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Transposable elements in genomic architecture of *Monilinia* fungal phytopathogens and TE-driven DMI-resistance adaptation

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Abstract

Background Fungicide resistance poses a significant challenge to plant disease management and influences the evolutionary dynamics of fungal pathogens. Besides being important phytopathogens, *Monilinia* species have become a model for discovering many fundamental questions related to fungal pathosystems. In this study, DMI-propiconazole sensitivity was investigated in view of transposable element (TE) dynamics in *M. fructicola* and *M. laxa*.

Results Propiconazole-sensitivity of 109 *M. fructicola* and 20 *M. laxa* isolates from different regions of Türkiye was assessed. Comprehensive TE identification within the species revealed that Class I elements were predominant, and TEs constituted approximately 9% of the genome for both *M. fructicola* and *M. laxa*, with a total of 15,327 and 10,710 TEs, respectively. An experimental evolution plan was developed for *Monilinia* that allows observing phenotypic and genotypic changes over successive generations under controlled selection pressures. Dynamic changes in TE content were discovered throughout the experimental evolution of *M. fructicola* under propiconazole pressure. With a net change of 187 TEs, the evolved strain showed an expansion of TE sequences, whereas different TE classes displayed diverse patterns of increase/decrease. Additionally, the presence of a nested TE upstream of the *CYP51* gene was observed in less-sensitive *M. fructicola* isolates but absent in highly-sensitive ones. Gene expressions of *CYP51* differed significantly between TE-containing and TE-lacking isolates, strongly supporting the contribution of this TE to fungicide resistance.

Conclusion This study establishes a critical link between TEs and DMI fungicide resistance by associating a nested TE with reduced sensitivity to propiconazole. We introduce an innovative experimental evolution framework for studying genomic changes under selective pressure and provide a comprehensive characterization of *Monilinia* TEs. These findings significantly advance our understanding of molecular resistance mechanisms in fungal pathogens, offering insights for more effective disease management.

Keywords Transposable elements, *Monilinia* species, Fungicide resistance, Experimental evolution

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Background

Transposable elements (TEs) are mobile DNA sequences that can move and insert themselves into different regions of a genome. These elements have been shown to be present in almost all eukaryotic species since their discovery, except for some lower eukaryotes like *Plasmodium falciparum* [1]. All eukaryotic genomes examined thus far, with a few notable exceptions, are known to harbor transposable elements (TEs) [2]. The TE composition of fungal genomes varies from less than 1% (e.g., *Fusarium graminearum*) [3] to more than 90% (e.g., *Blumeria graminis*) [4]. While TEs provide plasticity due to their ability to move and replicate and play a significant role in epigenetic changes in genomes [5], the vast majority of TE activity comes with various costs to the host, including disruption of functional genes [6], cellular cost of replicating [7], and changing the expression profiles in the regions they have been inserted [8]. Despite this, some TEs have proven to be beneficial to the host under stressful conditions [9]. The mutational activity of TEs, often influenced by host defense mechanisms has the potential to increase genetic diversity and speed up adaptive evolutionary processes, regardless of their effects [10].

