The Current Epidemic of the Barley Pathogen *Ramularia collo-cygni* Derives from a Population Expansion and Shows Global Admixture

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ABSTRACT

Ramularia leaf spot is becoming an ever-increasing problem in main barley-growing regions since the 1980s, causing up to 70% yield loss in extreme cases. Yet, the causal agent *Ramularia collo-cygni* remains poorly studied. The diversity of the pathogen in the field thus far remains unknown. Furthermore, it is unknown to what extent the pathogen has a sexual reproductive cycle. The teleomorph of *R. collo-cygni* has not been observed. To study the genetic diversity of *R. collo-cygni* and get more insights in its biology, we sequenced the genomes of 19 *R. collo-cygni* isolates from multiple geographic locations and diverse hosts. Nucleotide polymorphism analyses of all isolates shows that *R. collo-cygni* is genetically diverse worldwide, with little geographic or host specific differentiation. Next, we used two different methods to detect signals of recombination in our sample set. Both methods find putative recombination events, which

indicate that sexual reproduction happens or has happened in the global *R. collo-cygni* population. Lastly, we used these data on recombination to perform historic population size analyses. These suggest that the effective population size of *R. collo-cygni* decreased during the domestication of barley and subsequently grew with the rise of agriculture. Our findings deepen our understanding of *R. collo-cygni* biology and can help us to understand the current epidemic. We discuss how our findings support possible global spread through seed transfer, and we highlight how recombination, clonal spreading, and lack of host specificity could amplify global epidemics of this increasingly important disease and suggest specific approaches to combat the pathogen.

Keywords: ecology and epidemiology, population biology

The Dothidiomycete *Ramularia collo-cygni* was originally described as a parasitic agent on grasses as early as 1893 (Cavara 1893). Yet, it was only in the mid-1980s that it was rediscovered as the causal agent of the *Ramularia* leaf spot (RLS) disease of barley, and it is recognized as an important pathogen for barley worldwide (Havis et al. 2015). The development of molecular diagnostics has confirmed the unsymptomatic presence of the pathogen throughout the lifecycle of its host barley (Havis et al. 2015). The pathogen has become increasingly problematic in many of the world's barley-growing regions, affecting the yield often by up to 25% because of reduced kernel size and limited growth. In extreme cases, up to 70% yield reduction has been observed. These problems are intensified by the fact that *R. collo-cygni* resistance in existing barley cultivars is often of quantitative nature and remains limited. In fact, certain cultivars already seem to have lost resistance against the pathogen. Additionally, *R. collo-cygni* shows increasing fungicide resistance (Havis et al. 2015).

The reasons for the emergence of RLS remain largely unknown. The current epidemic of RLS has followed the intensive use of mildew-resistant *mlo* genotype cultivars. McGrann et al. (2014) suggested a tradeoff between the barley *mlo* mutation-mediated

powdery mildew resistance and susceptibility to RLS. However, the disease is not restricted to *mlo* genotypes, and analysis of near-isogenic *MLO* and *mlo* barley did not support a causal link between the *mlo* mutation and the amount of *R. collo-cygni* DNA detected in barley seeds (Hofer et al. 2015). Additional physiological properties of modern barley varieties, climate change, and an extended growing season might have increased the host susceptibility (West et al. 2012).

The first draft genome of the pathogen was published in 2016 and showed a low number of cell wall-degrading enzymes (CWDEs) as well as a large amount of secondary metabolite clusters (McGrann et al. 2016). These findings could partly be explained by the lifestyle of *R. collo-cygni*, because the fungus could have evolved these as a strategy to avoid detection of cell wall elicitors by the plant during the relatively long asymptomatic growth. However, large-scale comparative analysis of CWDEs in different pathogenic and nonpathogenic fungi revealed overall large variation and no correlation to pathogenicity or host (Zhao et al. 2013).

