Extraordinary Genome Instability and Widespread Chromosome Rearrangements During Vegetative Growth

Mareike Möller,*,† Michael Habig,*,† Michael Freitag,‡ and Eva H. Stukenbrock*,†
*Environmental Genomics, Christian-Albrechts University, D-24118 Kiel, Germany, †Max Planck Fellow Group Environmental Genomics, Max Planck Institute for Evolutionary Biology, D-24306 Plön, Germany, and ‡Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-7305
ORCID IDs: 0000-0002-2146-5507 (M.M.); 0000-0002-8059-806X (M.H.); 0000-0003-1174-8252 (M.F.); 0000-0001-8590-3345 (E.H.S.)

ABSTRACT The haploid genome of the pathogenic fungus *Zymoseptoria tritici* is contained on "core" and "accessory" chromosomes. While 13 core chromosomes are found in all strains, as many as eight accessory chromosomes show presence/absence variation and rearrangements among field isolates. The factors influencing these presence/absence polymorphisms are so far unknown. We investigated chromosome stability using experimental evolution, karyotyping, and genome sequencing. We report extremely high and variable rates of accessory chromosome loss during mitotic propagation *in vitro* and *in planta*. Spontaneous chromosome loss was observed in 2 to >50% of cells during 4 weeks of incubation. Similar rates of chromosome loss in the closely related *Zymoseptoria ardabiliae* suggest that this extreme chromosome dynamic is a conserved phenomenon in the genus. Elevating the incubation temperature greatly increases instability of accessory and even core chromosomes, causing severe rearrangements involving telomere fusion and chromosome breakage. Chromosome losses do not affect the fitness of *Zymoseptoria tritici in vitro*, but some lead to increased virulence, suggesting an adaptive role of this extraordinary chromosome instability.

KEYWORDS chromosome loss; mitosis; fungal pathogen; experimental evolution

PATHOGENIC fungi pose global threats to agriculture, human, and animal health. Pathogens infecting plants and animals have been shown to rapidly adapt to changing environments, including industrial agriculture and medical treatments, usually in response to adaptive pressure caused by the widespread use of fungicides and pharmaceuticals (Selmecki *et al.* 2010; Bennett *et al.* 2014). The genomes of many prominent fungal plant pathogens exhibit high levels of structural variation, including isolate- or lineage-specific regions often characterized by high repeat contents, and accessory chromosomes varying in frequency among individual isolates (de Jonge *et al.* 2013; Persoons *et al.* 2014; Plissonneau *et al.* 2016). Structural variation is often associated with meiotic recombination, but highly variable regions are also found in asexually and mostly asexually reproducing species (Wang *et al.* 2006; Chuma *et al.* 2011; Faino *et al.* 2016). In several plant pathogenic fungi, determinants of virulence, so-called effector proteins, are located in dynamic regions of the genome (Miao *et al.* 1991; Coleman *et al.* 2009; Ma *et al.* 2010). Accessory chromosomes often carry genes that encode virulence determinants and are linked to pathogenicity. Therefore, the absence of a particular chromosome in some species results in avirulent phenotypes (Ma *et al.* 2010; Tsuge *et al.* 2016; Vlaardingerbroek *et al.* 2016; van Dam *et al.* 2018). Little is known about the mechanisms that drive the dynamics of accessory chromosomes and highly variable regions in genomes of eukaryotic pathogens.

The plant pathogenic fungus *Zymoseptoria tritici* reproduces both sexually and asexually and causes disease on wheat, especially in Northern Europe and North America, resulting in extensive annual yield loss (Fones and Gurr 2015; Torriani *et al.* 2015). The *Z. tritici* reference isolate IPO323 contains eight accessory chromosomes with sizes ranging from 0.4 to 1 Mb, accounting for 12% of the entire

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[1]Corresponding author: Max Planck Institute for Evolutionary Biology, August-Thienemann-Str. 2, 24306 Plön, Germany. E-mail: stukenbrock@evolbio.mpg.de

genome (Goodwin *et al.* 2011). Chromosome rearrangements, including complete loss of accessory chromosomes, are a frequent phenomenon in this fungus and have been demonstrated to occur during meiosis (Wittenberg *et al.* 2009; Croll *et al.* 2015; Fouché *et al.* 2018). Infection experiments with *Z. tritici* strains in which single or multiple accessory chromosomes had been deleted showed that at least some accessory chromosomes encode virulence factors that determine host specificity (Habig *et al.* 2017). In contrast to the core chromosomes, accessory chromosomes are enriched with transposable elements and have low gene density (Goodwin *et al.* 2011). Chromatin of accessory chromosomes shows hallmarks of constitutive and facultative heterochromatin, consistent with the observed transcriptional silencing of genes present on these chromosomes (Kellner *et al.* 2014; Rudd *et al.* 2015; Schotanus *et al.* 2015). The centromeres, subtelomeric regions, and telomeric repeats of accessory chromosomes are indistinguishable from those of core chromosomes (Schotanus *et al.* 2015), suggesting that accessory chromosomes contain all the required regions for proper chromosome segregation. The unusual high number of accessory chromosomes, in combination with the extreme variability in chromosome content among field isolates or progeny from controlled crosses, make *Z. tritici* an excellent eukaryotic model to study accessory chromosomes and their dynamics (Mehrabi *et al.* 2007; Wittenberg *et al.* 2009; Stukenbrock *et al.* 2010; Goodwin *et al.* 2011). Here, we provide evidence for unexpectedly high rates of chromosome loss and changes during asexual propagation, both *in vitro* and *in planta*. We describe the types of structural rearrangements and overall variation in chromosome stability in this important crop pathogen.

Materials and Methods

Short-term in vitro growth experiment in liquid culture

Zymoseptoria strains were diluted from glycerol stocks (−80°), plated on yeast-malt-sucrose (YMS) agar (4 g yeast extract, 4 g malt extract, 4 g sucrose, and 20 g agar per 1 liter) plates and grown for 7 days at 18° to obtain single colonies. One single colony was picked and suspended in 100 μl of YMS. Three replicate cultures were inoculated with 20 μl of cells from the single colony (∼50,000 cells). Cells were grown in 25 ml YMS medium at 18 or 28° shaking at 200 rpm and 900 μl of the cultures were transferred to fresh medium after 3–4 days of growth. In total eight transfers were conducted, for a total time course of 4 weeks.

Short-term in vitro growth experiment on agar plates

A single colony derived directly from a plated dilution of frozen stock for Zt09 (IPO323Δ18) was resuspended in 1000 μl YMS including 25% glycerol by 2 min vortexing on a VXR basic Vibrax at 2000 rpm, and 10–50 μl were replated onto a YMS agar plate. Forty replicates were produced. Cells were grown for 7 days at 18° whereby a random colony (based on vicinity to a prefixed position on the plate) derived from a single cell was picked and transferred to a new plate as described above. The transfer was conducted for a total of four times before a randomly chosen colony of each replicate was PCR screened and their complement on accessory chromosomes characterized as described below.