Fungicides are chemical compounds that have been successful in preventing fungal diseases for many years [11]. Modern fungicides act as inhibitors for several biochemical processes and bind to specific protein targets, known as single-site or site-specific, in contrast to earlier fungicides which act on multiple sites (FRAC, https://www.frac.info/). Demethylation Inhibitors (DMIs), comm only used site-specific fungicides, act on the biosynthesis of ergosterol, which is a major component of the cell membrane for fungal pathogens [12]. These site-specific fungicides are highly active and often distributed along the plant tissues even with low doses, thus providing efficient disease management [13]. However, the combinations of pathogen biology (e.g., short generation time, large population size), environmental conditions and mode-of-action (MOA) and/or the dose of fungicide may direct the selection of resistant phenotypes resulting in resistance in natural populations [14]. Strong selection may result in increased frequency of resistant genotypes while eliminating susceptible genotypes in most pathogen populations or may trigger the emergence of *de novo* resistance mechanisms in pathogens in the long run. Resistance at the molecular level may be conferred either by the selection of variants at the target protein or the selection of variants at the non-target sites. Even the selection of a single nucleotide polymorphism (SNP) with a large effect on the target protein that results in decreased efficiency between the fungicide and the target may confer a high level of resistance in site-specific fungicides [14]. As stated above, resistance phenotypes may also arise from alterations at multiple targets/non-target sites, referred to as quantitative resistance. For example, many studies have revealed the association between the increased activity of efflux pumps and fungicide resistance, underlying the crucial and common relevance of these transporters in resistance occurrence [15]. ABC superfamily, with their ability to transport a wide range of compounds based on their low substrate specificity, is the most attributed transporter that takes place in fungicide resistance [14]. Thus, the alterations that occurred at non-target sites may confer resistance to not only the fungicide used but to other fungicides that will be used in nature as well. Along these lines, knowledge of a pathogen's sensitivity to a specific fungicide is essential in preventing significant losses in plant yield and quality, making it a vital piece of information for disease control.

Brown rot disease, caused by certain species of *Monilinia* genus, primarily *M. fructicola* and *M. laxa*, is a significant constraint in stone fruit production worldwide [16, 17], presenting a considerable challenge to peach fruit production in Türkiye [18]. Given the widespread use of site-specific fungicides, the emergence of resistance in *Monilinia* populations has become a significant concern for disease management and resistance to certain site-specific fungicides has been reported in different *Monilinia* populations worldwide. For instance, resistance to methyl benzimidazole carbamates (MBCs) has been observed in the U.S.A, and China [19, 20], and resistance to respiratory inhibitor (RI) fungicides has been reported in the U.S.A, Brazil, and Türkiye [21–23]. Moreover, resistance to DMI group fungicides has been reported in *Monilinia* populations from the United States and Brazil [24, 25]. To date, two main resistance mechanisms against DMI group fungicides have been proposed for *M. fructicola*, and no resistance mechanism for *M. laxa* has been identified, to our knowledge. In one of these mechanisms, it has been reported that the presence of a 65 bp long genetic element called "Mona" upstream of the *CYP51* gene results in an increase in *CYP51* gene expression, resulting in a resistance response [26]. While Mona is not definitively confirmed to be a TE, its multicopy nature suggests it may belong to this category [26]. Additionally, although this element was also found in sensitive *M. fructicola* isolates [25], the association with resistance is drawn from existing literature and remains to be further validated. In the second proposed resistance mechanism, a substitution at position 1492 of the *CYP51* gene, which causes an amino acid substitution from glycine (G) to serine (S), has been associated with resistance to DMI group fungicide tebuconazole in *M. fructicola* populations from Brazil [24]. In addition to these resistance mechanisms, a study conducted on *M. fructicola* has shown that the expression level of the ABC transporter gene might also be a determinant of DMI resistance based on a non-target mechanism [27]. To

date, no genome-wide investigation has been conducted to study the fungicide resistance mechanism for *Monilinia* species, and genome-wide studies attempting to understand fungicide resistance are carried out on some fungal organisms, including clinically relevant *Candida albicans* [28], wheat pathogen *Zymoseptoria tritici* [29], and barley pathogen *Rhynchosporium commune* [30]. It is important to study fungicide resistance mechanisms from multiple perspectives since they are complex traits involving multiple loci. Furthermore, the dynamics/ distributions of TEs and how these elements are shaping the evolution are well characterized in plant fungal pathogens such as *Zymoseptoria tritici* [31] and *Colletotrichum higginsianum* [32]. Although studies on TEs in many fungal plant pathogens are limited, they are essential as these elements have been associated with phenotypic changes [33]. Among the important demonstrated effects of TEs on genome structures, they may also contribute to fungicide resistance responses (e.g [34]).,. In a recent study conducted by [35], it has been shown that approximately 7% of the genomes of three prominent species of the *Monilinia* genus are constituted with TEs. Therefore, the first important step should be the discovery of TEs in a sample group of different species rather than a representative isolate, and then investigating their contribution to specific adaptation responses.