We generated an independent draft genome for another isolate (urug2) with improved scaffold length and showed that *R. collo-cygni* strongly diverged from *Zymoseptoria tritici*, its nearest sequenced sister species, about 27 million years ago (Stam et al. 2018). The *R. collo-cygni* genome does not show many of the typical plant pathogen characteristics, like transposable element islands or elevated rate of nonsynonymous over synonymous substitutions (dN/dS) in secreted or effector proteins, which would indicate selective pressure on such genes. We hypothesized that these genome characteristics are linked to the largely endophytic lifestyle of the pathogen. Yet again, they do not explain its emergence as an epidemic threat.

In addition to the lack of basic genomic knowledge, questions also remain regarding the transmission of the fungus itself. Some authors hypothesize that, based on the regular detection of *R. collo-cygni* from grain and microscopy studies of seedlings, seed

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transmission plays an important role in the current epidemic (Havis et al. 2014; Kaczmarek et al. 2017). Others have largely attributed the spread of epidemics to wind dispersal (Schützendübel et al. 2008). It has also been suggested that seed transmission would facilitate long-distance movement, whereas wind dispersal is responsible for local spread (Havis et al. 2015).

Other than being found on barley, *R. collo-cygni* has also been isolated from wheat, oat, maize, and a number of uncultivated grasses, such as *Agropyron repens*, suggesting a broad host range (Salamati and Reitan 2006). The compatible interactions with many of these grasses are microscopically similar to the infection of barley (Kaczmarek et al. 2017). Despite a similar initial stage, the reaction on dicotyledonous plants is found to be incompatible, which shows some degree of host specialization for grasses. It is so far not known whether isolates of *R. collo-cygni* generally are able to infect a broad range of host species or if host specialization (e.g., to barley) occurs within local populations. Additionally, there are limited insights about *R. collo-cygni* genetic diversity and spatial structuring of populations. An amplified fragment-length polymorphism (AFLP) study investigating the population structure of samples from barley in the Czech Republic, Germany, Slovakia, and Switzerland showed variability between the samples and rejected the hypothesis of random mating in the field, but it suggested that mixed reproduction is likely (Leisova-Svobodova et al. 2012).

No teleomorph of *R. collo-cygni* has been described. However, data from two studies, one using AFLPs and another using microsatellites (Hjortshøj et al. 2013; Piotrowska et al. 2016), hint that sexual reproduction might take place in the field. Moreover, both studies show considerable variation between their two sample locations (Scotland and Denmark or Scotland and Czech Republic, respectively), but they do not reveal clear substructures within the populations (e.g., within one country).

Here, we use the our urug2 reference genome for a population genetics approach to get a deeper insight in the biology of *R. collo-cygni* and better understand the lifecycle. We compare two previously published genome sequences and 17 additional sequenced isolates from a global collection from different hosts. Our aims are (i) to infer the pathogen's population structure and its global diversity, (ii) to detect breakage points on the genome and assess whether *R. collo-cygni* is indeed undergoing sexual reproduction in the field, and (iii) to infer the past demographic history (i.e., changes in population sizes) of the pathogen.

MATERIALS AND METHODS

Cultures, media, and incubation conditions. For this study, 19 different isolates were used. We used 14 isolates from different geographical locations and 5 isolates from different hosts (Table 1). Isolates were selected to include representatives of all different epidemiological zones of Europe, where the species was first described and where the first “reoccurrence” as a pathogen was reported. Moreover, Europe is the continent with the most frequent observations of strong epidemics, and thus, it is possibly the center of the emergence. For the comparison, several geographical outliers and a selection of isolates from alternative hosts were obtained.

Isolation and storage were carried out according to the method developed by Peter Frei (Agroscope Switzerland, *personal communication*). Spores were transferred with a needle from conidiophores of symptomatic leaves onto a petri dish with clear water agar. With the aid of sterile water, the spores were plated out over the surface of the dish and incubated at room temperature under 12-h light conditions. After 24 h, germinating single spores were picked and transferred to a new dish with potato dextrose agar (PDA; 4 g of potato extract and 20 g of dextrose) with antibiotics (aureomycin, 25 mg/ml). After 7 days of additional incubation at room temperatures, typical cultures were selected and transferred to dishes of 1/4-strength PDA for additional growth of single cultures. For long-term storage, mycelium was cut into small plugs and transferred into sterile screw-top tubes containing 1/4-strength potato dextrose broth (without agar). The tubes were stored at 4 to 10°C in the dark until further use.