Screening by PCR

Cells from transfer eight of liquid cultures were diluted and plated on YMS-agar plates to obtain single colonies. To extract DNA, single colonies were suspended in 50 μl of 25 mM NaOH and boiled at 98° for 10 min; 50 μl of 40 mM Tris-HCl (pH 5.5) were added and 4 μl were used as template for the PCR. Primers for the right and left subtelomeric regions and close to the centromere were used for the chromosome-loss screening in our *Z. tritici* isolates, for *Zymoseptoria ardabiliae* primers in the center of candidate accessory chromosome unitigs were used. Primers and expected fragment lengths are listed in Supplemental Material, Table S1. Primers were designed with Clonemanager (Sci-Ed Software, Denver, CO) and MacVector and ordered from eurofins Genomics (Ebersberg, Germany).

Plant experiments and pycnidia isolation

Growth conditions for plants (*Triticum aestivum*) were 16 hr at light intensity of ∼200 $\mu mol/m^{-2}s^{-1}$ and 8 hr darkness in growth chambers at 20° with 90% humidity. Seeds of the *Z. tritici* susceptible wheat cultivar Obelisk (Wiersum Plantbreeding BV, Winschoten, The Netherlands) were germinated on wet sterile Whatman paper for 4 days at growth conditions before potting. After potting, plants were further grown for 7 days before infection.

For the phenotypic analyses, three independent experiments were conducted and 21 plants were used per strain per experiment. An ∼5 cm long section on the second leaf of each plant was infected by brushing a cell suspension with 10^7 cell/ml in H_2O + 0.1% Tween 20 on the abaxial and adaxial side of the second leaf. Plants were placed into sealed bags containing ∼1 liter of water for 48 hr to facilitate infection at maximum air humidity. Infected leaf areas were harvested 21 days postinfection for further phenotypic analysis or prepared for pycnidia isolation by surface sterilization with 1.2% NaClO for 2 min, followed by 70% ethanol for a few seconds, and washed twice with H_2O. Leaves were placed into a sterile environment with maximum air humidity and incubated for 7–14 days at plant growth conditions. Spores that had been pressed out from pycnidia were isolated using a sterile syringe needle and resuspended into 50 μl YMS medium with 50% glycerol. Cells were suspended by 30 min vortexing on a VXR basic Vibrax (IKA, Staufen, Germany) at 2000 rpm, plated on YMS agar, and incubated for 7 days at 18°. Colonies arising from single cells were PCR screened and their complement on accessory chromosomes characterized as described below.

Phenotypic characterization of infected leaves

Harvested leaves were taped to a sheet of paper and scanned using a flatbed scanner at a resolution of 2400 dpi. The scanned leaves were analyzed using ImageJ (Schneider

et al. 2012) and a plug-in described previously (Stewart *et al.* 2016). The measurement of pycnidia/cm^2 was used for further statistical analyses in R (Ihaka and Gentleman 1996). The Wilcoxon rank-sum test and the Holm's correction for multiple testing were applied to assess statistical differences between the samples.

In vitro phenotype assay

A spore solution containing 10^7 cells/ml and 10-fold dilution series to 1000 cells/ml was prepared. To test for responses to different stress conditions *in vitro*, YMS plates containing NaCl (0.5 and 1 M), sorbitol (1 and 1.5 M), Congo Red (300 μg/ml and 500 μg/ml), H_2O_2 (1.5 and 2 mM), methyl methanesulfonate (0.01%); an H_2O-agar plate; and two plates containing only YMS were prepared. Three microliter of the spore suspension dilutions were pipetted on the plates and incubated at 18° for 7 days. One of the YMS plates was incubated at 28° to test for thermal stress responses.

In vitro growth assay

YMS liquid cultures containing 10^5 cells/ml of either Zt09 or the chromosome-loss strains Zt09Δ14 and Zt09Δ21 were prepared. Three replicates per strain were used in each experiment. The spores were grown for 4 days at 18° at 200 rpm in 25 ml YMS and the OD_{600} was measured at different time points throughout the experiment. The data were fitted using the R package growthcurver (Sprouffske and Wagner 2016) and the *r* values of each replicate used for statistical comparison. The Wilcoxon rank-sum test was used to compare the *r* values of the different strains.

Pulsed-field gel electrophoresis and Southern blotting

Fungal strains were grown in YMS medium for 5 days. Cells were harvested by centrifugation for 10 min at 3500 rpm. We used 5×10^8 cells for plug preparation. The cells were resuspended in 1 ml H_2O and mixed with 1 ml of 2.2% low-range ultra-agarose (Bio-Rad, Munich, Germany). The mixture was pipetted into plug casting molds and cooled for 1 hr at 4°. The plugs were transferred to 50 ml screw cap Falcon tubes containing 5 ml of lysis buffer (1% SDS, 0.45 M EDTA, 1.5 mg/ml proteinase K; Roth, Karlsruhe, Germany), and incubated in lysis buffer for 48 hr at 55°, replacing the buffer once after 24 hr. Chromosomal plugs were washed three times for 20 min with 1 × TE buffer before storage in 5 ml of 0.5 M EDTA at 4°. Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF-DR III pulsed field electrophoresis system (Bio-Rad). To separate the small accessory chromosomes, the following settings were applied: switching time 50–150 s, 5 V/cm, 120° angle, 1% pulsed-field agarose (Bio-Rad) in 0.5 × TBE (Tris/borate/EDTA) for 48 hr. Separation of midsize chromosomes was conducted with the settings: switching time 250–1000 s, 3 V/cm, 106° angle, 1% pulsed-field agarose in 0.5 × TBE for 72 hr. *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad) was used as size marker for the accessory chromosomes, and *Hansenula wingei* chromosomal DNA (Bio-Rad) for mid-size chromosomes. Gels were stained in ethidium bromide staining solution (1 μg/ml ethidium bromide in H_2O) for 30 min. Detection of chromosomal bands was performed with the GelDocTM XR+ system (Bio-Rad).

Southern blotting was performed as described previously (Southern 1975) using digoxigenin-labeled probes generated with the PCR digoxigenin labeling Mix (Roche, Mannheim, Germany), following the manufacturer's instructions.

Sequencing and genome comparison

DNA for whole genome sequencing was prepared as described in Allen *et al.* (2006). Library preparation with a reduced number of PCR cycles (four cycles) and sequencing were performed by the Max Planck Genome Center, Cologne, Germany (http://mpgc.mpipz.mpg.de/home/) using an Illumina HiSeq3000 machine, obtaining ∼30× coverage with 150 nt paired-end reads. Raw reads were quality filtered using Trimmomatic and mapped to the reference genome of *Z. tritici* or *Z. ardabiliae* using bowtie2. SNP calling was conducted with samtools and bcftools and further quality filtered. The filtered SNPs were validated by manual inspection. The data were visualized in Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/) (Thorvaldsdóttir *et al.* 2013). Detailed commands for each step of the data processing pipeline can be found in the supplemental material.

Data availability

The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Strains are available upon request. Table S1 is a list of primers used for screening accessory chromosomes. Table S2 is a list of identified chromosome-loss strains. Table S3 shows chromosome variation of the sequenced strains grown at 28°. Table S4 lists location and annotation of SNPs and INDELs found in the sequenced strains. Table S5 displays sequence annotation of chromosomal breakpoints in the sequenced strains derived from the temperature stress experiment.

Supplemental figures are available in supporting figures and legends file. File S1 contains additional methods section including code used to analyze the genomic data. Genomic sequencing raw reads (FASTQ files) are available online at Sequence Read Archive under BioProject ID PRJNA428438. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.6854819.