Exposure to an important selection factor such as a fungicide effect might result in genome structural changes, resulting in resistance responses, and experimental evolution studies in this direction are expected to be quite informative. Over the years, experimental evolution has proven to be a powerful approach for studying the genetic mechanisms and evolutionary dynamics involved in the development of fungicide resistance. For instance, the adaptation of a resistant genotype has been observed in the fungal pathogen *Fusarium graminearum* after continuous exposure to DMI-group fungicide tebuconazole [36]. In another experimental evolution study, continuous exposure to fungicide azoxystrobin in *M. fructicola* isolates was shown to increase the transposition rate of a transposon named Mftc1, which has 100% sequence similarity to the Mona element [34]. Experimental evolution scenarios could be very informative in understanding when and how fungicide resistance gains occur, especially in the pathogens determined to be susceptible to an antifungal as given in this research. Although there are many successful experimental evolution studies in bacteria and yeast groups [37–40], the number of examples in this direction in mycelial fungi is quite limited. The development and application of experimental evolution protocols based on mycelial transfer are also required to understanding many important fungal adaptations.

In this study, we aimed to investigate the content and evolutionary dynamics of TEs in two closely related fungal plant pathogen species, *M. fructicola* and *M. laxa* and to investigate the effect of TE on the resistance adaptation to propiconazole from the DMIs in the populations of these species. The pathogens for which we addressed research questions were populations from Türkiye. We have detailed characterizations and have examined many characteristics of these populations [18, 22, 41–43]. To achieve our research goals, detailed and multidimensional analyzes such as determination of TE content in the genome by intra- and inter-species comparative analyzes, experimental evolution for resistance adaptation by determining propiconazole phenotype, evaluation of resistance response with comparative genome analyzes, and confirmation of resistance gain by *CYP51* gene expression levels were carried out. The study provides both TE information for these important phytopathogens from evolutionary perspectives, new discoveries of the role of TE in fungicide resistance adaptations, and an important model study in understanding fungal adaptations with experimental evolutionary applications.

Methods

Fungal isolates and genome data used in the analyses

A total of 129 isolates consisting of 109 *M. fructicola* and 20 *M. laxa* isolates collected from peach orchards in six provinces located in five geographical locations of Türkiye were used. All these isolates were collected and described in our previous study, and detailed information on the sampling was provided by [18]. The pure original isolates are stored obtained on Whatman filter paper no. 1, at -20˚C. Thus, fresh samples are cultured from these stocks for any research questions to avoid changes in the isolates due to multiple subculturing procedures. Culture processes are carried out in potato dextrose agar (PDA) medium with 23˚C dark incubation. The whole isolate collection [18] was used for in vitro propiconazole fungicide susceptibility experiments as described below. Additionally, previously generated whole genome sequence data of nine *M. fructicola* isolates and nine *M. laxa* isolates [41] from the same collection of 129 isolates were used for all genomic analyses in this study. The genomic data of the isolates exhibit a notably high quality, and comprehensive information regarding the analyses of the genome data can be found in our prior publication [41]. In general, genomes of *M. laxa* presented an average of 155 contigs and an N50 length of 552 kilobases (kb), with total genome sizes ranging from 41.99 to 42.16 Mb. On the other hand, the genomes of *M. fructicola* isolates presented an average of 679 contigs and an N50 length of 131 kb, with genome sizes ranging between 43.84 and 44.51 Mb.