DNA, library preparation, and sequencing. Mycelia from above-mentioned cultures were harvested, and finely ground mycelium was used for DNA extraction as described in detail before (supplementary methods of Stam et al. 2018); in short, mycelium was flash frozen, and DNA was extracted using a classical cetrimonium bromide (CTAB) buffer and phenol/chloroform extraction. All samples were treated with ribonuclease during the first extraction step, and the DNA was washed and precipitated twice using 70% ethanol before resuspending in Tris buffer.

A sequencing library was made using the TruSeq DNA HT prep Kit with paired end chemistry (2 × 100 bp; insert size, 180 bp) following the manufacturer's protocols. Sequencing was performed by NGS@TUM using two lanes on an Illumina HiSeq 2500 sequencer.

Single-nucleotide polymorphism calling and summary statistics. Sequence data were quality checked and trimmed using

TABLE 1. Overview of the sequenced samples, including information on origin (geographic coordinates: latitude and longitude in decimal degrees), as well as obtained read depth and number of polymorphisms per sample after stringent filtering (low read quality [qual < 35]; low mapping quality [mq < 40]; Fischer strand difference [fs > 60]; low read depth [depth < 10]; and clustering of polymorphisms [cluster window 10; cluster size 3])^a

Strain code	Strain name	Origin	Host	Coordinates	Average read depth	SNPs after filtering
100413	Switzerland	Switzerland	Barley	47.609577, 9.146538	89	30,800
100414	Czech Republic	Czech Republic	Barley	49.237393, 17.538407	88	29,005
100415	Germany	Germany	Barley	48.400360, 11.714086	71	29,387
100416	Poland	Poland	Barley	53.282751, 17.362838	114	25,128
100417	Russia	Russia	Barley	45.090536, 38.912033	97	26,450
100418	Hungary	Hungary	Barley	47.1625, 19.5033	109	27,967
100419	New Zealand	New Zealand	Barley	-43.33000, 172.28000	108	33,683
100420	Argentina	Argentina	Barley	-33.891281, -60.574599	98	42,167
100421	Scotland	Scotland	Barley	55.86090, 3.20640	97	25,702
100422	Denmark	Denmark	Barley	55.316667, 11.400000	93	26,770
100423	France	France	Barley	47.87950, 2.773600	87	26,095
100424	England	England	Barley	50.864600, 0.255200	95	23,510
100425	Norway	Norway	Barley	63.430500, 10.395100	97	44,390
100426	Wheat	Germany	Winter wheat	48.400360, 11.714086	102	23,233
100427	Tritordeum	Germany	<i>Tritordeum</i>	48.400360, 11.714086	102	26,582
100428	Oat	Germany	Oat	48.400360, 11.714086	97	28,866
100429	Agropyron	Switzerland	<i>Agropyron repens</i>	46.398888, 6.233293	94	28,323
100430	Poa	Switzerland	<i>Poa annua</i>	46.398888, 6.233293	88	29,665
Urug2	Uruguay	Uruguay	Barley	-34.334326, -57.691932	190	57

^a The variant call file with all single-nucleotide polymorphisms (SNPs) and their assigned quality can be found on the Zenodo data repository (Stam et al. 2019).

