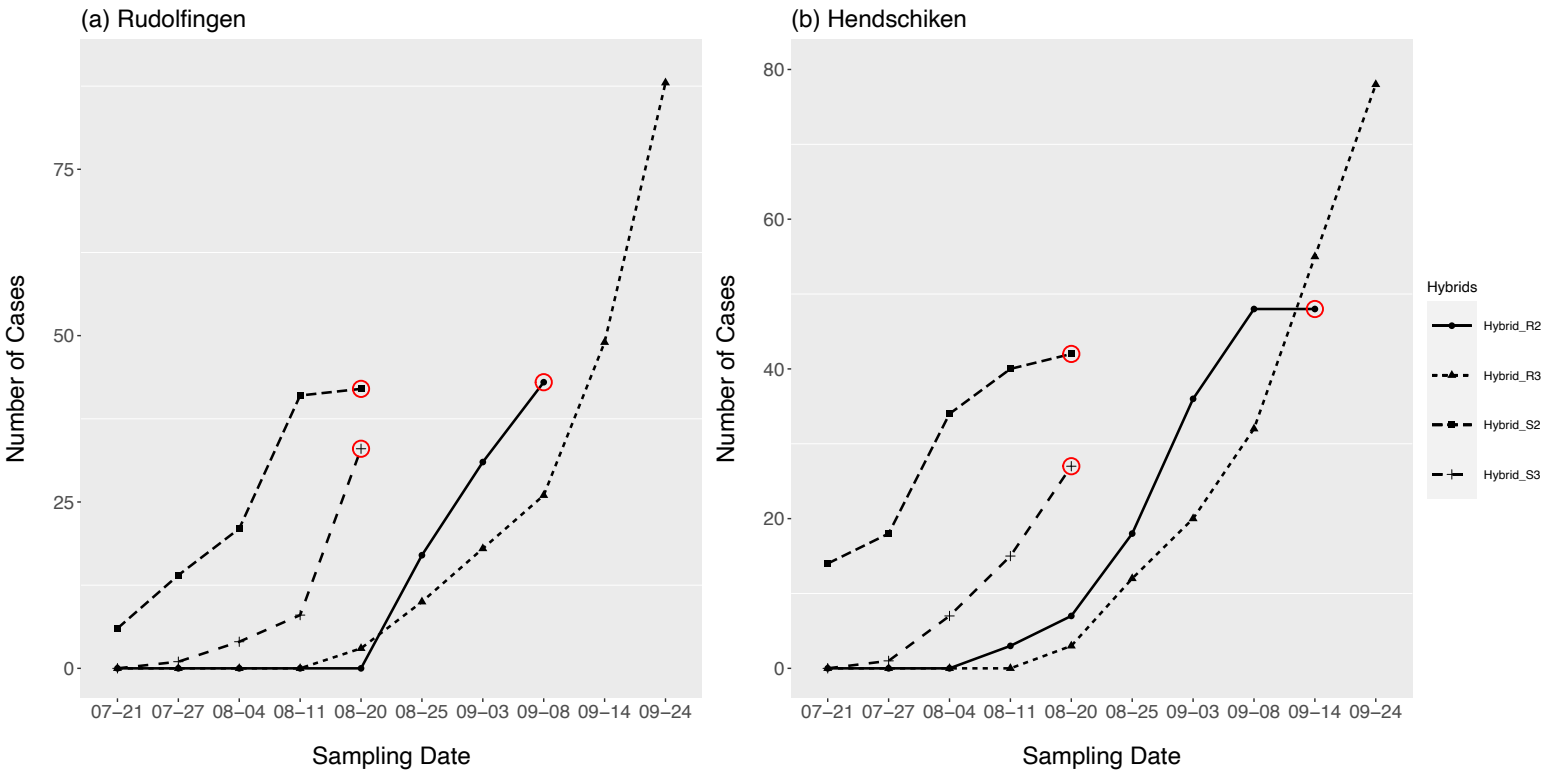

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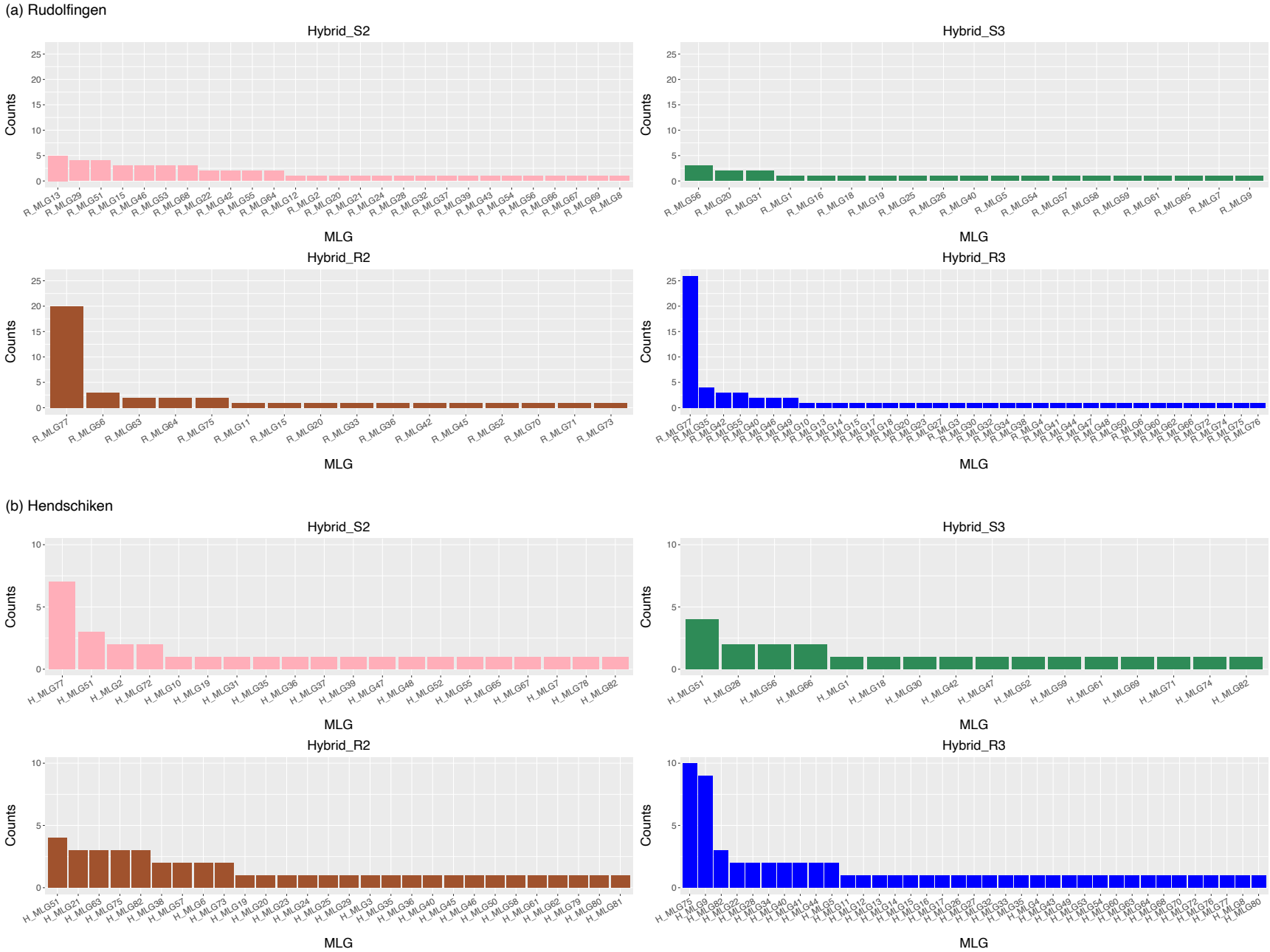