Results

Accessory chromosomes are lost at a very high rate in vitro

To assess the stability of accessory chromosomes in *Z. tritici* we conducted an *in vitro* long-term growth experiment. We used the *Z. tritici* isolate IPO323Δchr18, derived from the reference strain IPO323 for which there is a completely assembled genome (Goodwin *et al.* 2011). IPO323Δchr18 lost chromosome 18 during previous *in vitro* propagation, and is here referred to as Zt09 (Kellner *et al.* 2014).

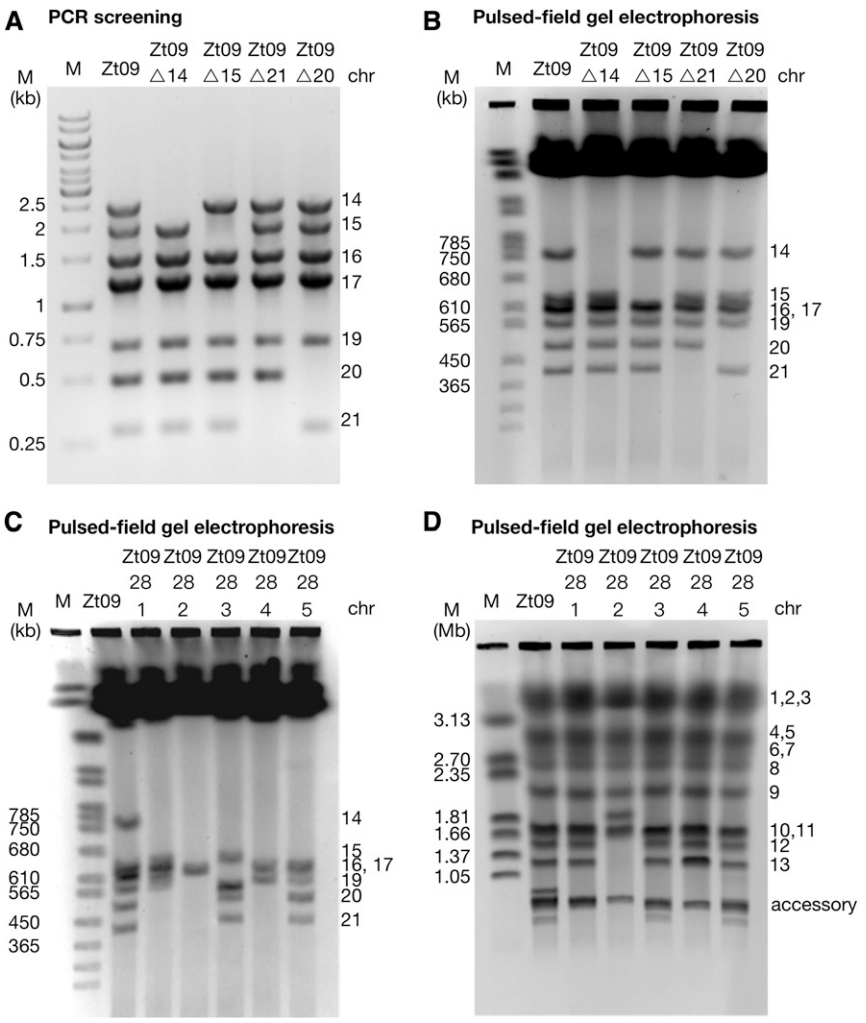

Figure 1 Screening by PCR and pulsed-field gel electrophoresis (PFGE) to identify accessory chromosome-loss strains in *Z. tritici*. (A) Screening by PCR of the progenitor Zt09 strain and evolved strains to assess chromosome losses using primer pairs located close to the centromeric region of the respective accessory chromosome. Multiplex PCR was used to simultaneously screen for the presence or absence of all accessory chromosomes in one strain. Absence of a PCR product indicates the absence of the chromosome; chromosome 18 is not present in Zt09. (B) PFGE was conducted to validate the absence of accessory chromosomes in chromosome-loss candidates identified by the initial PCR. Here, the separation of small accessory chromosomes of the progenitor strain Zt09 and four chromosome-loss strains is shown. The corresponding chromosome to each band is indicated on the right. The absence of chromosomal bands confirms the loss of the respective chromosome; chromosome 18 is similar in length to 16 and 17 but is absent in Zt09. PFGE of the accessory (C) and mid-size (D) chromosomes of strains originating from the *in vitro* temperature stress experiment confirms multiple accessory chromosome losses, size alterations, and chromosome fusions (see also Figure 3, Figure S1, and Table S3). The bands for chromosomes 3, 12, 13 and 21 are absent in strain 28-2; however, genome sequencing confirms that the chromosomes are still present in the genome (Figure 2B). The absence of bands on the PFGE can be explained by chromosome size changes whereby chromosomes 3 and 13, and 12 and 21 have experienced chromosome fusions (Figure 3 and Figure S1). Note a new chromosome band with a size of ~1.8 Mb resulting from the fusion of chromosomes 12 and 21. In the strain 28-3, the accessory chromosomes 17 and 19 are shorter than the reference chromosomes due to chromosome breakage (Figure 2B and Table S3). Strain 28-4 has an additional band of ~1.2 Mb, representing the fusion of a duplicated chromosome 17 (Figure S1). All images of stained gels are color-inverted to make differences more obvious.

We propagated fungal cells in liquid culture at 18°, including eight transfers to fresh medium. After 4 weeks (representing ~80 cell divisions per cell), 576 single strains originating from three replicate cultures were tested for the presence of the seven accessory chromosomes from the Zt09 progenitor by a PCR assay. The presence of marker regions, located close to the centromere, and the right and left telomere repeats of each accessory chromosome, were tested (Table S1). When the screening by PCR suggested absence of an accessory chromosome, we further validated the result by electrophoretic separation of the fungal chromosomes by PFGE (Figure 1, A and B). Thirty eight of the 576 (~7%) tested strains lacked one accessory chromosome. We did not find strains that lacked >1 chromosome, but observe a clear trend where accessory chromosomes 14, 15, and 16 were lost more frequently than smaller accessory chromosomes (Table 1). For example, chromosome 14 was absent in 18 strains, chromosome 15 in eight, and 16 in nine strains. The small chromosomes 20 and 21 were lost only in two and one strain, respectively, whereas chromosomes 17 and 19 were never absent following the *in vitro* propagation (Table S2 and Table 1).