trimmomatic (recommended settings) (Bolger et al. 2014). All samples were mapped to our reference genome (Stam et al. 2018) using the Burrows–Wheeler alignment tool (Li and Durbin 2009). The duplicated reads were removed, and single-nucleotide polymorphism (SNP) calling was performed using GATKs Haplotype-Caller (McKenna et al. 2010) with ploidy set to one. SNPs were subsequently filtered using GATKs recommended filtering criteria for read and mapping quality. SNPs were filtered out when they had low quality ($qual < 35$), low mapping quality ($mq < 40$), and high Fisher strand difference ($fs > 60$). Also, regions with very low overall reads mapped ($depth < 10$) were removed. In addition, we removed SNP around indels and SNPs occurring in clusters (more than three SNPs within a 10-bp window). The variant call file (vcf) with all SNPs and their assigned quality can be found in the Zenodo data repository (Stam et al. 2019). Vcftools (Danecek et al. 2011) was used to extract the summary statistics, calculate the transition to transversion ratio, and convert the vcfs to PLINK phenotype files that can be used for additional analysis. Data for the site frequency spectrum were directly extracted from the vcfs and visualized in R (R Core Team) using the package ggplot2 (Wickham 2009). Tajima's D and nucleotide diversity (π) were calculated over the genome using the R package PopGenome (Pfeifer et al. 2014) and reported per gene as average per site value.

Population structure and phylogeny. To assess the population structure, we used the LEA package (Frichot and François 2015) in R. We analyzed basic population structure by drawing a principal component analysis (PCA) for all data using the `pca()` function. We calculated the minimal cross-entropy (mce) for the dataset; mce is lowest when using low K values, and thus, we choose $K = 2$ to 4 for additional analysis. We performed 10 runs with α set to 10 and 100. Both analyses were run for both all SNPs as well as SNPs in coding regions only. Differences between all of these runs were minimal; thus, only results for a single run (with $\alpha = 10$, SNPs in coding regions only) were plotted. To construct a population phylogeny, we extracted all 162,045 SNPs as alternative reference genomes using GATK and drew an unrooted neighborhood joining tree using PhyML in Seaview (using the general time-reversible [GTR] model and allowing nearest neighbor interchange [NNI] with BioNJ and 1,000 bootstraps) (Gouy et al. 2010; Guindon and Gascuel 2003).

Linkage disequilibrium analysis using LDHelmet. We calculated the recombination rate in ρ per kilobase using the software LDHelmet (Chan et al. 2012). LDHelmet requires long scaffolds to calculate breakage points. We performed the analysis on scaffolds 1 to 12, because the others are shorter than 1 Mb. The software was run using the guidelines provided by the authors. We used a window size of 50 and recommended parameters for ρ (recombination) search space. We specified a burn in of 100,000 iterations and 1,000,000 true iterations. Block penalty ($-b$) was set to 50. The results for the upper and lower 2.5 percentile as well as the median were extracted from the binary results file and imported into R for visualization. The histogram with ρ values per bin was plotted using the R package ggplot2. Linkage disequilibrium graphs were plotted using the base plot function. These analyses were done once for all samples and once for a subset of the samples excluding the three outliers as identified by the phylogeny (Argentina, Norway, and New Zealand).

Estimation of recombination events with ClonalFrameML. The genomic sequences for scaffolds 1 and 2 were extracted for each strain. A phylogenetic tree was constructed using PhyML in Seaview (GTR, NNI, BioNJ, and 100 bootstraps) (Gouy et al. 2010; Guindon and Gascuel 2003). Next, we used ClonalFrameML (Didelot and Wilson 2015) to estimate recombination events. As prior for the transition to transversion ratio, we used the calculated rate of 2.3. Other priors were set as follows: $1/\rho$ was taken from our LDHelmet analysis, the minimum length for each recombination was set to 10 kb, v (mean divergence of imported DNA) was set to 0.1 as recommended in the manual, and simulations were run

three times, each time with different genomic mutation rates of 1×10^{-7} , 1×10^{-7} , 1×10^{-8} , and 1×10^{-9} . As recommended in the manual, we set the allowed standard deviation for the estimations such that we allowed several orders of magnitude variation in our estimation. The number of recombination events was extracted from the result file and visualized as events per megabase using ggplot2 in R.