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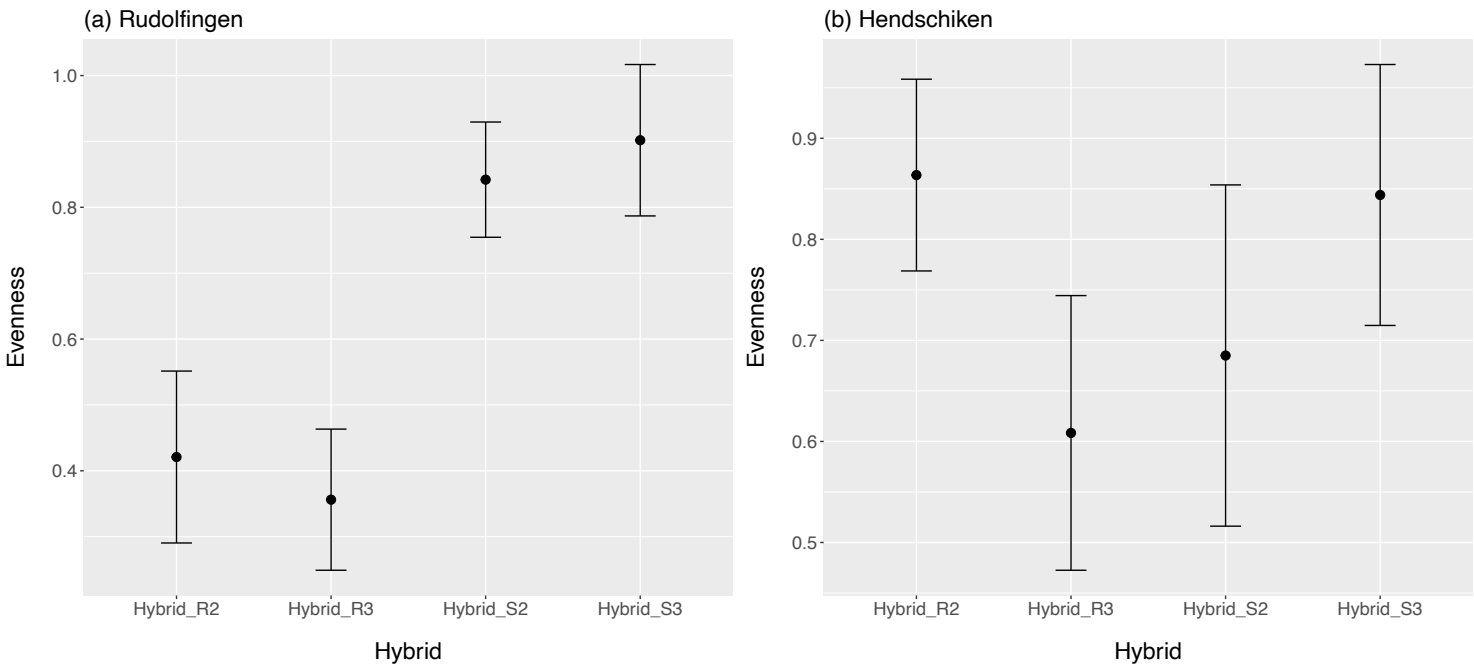

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