We considered the possibility that natural selection acted on a large population of cells in the liquid *Z. tritici* cultures and thereby contributed to the observed nonrandom chromosome losses. To test this, we designed a second evolution experiment to propagate individual cell lineages in the absence of selection. For this experiment, 40 strains originating from one common progenitor (Zt09) were cultivated on plates. Every week, randomly selected colonies derived from single cells were transferred to a fresh plate. These repeated strong bottlenecks allowed us to propagate single lineages of *Z. tritici* without an effect of natural selection (Lynch *et al.* 2008). After 4 weeks of growth (including four transfers to new plates), the 40 evolved strains were tested for the presence of accessory chromosomes as described above. Five out of 40 (~13%) strains, twice as many as in the previous

Table 1 Summary of chromosome losses in *Z. tritici* during evolution experiments *in vitro* and *in planta*

Chromosome (kb)	Zt09 *in vitro* culture	Zt09 *in vitro* plate	IPO323 *in planta*	Zt09 *in vitro* temperature stress
14 (773)	18	0	1	108 (33)
15 (639)	8	0	6	2 (8)
16 (607)	9	4	1	0 (9)
17 (584)	0	0	0	0
18 (574)	NA	NA	7	NA
19 (550)	0	0	2	2 (2)
20 (472)	2	0	0	1 (17)
21 (409)	1	1	0	1 (7)
Total chromosome-loss strains	38	5	17	114 (34)
Total strains tested	576	40	986	188

Listed are the number of strains that lost an accessory chromosome and which accessory chromosomes were lost. Sizes of the accessory chromosomes are listed next to the chromosome number. Strains with >1 chromosome lost are listed in brackets.

experiment, lacked an accessory chromosome suggesting that selection in a larger population of cells indeed had removed some of the spontaneously occurring chromosome losses. Interestingly, in this experiment we observe the loss of only two different chromosomes: chromosome 16 was lost in four strains, and chromosome 21 in one strain. Our two *in vitro* experiments suggest that chromosome loss is not entirely random, and that specific chromosomes are lost at a higher rate (Table 1), depending on the *in vitro* growth conditions. The frequency of chromosome losses during asexual growth *in vitro* is extremely high in *Z. tritici* and exceeds previously reported spontaneous chromosome losses in *Fusarium oxysporum* f. sp. *lycopersici* by far (Vlaardingerbroek *et al.* 2016).

Accessory chromosomes are unstable in planta

The growth of *Z. tritici* in rich medium *in vitro* is highly distinct from growth of the fungus in its natural environment. *Z. tritici* is a hemi-biotrophic pathogen infecting the mesophyll of wheat leaves (Goodwin *et al.* 2011; Ponomarenko *et al.* 2011), where it forms asexual fruiting bodies, pycnidia, containing presumably clonal conidiospores (pycnidiospores). Pycnidia develop in the substomatal cavities, where the environment and nutrient availability differ from our tested *in vitro* conditions (Rudd *et al.* 2015; Haueisen *et al.* 2017). To test whether chromosome loss occurs only *in vitro* or also during the natural lifecycle of *Z. tritici*, we assessed the loss of accessory chromosomes *in planta*. We collected pycnidia from wheat leaves infected with the *Z. tritici* strain IPO323 and tested single pycnidiospores for the presence of accessory chromosomes. A total number of 968 pycnidiospores originating from 42 separate pycnidia were screened by PCR. In total, 17 strains missing an accessory chromosome were identified (~1.7%). Chromosomes 15 and 18 were the most frequently lost chromosomes, while chromosomes 17, 20, and 21 were not lost in any of the strains (Table S2 and Table 1). As for the *in vitro* experiments, no strains lacking >1 chromosome were found. Interestingly, chromosome 18, which was found to be lost at high rates in our plant experiments, was shown to be frequently absent in field isolates *of Z. tritici* (Croll *et al.* 2013; McDonald *et al.* 2016). In summary, our findings show that chromosome loss in *Z. tritici* not only results from the nondisjunction of homologous chromosomes during meiosis II, as proposed previously (Wittenberg *et al.* 2009), but also from frequent chromosome losses during asexual growth and mitotic spore formation *in planta*. The observed frequency of chromosome losses *in planta* is not as high as during *in vitro* growth; however, the number of mitotic divisions to develop pycnidia is presumably lower than during 4 weeks of *in vitro* growth. Therefore, we propose that the reduced number of chromosome losses *in planta* rather reflects the reduced number of cell divisions than possible fitness effects of chromosome losses.

Chromosome instability is greatly increased during exposure to heat stress

In their natural environments, pathogens are exposed to various kinds of biotic and abiotic stresses. For example, the local environment on the leaf surface can fluctuate severely in temperature and humidity conditions (Zhan and McDonald 2011). We assessed the effect of an increase in temperature on chromosome stability of *Z. tritici*. To this end, we cultivated Zt09 *in vitro* at elevated temperatures, namely an increase of 10° from 18 to 28° during 4 weeks of incubation. Subsequent screening by PCR for accessory chromosomes revealed severe chromosome losses in the tested strains. Out of 188 evolved and tested strains, 148 (~80%) were missing at least one accessory chromosome. Chromosome 14 was the most frequently lost chromosome and 34 evolved strains were lacking >1 chromosome. The maximum number of missing chromosomes was six in one strain (Table S2 and Table 1). PFGE revealed that karyotype alterations were not restricted to simple chromosome loss as observed in the *in vitro* experiments at 18° and the *in planta* experiments. In contrast, we observed frequent size variation of both core and accessory chromosomes as a consequence of chromosome breakage, fusions, and duplications (see below) (Figure 1, C and D, Figure S1, and Table S3).

Genome sequencing of chromosome-loss strains reveals chromosome breakage and fusion, but a low number of SNPs

In total, we sequenced 19 genomes of strains that had lost different chromosomes or displayed rearrangements of

chromosomes as revealed by PFGE. The strains represented derived strains from the different *in vitro* and the *in planta* experiment and the respective progenitor strains; all sequencing results have been deposited in the Sequence Read Archive under BioProject ID PRJNA428438. Overall, we confirmed the absence of complete accessory chromosomes (Figure 2A), and we found very few additional changes. After filtering to exclude reads of poor quality and coverage (see *Materials and Methods* and Supplemental Material) we identified a total number of nine SNPs in eight sequenced strains originating from the *in vitro* experiment in liquid culture at 18° and the *in planta* experiment. Of these, three SNPs were located in coding regions (Table S4). Besides the complete loss of accessory chromosomes and the identified SNPs, we found no other mutations when comparing the progenitor and evolved strains. However, we identified SNPs distinguishing Zt09 from our IPO323 reference strain, and the published genome sequence; most of these SNPs were in noncoding sequences (Table S4). As we show chromosome losses in different strains, we conclude that the few point mutations do not have a measurable effect on chromosome loss.