Historic effective population size estimation. For the estimation of the historic effective population size (N_e), we used the software PopSizeABC (Boitard et al. 2016). PopSizeABC works best with long scaffolds; therefore, we calculated summary statistics for the first 12 scaffolds (each >1-Mb long). We performed 200,000 simulations with each of 50 independent fragments of 2 Mb. Parameters for p were derived from the LDHelmet results, with a minimum cutoff at 1×10^{-8} and a maximum at 1×10^{-7} set as priors. Minimum allele frequency was set at 2, and the maximum N_e was set at 1,000,000. Summary statistics were extracted using the same parameters, with the tolerance, as recommended, set to 0.0005.

The ABC method was repeated for generation times of 0.25, 0.5, 1, 3, and 5 years. The graphs were plotted in R using a modification of the script provided by PopSizeABC to allow for visualization of 100 individual runs.

Data availability. Sequencing data are available from the European Nucleotide Archive (accession no. PRJEB12938; <https://www.ebi.ac.uk/ena/data/search?query=PRJEB12938>). The vcf is available in Zenodo at <https://zenodo.org/record/3256422> (Stam et al. 2019).

RESULTS

Sequencing *R. collo-cygni* strains. All reads from the sequenced isolates were mapped to our reference genome. Average read depth ranged from 64× to 113× after deduplication (Table 1). SNPs were called using GATK (McKenna et al. 2010). In total, we obtained 319,477 SNPs. To verify robust SNP call, we used stringent settings for mapping and SNP quality; 157,433 SNPs were filtered out, 128,943 of which because of low mapping quality. We kept 162,045 high-confidence SNPs between all samples, corresponding to approximately one SNP in every 200 bases. The file with all SNP calls and their quality is available online (Stam et al. 2019). The number of SNPs called in each of the individual samples ranged from 23,233 to 44,390, with a mean of 29,317 SNPs (Table 1). Thus, on average, each sample has one SNP every ~1,090 bp compared with the reference. In the whole dataset, 79,949 SNPs occurred within coding regions. We calculated a transition to transversion ratio of 2.36 for the whole genome and 2.31 for the coding sites, suggesting no anomalies in the SNP calling. Of all SNPs in the coding regions, 56% occur as singletons within the sample set (Supplementary Fig. S1). Average nucleotide diversity per site (π) over all coding sites was 4.7×10^{-4} , and Tajima's D was -1.186 . When looking at per gene statistics, median value of π per site is 4.7×10^{-4} , and Tajima's D was -1.1630 . Such overall low diversity, high singleton rate, and low Tajima's D are indicative of a population expansion, strong selective constraints, or pervasive positive selection (selective sweeps).

***R. collo-cygni* population structure and phylogeny.** We further used several approaches to assess the population structure of *R. collo-cygni*. We first reduced the complexity of the data and constructed a PCA for the SNPs using LEA, an R package used to calculate admixture (Frichot and François 2015). Supplementary Figure S2 shows that the first two components explain 17% (12 and 5) of the variation. When we drew the two first components, most isolates grouped very closely together in one cluster. Only three outliers (Norway, Argentina, and New Zealand) could be observed. Similar patterns appeared when using the coding regions of the genome only as well as in the whole genome. Next, we estimated the population structure from the SNP data. The previous PCA results

and minimum entropy analysis (Supplementary Fig. S3) suggested the optimal cluster K value to be between one and three. We ran LEA, for K values = 2 to 4, in 10 separate runs. As expected from the PCA, Figure 1A shows that, with these K values, two or three isolates could be marked as outliers, being the isolates from Norway, Argentina, and possibly, New Zealand. All other samples grouped together with $K = 3$ and showed a transient pattern for $K = 4$. Segregation of isolates could not be seen on a geographical basis or based on the host of origin.