Whole genome sequencing of five strains evolved at 28° verified the losses of several accessory chromosomes. Furthermore, comparison of the resequenced genomes of the progenitor and the evolved strains revealed substantial size variation resulting from chromosome breakage of core and accessory chromosomes, and duplications of accessory chromosomes (Figure 2B). Chromosome breaks located close to the ends of chromosomes resulted in shortened chromosomes caused by subtelomeric deletions of ~0.2–60 kb (Table S3). Detailed analyses of the distribution of discordant paired-end reads mapping to different chromosomes revealed a high number of reads at the chromosome breakpoints with the respective read mates mapping to telomeric repeats (Figure 3). This suggested that most chromosome breaks resulted in shorter chromosomes to which telomeres were added *de novo*. Besides *de novo* telomere formation, we found evidence for fusion of core chromosomes in strain Zt09 28-2, where discordant read mapping indicated fusion of the right arms of chromosomes 3 and 13 (Figure 3 and Table S3). PFGE and Southern blots further showed fusion of chromosomes 12 and 21 in Zt09 28-2 (Figure S1). In a previous study, we reported evidence for the likely fusion of an ancestral accessory chromosome and core chromosome 7 in the reference isolate IPO323 and Zt09 (Kellner *et al.* 2014; Schotanus *et al.* 2015). Here, we deduced a fusion of the duplicated chromosome 17 in strain Zt09 28-4 (Figure 1, C and D and Figure S1). Similar fusions of the same accessory chromosome 17 following meiosis had been suggested previously, and "breakage-fusion bridge" cycles (McClintock 1938, 1941) were invoked as a mechanism to form a new accessory chromosome (Croll *et al.* 2013). Based on the position of centromeres in *Z. tritici* (Schotanus *et al.* 2015), all three chromosome fusion events that we detected, resulted in the formation of dicentric chromosomes. On two chromosomes involved in the fusion events, we identified the "internal" breakpoints (Table S3) likely reflecting the consequences of chromosome breakage of a dicentric chromosome during mitosis. This indicates that breakage-fusion bridge cycles also occur during mitosis in *Z. tritici* and can be a mechanism involved in the structural variation detected in our experiments. In total, in the five analyzed genomes derived from strains grown at 28°, we identified the following spontaneous changes: 15 chromosome breakages (12 with *de novo* telomere formation and three without evidence for new telomeres), three chromosome fusions, three chromosome duplications, and 16 chromosome losses (Table S3). The breakpoints of the structural rearrangements often colocalize with annotated transposons, predominantly class I elements (Table S5). Transposable elements are often associated with structural rearrangements (Faino *et al.* 2016; Plissonneau *et al.* 2016) and their activation under stress conditions might contribute to the observed genome instability (Chadha and Sharma 2014). A key observation from our genome data analyses is the frequent involvement of *de novo* telomere formation and chromosomal fusion as a mechanism to heal broken chromosome ends. While this has been reported for cancer cells (Murnane 2012), it is generally considered a rare event in normal, nontransformed cells. Here we show that *de novo* telomere formation and chromosome fusion readily occur in a filamentous fungus during growth at temperature stress.

Accessory chromosome instability also occurs in Zymoseptoria sister species

To assess whether the high frequency of mitotic chromosome loss is specific to *Z. tritici*, we conducted a short-term *in vitro* evolution experiment on the fungus *Z. ardabiliae*, a closely related sister species of *Z. tritici* that infects wild grasses. We used the previously characterized isolate STIR04 1.1.1 (Stukenbrock *et al.* 2011), here called Za17, in our experiments.

We first generated a high-quality reference genome based on long-read single molecule real time (SMRT) sequencing (Supplemental Material). Analyses of a population genomic dataset revealed at least four accessory chromosomes varying in their frequency among 17 *Z. ardabiliae* isolates (Stukenbrock *et al.* 2011; Stukenbrock and Dutheil 2018). Based on PFGE and homology of *Z. ardabiliae* unitigs to fragments of *Z. tritici* IPO323 accessory chromosomes (Soderlund *et al.* 2011), we designed primers to amplify six loci located on putative accessory chromosomes of Za17 (Table S1).

Next, we conducted an *in vitro* evolution experiment at 18° for 4 weeks in liquid culture with Za17 as the progenitor strain. Screening of 288 single clones by PCR identified five strains lacking one of the accessory chromosomes (~1.7%). We further verified the absence of accessory chromosomes by PFGE for all five strains and whole genome sequencing for three of the five *Z. ardabiliae* strains (Figure 2C). By combining PFGE and genome sequencing of chromosome-loss strains we could confirm unitigs 24, 34, 49 and 51 to be accessory sequences pertaining to three accessory chromosomes (Figure 2C).

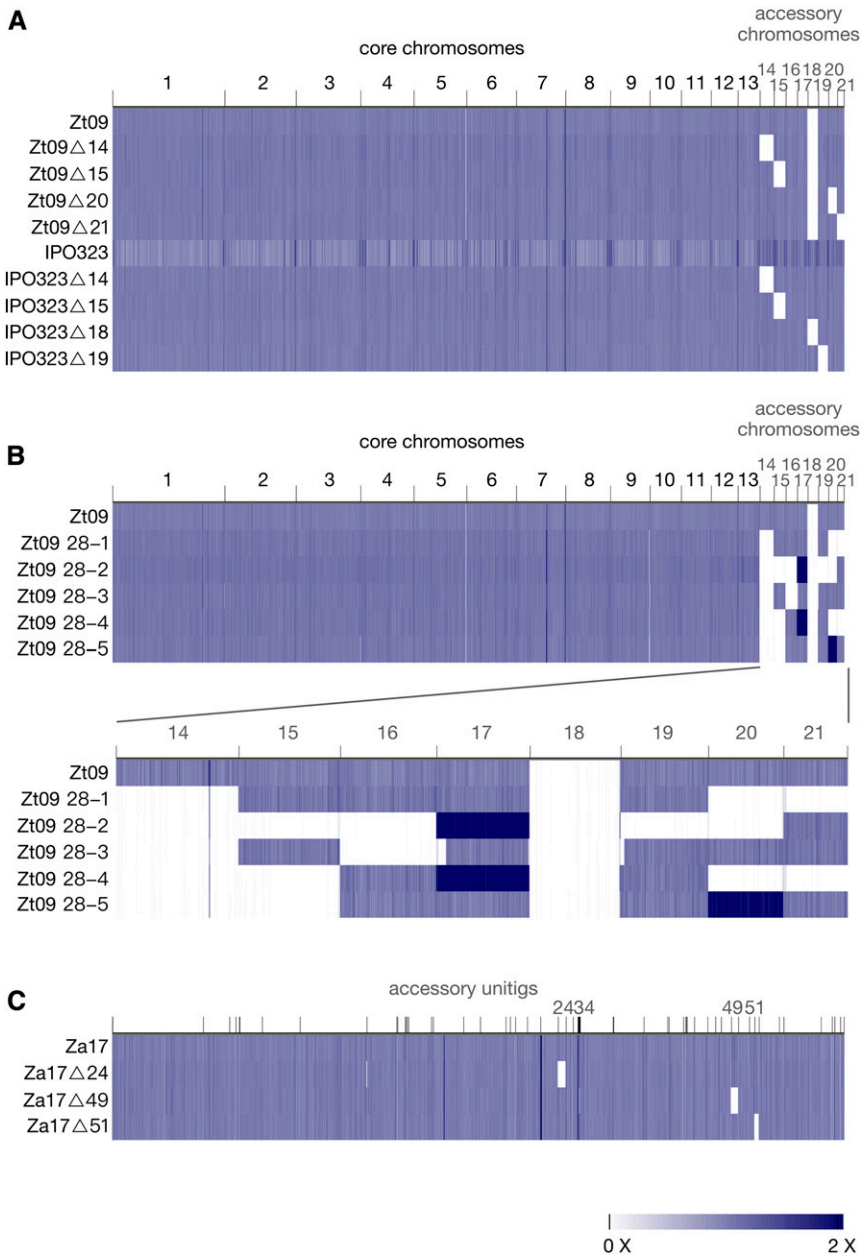

Figure 2 Whole genome sequencing confirms loss of accessory chromosomes in *Z. tritici* and *Z. ardabiliae*. The genomes of chromosome-loss strains derived from the *Z. tritici in vitro* evolution experiment, the *in planta* pycnidiospore isolations, the *Z. ardabiliae in vitro* evolution experiment, and the respective progenitor strains were sequenced by paired-end Illumina sequencing and mapped to the respective reference isolates, (A and B) IPO323 (*Z. tritici*) or (C) Za17 (*Z. ardabiliae*). The losses of entire chromosomes (white boxes) were verified for each strain, but few mutations in form of SNPs, indels, or copy number variation were detected (Table S4). The *Z. tritici* reference genome consists of whole chromosomes, while the *Z. ardabiliae* reference genome is composed of unitigs obtained from the SMRT sequencing assembly (ordered by name of the unitig). The read depth is here normalized to 1 X per strain. (B) Genome sequencing of strains derived from the temperature stress experiment revealed, besides verification of accessory chromosome losses, chromosome breaks at the ends of core and accessory chromosomes, and accessory chromosome duplications. Zooming in on the coverage of accessory chromosomes highlights the duplications of chromosomes 17 (Zt09-28-2, Zt09-28-4) and 20 (Zt09-28-5), and the chromosome breakage of chromosomes 17 and 19 (Zt09-28-3). Darker blue shading indicates higher coverage. Most repetitive sequences have a higher coverage than single copy regions, resulting in different shades of blue in the coverage graph. The intense dark blue lines indicate high coverage regions on ribosomal DNA (rDNA) clusters or repetitive DNA due to underestimation of repeats in the reference assembly (for example, the thin black line in chromosome 7 indicates the location of several rDNA cluster repeats, the only such repeats in the annotated genome sequence, but the expected true number of rDNA repeats is ~50). Accessory chromosomes or unitigs are highlighted in grey.

These findings resemble the rapid loss of chromosomes in *Z. tritici* and suggest a common phenomenon, most likely related to mitotic cell division in the two *Zymoseptoria* species.

Accessory chromosome losses affect in planta phenotypes

We next addressed the effect of spontaneous chromosome loss by comparing the fitness of the progenitor and evolved *Z. tritici* strains under different *in vitro* growth conditions, and during infection of a susceptible wheat variety by comparing growth and pycnidia formation. *In vitro*, the growth rate of strains lacking an additional accessory chromosome (Zt09Δ14 and Zt09Δ21) was comparable to the growth rate of the progenitor, Zt09, but with a slight tendency to slower growth of the derived chromosome-loss strains (Figure S2). For a more detailed phenotypic characterization, we used the five chromosome-loss strains isolated from pycnidia from the *in planta* experiment (Table S2). Several stress conditions including osmotic, oxidative, temperature, and cell wall stresses were tested *in vitro*. We compared the fungal phenotypes of the chromosome-loss strains and the progenitor strain IPO323 and observed no difference in growth rate or colony morphology between any chromosome-loss strain and IPO323 (Figure 4A). *In planta*, however, we observed phenotypic differences between the evolved chromosome-loss strains and their progenitor. We measured fitness by counting the number of asexual fruiting bodies formed in stomata on infected wheat leaves and found a slightly higher fitness of the chromosome-loss strains compared to the progenitor strain (Figure 4B). The difference was most pronounced for

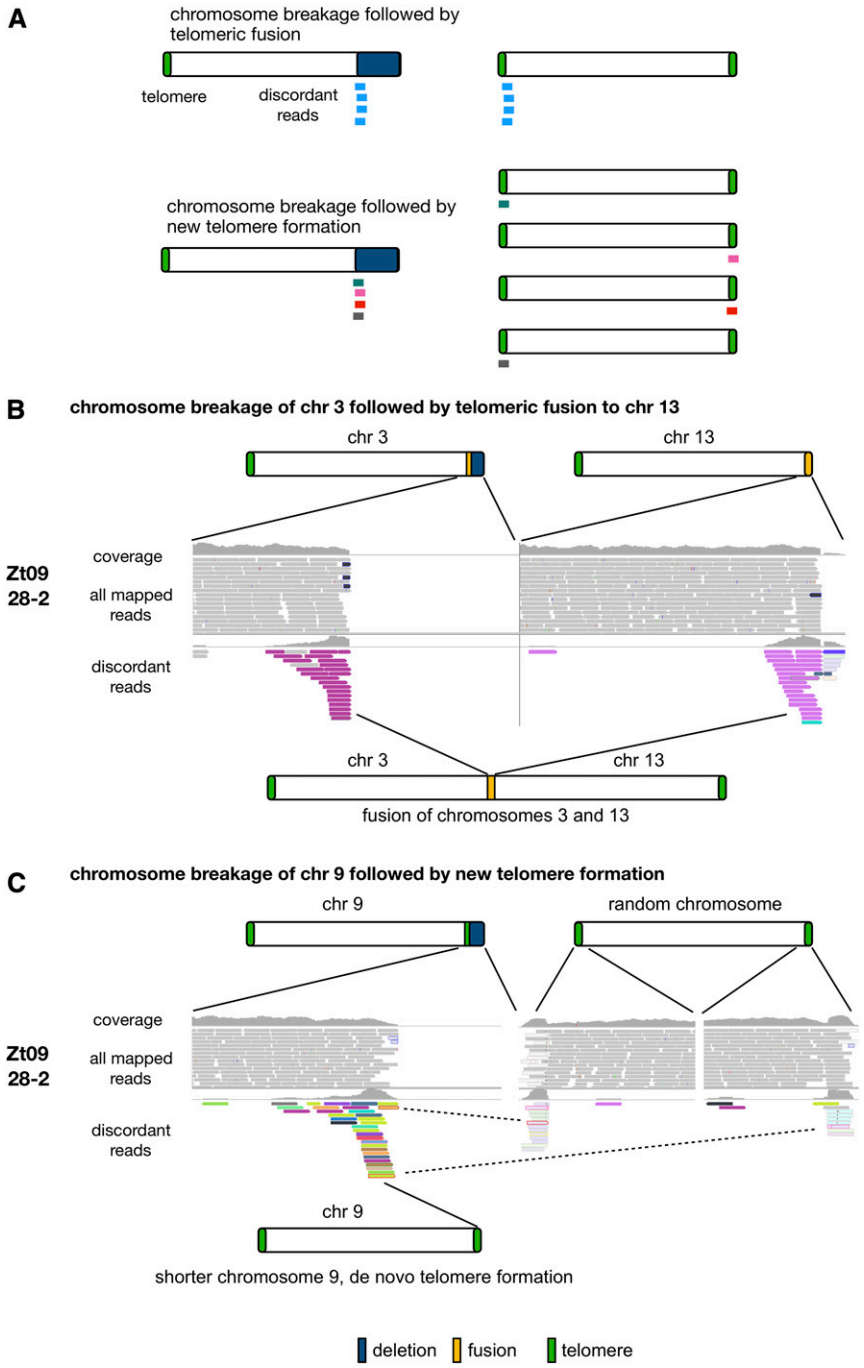

Figure 3 Discordant read analysis reveals chromosome fusion and chromosome breakage. (A) Schematic illustration of discordant read mapping in the case of chromosome breakage and fusion. If chromosome breakage is followed by fusion to a different chromosome, the discordantly mapping reads at the breakpoint will have their respective read mate on the chromosome that is fused to the breakpoint. If breakage is followed by *de novo* telomere formation, the discordant reads have their respective read mates on telomeric repeats on random chromosomes. (B and C) Read mapping to the genome of the reference isolate IPO323. (B) In the experimentally evolved strains of *Z. tritici* at 28°, the fusion of chromosomes 3 and 13 in the strain Zt09 28-2 is indicated by the increased occurrence of discordant reads at the chromosome breakpoint of the right arm of chromosome 3 and the right arm of chromosome 13. The color of the reads represents the chromosome their respective mate is mapping to. (C) New telomere formation at the breakpoint, here, as example, shown for chromosome 9 in the strain Zt09 28-2. Telomere formation is indicated by a high number of discordant reads, where the read mates are mapping to the telomeric repeats of different random chromosomes (dashed line). A total of 12 breakage events that were followed by *de novo* telomere formation were detected in the five analyzed strains derived from the experiment at elevated temperature.

the strains lacking chromosome 14 and chromosome 19 where the density of pycnidia was found to be significantly higher (*P* values of 0.0003 and 0.015, Wilcoxon rank-sum test and Holm's correction for multiple testing) compared to the IPO323 progenitor (Figure 4B).