To confirm these findings, we extracted all high-confidence polymorphic sites from the reconstructed genome sequence for each of the isolates. The resulting alignment was used to construct a phylogeny using PhyML (NNI, 1,000 bootstraps). Figure 1B shows that isolates from Norway and Argentina were indeed the two main outliers followed by New Zealand. Using the same shading in both panels revealed very similar internal structures. The low bootstrap values at some of the internal branches of the phylogeny indicate that no clear subgroups can be defined within the inner branches of

Fig. 1. Global *Ramularia collo-cygni* substructure. **A**, Admixture plots for $K + 2$ to 4. Each vertical bar (x axis) represents a different isolate. Gray shading illustrates the different assigned genotypes or admixture thereof when a bar has more shades on the y axis. **B**, Phylogenetic reconstruction based on all single-nucleotide polymorphisms identified between the 19 sequenced *R. collo-cygni* isolates of our study. The phylogeny was made using PhyML (general time-reversible model and nearest neighbor interchange; BioNJ; 1,000 bootstraps). Bootstrap values are indicated on the individual branches. Branches are shaded as in **A**.

the tree. Thus, except for three outliers, we did not find a clear population structure for *R. collo-cygni* in our dataset.

Analyses of sexual recombination. Next, we investigated whether there are signatures of sexual recombination within the species. We used LDHelmets (Chan et al. 2012) to estimate the linkage disequilibrium within the species on the longest scaffolds of our assembly (those >1-Mb long). Figure 2 and Supplementary Figure S4 show that, when analyzing all 19 isolates, rare but very clear changes in linkage disequilibrium events could be observed. We calculated p values across the genome that were mostly $<10^{-6}$ /kbp. However, in small windows, these values ranged up to eight (Supplementary Fig. S5). When we repeated the LDHelmets analysis without the three aforementioned outliers, these peaks in p became lower, but they remained present (Fig. 2).

To confirm our findings, we used ClonalFrameML (Didelot and Wilson 2015) to analyze recombination on the first two scaffolds within our set of *R. collo-cygni* isolates. ClonalFrameML uses genome alignments to screen for breakpoints that are indicative of recombination events. We took the priors $1/p$ and transition to transversion ratio from our analyses above. The expected mutation rate of the genome of *R. collo-cygni* is unknown, but genomic mutation rates in related eukaryotic microorganisms generally range from 1×10^{-6} to 1×10^{-9} per 1 bp, with 1×10^{-7} to 1×10^{-8} the most likely rate for multicellular fungi, like *R. collo-cygni* (Drake et al. 1998). For each of the 19 sampled strains, we calculated the expected number of recombination events. The number of events varied per strain. In all cases, we saw strong evidence for low rates of recombination, with median rates ranging from six to four recombination events per 1 Mb on both scaffolds 1 and 2 (Fig. 3). This indicates that sexual reproduction indeed happened to a certain extent through the whole sample set.

Estimation of the N_e . All our estimates of N_e revealed one main fluctuation (Supplementary Fig. S6): a historically large N_e of >100,000, with a gradual decline over time, where N_e dropped as low as 100. After that, N_e gradually increased again, and it has remained relatively stable in the three latest iterations of the N_e size estimations. With a generation time set to 1 year (a realistic scenario

Fig. 2. Recombination analyses in *Ramularia collo-cygni* using LDHelmets. Recombination in p per kilobase (y axis) was calculated per position on the scaffold for the 12 longest scaffolds (>1,000,000 bp). Scaffolds 1 and 2 are shown. Left panels show the results for all samples, and right panels show the results excluding the three outlier samples. The x axis shows the scaffold length in base pairs.

seeing the endophytic growth and symptom appearance late in each season), we estimated that *R. collo-cygni* saw a peak in population growth about 10,000 years ago; then, it underwent a bottleneck, with the lowest N_e between 300 and 1,500 years ago. Since then, the N_e has increased 100-fold to about 100,000 (Fig. 4).