Discussion

Consequences for evolution of accessory chromosomes

Accessory chromosomes were proposed to serve as an "evolutionary cradle" for creating novel virulence genes without risking the disruption of essential genes on the core chromosomes (Croll and McDonald 2012). Indeed, signatures for accelerated evolution were shown by overall higher dN/dS ratios of genes on the accessory chromosomes compared to the core chromosomes (Stukenbrock *et al.* 2010). However, natural selection acts at the level of individuals and cannot maintain "a playground" for future beneficial effects. Rather, we hypothesize that the dynamic of accessory chromosome loss reflects a dynamic in the selective environment of *Z. tritici*, where under certain conditions these small chromosomes confer a fitness advantage, while under different

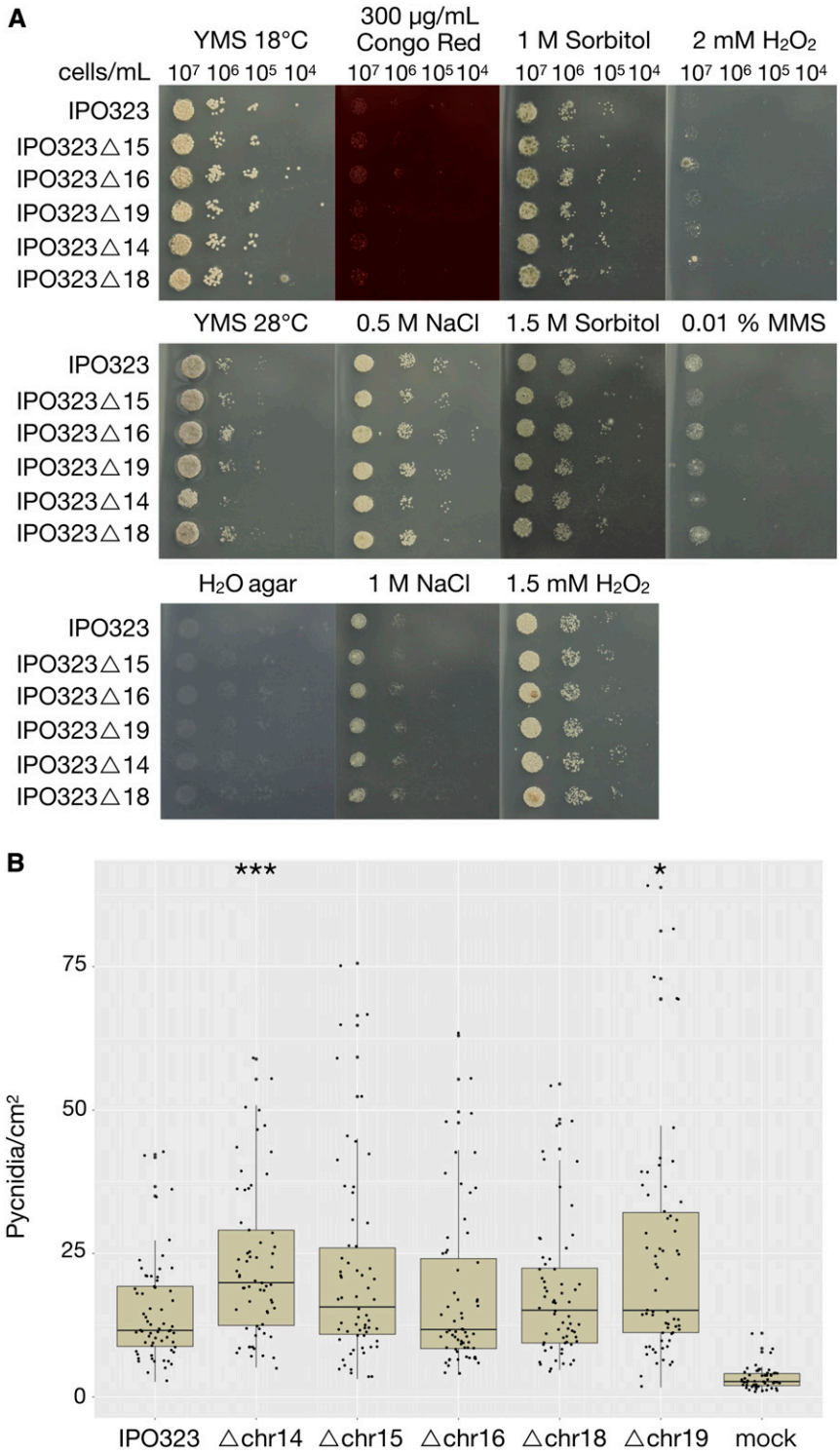

Figure 4 *In vitro* and *in planta* phenotype assays of the *Z. tritici* reference isolate IPO323 and the *in planta* chromosome-loss strains. (A) Several *in vitro* stress conditions were tested to assess the effect of accessory chromosome losses on fitness. Five *in planta* chromosome-loss strains were tested, IPO323 was used as the reference strain. We observed no noteworthy differences in growth rate or colony morphology between reference and chromosome-loss strains. (B) To investigate the effect of accessory chromosome losses on *Z. tritici* infection of wheat, three independent experiments with the reference strain IPO323 and five chromosome-loss strains were conducted. The fitness of each strain was measured by counting the number of pycnidia per square centimeter on the leaf surface. Statistical analysis (Wilcoxon rank-sum test and Holm's correction, $*P \leq 0.05$, $***P \leq 0.001$) identified two chromosome-loss strains (chromosomes 14 and 19 *P*-values 0.0003 and 0.015) that show a significantly higher number of pycnidia compared to the reference strain IPO323 under the conditions used in this experiment.

conditions they cause decrease in fitness. A fitness advantage of chromosome-loss strains *in planta* may indicate the presence of avirulence factors on at least some accessory chromosomes. Avirulence factors can be recognized by the host to induce a resistance response resulting in reduction in virulence or even abortion of infection (Petit-Houdenot and Fudal 2017; Zhong *et al.* 2017). Our previous findings support this notion, as we found small but significant negative effects of accessory chromosomes during host infection (Habig *et al.* 2017). In contrast, Fouché *et al.* (2018) detected small, quantitative increases of virulence traits if specific accessory chromosomes were present. However, so far no avirulence factors and very few potential effectors were identified on accessory chromosomes in *Z. tritici* and the vast majority of annotated genes on accessory chromosomes does not have a predicted function (Goodwin *et al.* 2011;