DISCUSSION

R. collo-cygni was discovered as a saprophyte of grasses in the 1890s (Cavara 1893). Since the 1980s, it has attracted increasing attention as the causal agent for RLS. To obtain a better understanding of the population structure and the role of sexual reproduction in the current worldwide emergence of *R. collo-cygni*, we analyzed 19 *R. collo-cygni* strains. We reveal a relatively low genetic diversity. We found one SNP per 200 bases in 19 samples compared with, for example, one SNP per 20 bases in 106 global *Z. tritici* samples (Hartmann et al. 2017). We also observe a very similar transition to transversion ratio on coding regions and the whole genome (2.31 and 2.37, respectively). These findings are a first indication of a possible bottleneck and expansion of the species. There is very little evidence for geographical population substructure. With the exception of three possible outlier samples, all of our samples form one genetic cluster. In a previous study, populations from the Czech Republic and Scotland were found to form two moderately diverged populations that could already be part of the same genetic cluster (Piotrowska et al. 2016). With our additional sampling, we show that isolates from these populations belong within the same large main cluster that covers a large

geographic area. The main cluster can be divided in two putative genomic groups, but these do not show a strong geographical correlation. These results are partly surprising, because other cereal pathogens, such as the primarily windborne wheat yellow rust fungus *Puccinia striiformis* f. sp. *tritici* (Ali et al. 2014) and also, *Fusarium graminearum* (another partially seedborne cereal pathogen) (O'Donnell et al. 2000), do show geographically distinct lineages.

One explanation for lack of geographical clustering of the sample could be long-distance dispersal of the pathogen. As discussed, wind dispersal could be one means by which *R. collo-cygni* spores travel around the globe (Stabentheiner et al. 2009; Walters et al. 2008). Such a mode of dispersal is heavily reliant on stochastic processes (Brown and Hovmöller 2002), but that would only in part explain the lack of geographical clustering. In a wind dispersal scenario, geographically close areas are often most likely to be genetically more similar to one another. A more likely explanation for the lack of geographical structure could be seed transmission originating from contaminated seed shipment. Barley seeds are often shipped around the globe, and the effect could be amplified by maintenance of contaminated barley in winter nurseries. Such seed transmission of *R. collo-cygni* has been suggested before (Havis et al. 2014) and would be in line with observations made for cereal pathogens, like *Magnaporthe oryzae* (Islam et al. 2016) and *Rhynchosporium commune* (Linde et al. 2009). It also fits that *R. collo-cygni* is regularly detected inside the grain (Matusinsky et al. 2011). Interestingly, the most closely related sister species to *R. collo-cygni*, *Z. tritici*, is rarely detected in seed (Suffert et al. 2011).

Fig. 3. Recombination analyses using ClonalFrameML. The number of expected recombinations per megabase per isolate as calculated by ClonalFrameML (y axis) is plotted for scaffolds 1 and 2 (x axis). The analyses were done with four different assumed genomic mutation rates (μ) ranging from 1×10^{-6} (black) to 1×10^{-9} (light gray). Two are estimated for different genomic mutation rates (x axis).

Our analyses also show no sign of a clear host specialization, because none of the outliers stem from an alternative host. These results are in contrast with many other fungi. For example, the rice blast fungus *M. oryzae* is pervasive on rice, but specific strains can also be found on many other monocot hosts (Gladieux et al. 2018; Yoshida et al. 2016). *Z. tritici* contains a small set of genes that can be related to host preference (Poppe et al. 2015), and even the broad-host range, opportunistic pathogen *Botrytis cinerea* shows signs of host adaptation (Leyronas et al. 2015). It will be crucial to identify other factors in the biology of *R. collo-cygni* that determine its generalist properties. This is particularly relevant, because other wild gramineous hosts can be an alternative source of inoculum. This can result in more than one overwintering host being available that can provide a “green bridge” or “seed bridge” for *R. collo-cygni* to survive winter. Yet, it has to be noted that analyses from spore traps report very little spore movement until later in the season (Havis et al. 2015).

The lack of host adaptation on the fungal side could be further evidence for *R. collo-cygni* being a general endophyte of grasses. Pathogenesis would then derive from susceptibility factors from the host genetic side, likely triggered or at least enhanced by environmental factors. In *Piriformospora indica*, metabolic cues from its hosts result in different levels of colonization by directly affecting expression levels of genes related to specific lifestyles

(Lahrman et al. 2013). Similar specific interactions are described for symbiosis (Rodriguez and Redman 2008). In fact, early infection with *R. collo-cygni* can have beneficial effects on yield, suggesting that the host–parasite interaction switches from mutualistic via endophytic to parasitic (Newton et al. 2010). An early mutualistic interaction could also explain the lack of major resistance genes against *R. collo-cygni* in barley. The absence of epidemics in the related genus *Gramineae* and the high diversity in barley germplasm give hope that host susceptibility factors, quantitative resistance loci, or even resistance genes can be identified for future control of RLS. However, an integrated genome-wide analysis of the host and pathogen transcriptome landscape after inoculation of a susceptible barley cultivar displayed molecular signatures reminiscent of a pathogenic fungus, triggering classical defense response pathways to necrotrophic pathogens, like ethylene and jasmonic acid signaling, in the susceptible host (Sjokvist et al. 2019). This also suggests that *R. collo-cygni*, at least partially, suppressed host defense responses in the later symptomatic stages of infection, at least under artificial inoculation conditions. Understanding the fungal virulence factors during leaf infection in various barley cultivars will thus be important for the prevention of RLS disease.

Rapid expansion of a plant pathogen can arise from beneficial recombination events during sexual reproduction followed by

Fig. 4. Inference of the past demographic history of *Ramularia collo-cygni*. Overview of estimated historic population size back in time. Expected effective population size (N_e) was estimated by comparing observed summary statistics with 200,000 simulations using PopSizeABC. Minor variations could be observed for each run. Gray lines represent 100 individual runs, and the black line shows the median values for those. The y axis shows the N_e against the number of years (or generations) ago (x axis).

selection on susceptible hosts. Using linkage disequilibrium scans and recombination analyses, we show that indeed there is evidence of sexual recombination within the collection of isolates that we inspected. This supports previous hypotheses (Hjortshøj et al. 2013; Piotrowska et al. 2016). Interestingly, the linkage disequilibrium scan signal strength for recombination is lower in the main subpopulation (or main isolate cluster) that excludes Argentina, Norway, and New Zealand. This could indicate that the expansion of *R. collo-cygni* is possibly partly attributed to clonal propagation with episodic sexual recombination. The combination of high clonal reproduction and global transfer via seed was probably favored by modern intensification of cereal production and global cereal food, fodder and seed trade, and trafficking.

When calculating the effective population size over time, we see an increase shortly after domestication of barley (10,000 years ago) followed by a gradual decrease. The past 300 years show a gradual increase again. This coincides with the intensification and switch to monocultures of barley, suggesting that *R. collo-cygni* expanded over time with the increased availability of susceptible hosts (Hjortshøj et al. 2013; Piotrowska et al. 2016). We speculate that this could have happened after recombination of strains that occurred as endophytes in wild grasses; this is a scenario similar to tan spot on wheat, which was frequently found in wild grasses and transferred to wheat only in the 1940s (Friesen et al. 2006). Yet, additional sampling, particularly from wild grasses, would be required to confirm this scenario.

With its relatively large effective population size compared with *Z. tritici* (Stukenbrock et al. 2011), likely mixed reproduction, and global gene flow, *R. collo-cygni* meets the criteria to be classified as a high-risk pathogen (McDonald and Linde 2002). No major R genes have been identified against *R. collo-cygni* in barley, and the characteristics described above suggest that, even if R genes were to be identified, they are unlikely to provide durable resistance, even when introduced as stacks or pyramids (Stam and McDonald 2018). Similarly, fungicide resistance can evolve rapidly and spread throughout the global populations owing to strong positive selection combined with recombination and extensive gene flow. Thus, the best way to diminish the current epidemic would be to drastically reduce the effective population size. This could be achieved by measurements to reduce overwintering capacity and transfer of infected seed or more diverse breeding approaches, including identification of host factors that result in quantitative resistances or manipulation of host susceptibility factors.

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