Grandaubert *et al.* 2015). In other pathogenic fungi, however, the opposite effect has been demonstrated in several cases. A strong positive effect on virulence of accessory chromosomes has been observed in *F. oxysporum* f. sp. and *Nectria haematococca* (Coleman *et al.* 2009; Ma *et al.* 2010; van Dam *et al.* 2018). In these pathogens chromosome losses resulted in complete loss of pathogenicity. While these cases are currently considered the norm, our observations show that responses to accessory chromosome loss can be more varied. Even though this fungus is an important pathogen in all wheat-growing countries, the biology of *Z. tritici* is still not completely understood. Mating and overwintering in the soil or litter layer remain largely unknown lifecycle stages, and we postulate that they impose different selection pressures on the fungus. During these stages, accessory chromosomes may confer significant fitness advantages that have not been demonstrated yet. Nevertheless, any effects on fitness found to date are rather small. While beneficial effects, even if they are comparably small, might explain the maintenance of accessory chromosomes in *Z. tritici* populations, negative effects as observed in this study and reported by Habig *et al.* (2017) raise the question why these chromosomes have not been lost over time, especially as they can easily be lost during meiosis and mitosis. Accessory chromosome can be lost during meiosis (Wittenberg *et al.* 2009), but interestingly accessory chromosomes have also been shown to follow non-Mendelian segregation and to be transmitted at a significantly higher rate than expected, if one of the two mating partners lacks an accessory chromosome (Fouché *et al.* 2018; Habig *et al.*, 2018). While there is no comprehensive mechanistic explanation yet, chromosome conservation by re-replication during meiosis may counteract the chromosome loss during vegetative growth and therefore maintain accessory chromosomes in pathogen populations. In *Alternaria alternata*, the spontaneous loss of a conditionally dispensable chromosome during subculturing has been described for one isolate (Johnson *et al.* 2001). Furthermore, loss of a conditionally dispensable chromosome after meiosis has been observed in *N. haematococca* (aka *Fusarium solani* MPVI) (Miao *et al.* 1991). A recent study of the plant pathogenic fungus *F. oxysporum* f. sp. *lycopersici* describes the spontaneous loss of dispensable chromosomes *in vitro* in one cell out of 35,000 (Vlaardingerbroek *et al.* 2016). This rate is much lower than the chromosome-loss rate that we observe in *Z. tritici* and *Z. ardabiliae*, where, depending on the growth conditions, between ~2 and ~80% of the tested strains, lack an accessory chromosome. In combination, these results indicate that chromosome instability is a common phenomenon in fungi, but the stability of chromosomes can vary substantially between different species. Studies in *Saccharomyces* and *Candida* species have shown that chromosome loss resulting in aneuploidy is a relatively frequent phenomenon in diploid cells and is advantageous under certain conditions (Selmecki *et al.* 2010; Kumaran *et al.* 2013; Bennett *et al.* 2014; Zhu *et al.* 2014). The loss of chromosomes in haploid organisms, such as *Z. tritici*, is expected to have more severe consequences, as genetic information is lost from the cell. We considered, however, that the effect of rapid chromosome loss or instability may be advantageous if these traits are selected for in pathogens like *Zymoseptoria*.

The temperature stress experiment *in vitro* showed that genome instability in *Z. tritici*, at this temperature, is not restricted to the accessory chromosomes, but also involves the core genome. New chromosome formation induced by telomere to telomere fusion followed by breakage of the dicentric chromosome was reported in *Cryptococcus neoformans* (Fraser *et al.* 2005) but likely occurred during meiosis rather than mitosis. In the asexual *Candida glabrata*, clinical isolates frequently inhabit chromosomal rearrangements indicating that structural variation acts as a virulence mechanism (Poláková *et al.* 2009) and might be a response to stresses such as antifungal treatment or elevated temperature as observed here. The instable regions close to the chromosome ends on the core chromosomes, however, show structural characteristics similar to the accessory chromosomes, such as higher repeat content, lower gene density and specific histone modification patterns (Dhillon *et al.* 2014; Grandaubert *et al.* 2015; Schotanus *et al.* 2015).

The accessory chromosomes of *Z. tritici* do not differ in terms of centromere or telomere organization from the core chromosomes, but the chromatin is largely transcriptionally inactive and potentially more condensed due to an enrichment with the histone H3 that is trimethylated at lysine 27 (H3K27me3), which covers almost all of the accessory chromosomes (Schotanus *et al.* 2015). Enrichment of the facultative heterochromatin mark H3K27me3 seems to be a common feature of accessory chromosomes in several fungal pathogens but the functional relevance for stability and transcriptional regulation of these chromosomes has not been addressed so far (Galazka and Freitag 2014; Seidl *et al.* 2016). H3K27me3 is also enriched in subtelomeric regions of core chromosomes, that we show here as prone to instability. High-resolution microscopic analyses indicate an unusual localization of centromeres in the *Z. tritici* nuclei, suggesting a distinct spatial organization of different chromosomes (Schotanus *et al.* 2015). Heterochromatin has been found close to the nuclear periphery, and H3K27me3 has been shown to be involved in facilitating lamina-proximal positioning (Harr *et al.* 2015). We hypothesize that the heterochromatic structure reflects a distinct physical organization of core and accessory chromosomes and likely the subtelomeric regions in the nucleus of *Z. tritici*, and that this is correlated to the instability of heterochromatic regions and chromosomes. An Hi-C study on *Neurospora crassa* showed that absence of H3K27me3 resulted in movement of subtelomeric regions and centromeres from the nuclear periphery into the nuclear matrix (Klocko *et al.* 2016). Further experiments focusing on localization of specific *Zymoseptoria* chromosomes in the nucleus by cytology or Hi-C (Galazka *et al.* 2016) should be conducted to address this hypothesis.

In conclusion, using experimental evolution, electrophoretic karyotyping and genome sequencing we showed that

the accessory chromosomes of *Z. tritici* are highly unstable during asexual growth across numerous mitotic cell divisions ("mitotic growth") *in vitro* as well as *in planta*. Surprisingly, increasing the temperature from 18 to 28° dramatically increased overall genome instability in *Z. tritici*. Besides chromosome losses we observed structural variation in form of chromosome breakage, duplication, and fusion involving both core and accessory chromosomes, and all events were increased near telomeric sequences. In *Z. tritici*, all of these chromosome abnormalities have thus far been associated with meiosis (Wittenberg *et al.* 2009; Croll *et al.* 2013), however, our study highlights an important role of mitotic growth in generating genetic diversity. The variability in chromosome number and structure obtained over a relatively short period of time (during one infection event, or 4 weeks of vegetative growth) correlates with the genomic diversity observed in field populations of this important plant pathogen (McDonald and Martinez 1991; Linde *et al.* 2002; Zhan *et al.* 2003; Mehrabi *et al.* 2007). Our findings demonstrate that even sexual fungal pathogens can accelerate the generation of new genetic diversity by mitosis-associated structural variation. The mitotic events responsible for these rearrangements are still to be characterized.

Lastly, chromosome instability has been observed in various organisms and is a frequent phenomenon in cancer cells (McGranahan *et al.* 2012). Epigenetic factors and heterochromatin in particular have been shown to affect chromosome stability in human cancer cells (Slee *et al.* 2012). The accessory chromosome dynamics correlating with distinct chromatin patterns suggest that *Z. tritici* may provide a new model for mechanistic studies of chromosome instability in cancer cells.

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