In vitro mycelium growth inhibition assay for propiconazole

Technical grade of demethylation Inhibitor (DMI) group fungicide propiconazole (≥98%, Sigma Aldrich Co.) was used. Stock solutions were stored at 4˚C and acetone was used as a solvent for propiconazole. Ten-day-old cultures were utilized as starting material for all in vitro fungicide sensitivity assays. All the in vitro mycelium growth-based fungicide sensitivity assays were conducted using PDA. The method by [22] was followed. In short, 20 representative isolates (11 *M. fructicola* and 9 *M. laxa*) were selected to calculate the mean concentration required for 50% inhibition dose (IC_{50}) for each species separately. The in vitro sensitivity assays involved the assessment of concentrations of 0.05, 0.1, 0.3, 0.5, 0.7 and 1 µg/ml for the representative collection. Using GraphPad Prism version 6.00 [44], the IC_{50} value for each isolate in the representative group was calculated, and mean IC_{50} values were evaluated separately for *M. fructicola* and *M. laxa.* The IC_{50} values, confidence intervals, and mean IC_{50} concentrations of propiconazole for the representative collection were presented in Supplementary Table S1. Subsequently, the discriminatory concentration (defined as the corresponding mean IC_{50} concentration) was applied to the entire collection, and the relative growth (RG) value was calculated for each isolate by comparing its growth on the control to its growth on the discriminatory concentration [(Average growth value on discriminatory concentration x 100)/Average growth value on control].

For the in vitro assays to screen the entire collection with the discriminative doses, 20 mL of medium was poured equally into each Petri dish, and mycelial plugs (1 cm in diameter) from 10-day-old cultures were transferred to the fungicide-amended PDA. Fungicide-unamended media served as the control for all in vitro assays, and each isolate/concentration was tested three times. Cultures were kept in the dark at 23˚C for 7 days, and mean colony diameters (excluding the size of the initial 1 cm inoculum) were measured in two perpendicular directions at the end of the 7th day. No significant differences ($P > 0.05$) were found between the replicates of in vitro mycelial growth inhibition assays based on one-way ANOVA test; thus, the mean values of the replicates were used for IC_{50} and relative growth (RG) calculation.All calculations for in vitro assays and sensitivity level determinations were performed as described in [22]. Finally, a total of 16 isolates (8 isolates for each species) were selected for all further genomic analyses based on their sensitivity levels. Of the isolates selected for each species, the 4 were highly sensitive and the 4 were sensitive to propiconazole.

Genome assemblies

The genome assemblies used in this study were obtained from a previous publication [41]. All assemblies including the reference-based genome assemblies of one isolate per species (Ti-B3-A3-2 for *M. fructicola* and Yıldırım-1 for *M. laxa*), as well as the assemblies for 16 selected isolates (8 *M. fructicola* and 8 *M. laxa*), originate from this prior publication. In short, the reference-based genome assemblies obtained using the "Reference-based Genome Assembly and Annotation Toolkit (RGAAT)" [45], were utilized to establish the best possible ungapped genome per species. These assemblies served as a benchmark for TE characterization and enabled more detailed analysis of Kimura-2 distances for all detected TEs. The 16 isolates, selected based on their sensitivity to propiconazole (4 highly sensitive and 4 sensitive per species, as described in the previous section), were analyzed to investigate TE content at the individual isolate level, with all 16 assemblies generated using SPADES version 3.11.1 [46].

Transposable element identification, classification, and annotation

The genomes of *M. fructicola* (isolate Ti-B3-A3-2) and *M. laxa* (isolate Yıldırım-1) were analyzed for transposable elements (TEs) and other repeats using the RepeatModeler and RepeatMasker pipelines. A *de novo* repeat library was generated for the two reference genomes using RepeatModeler version 2.0.2 [47], which integrates RECON [48], RepeatScout [49], and Tandem Repeat Finder [50]. The obtained repeats were checked for duplicates and merged with the Repbase library, followed by classification using the RepeatClassifier tool implemented in RepeatModeler. RepeatMasker version 4.1.2 (Smit, et al. 2015) was then employed to annotate the repeats and TEs, using the custom library with the -no_is, -a, and -s parameters and RM_BLAST search model. The Kimura divergence values and repeat landscape were calculated using RepeatMasker scripts. Subsequently, repeat annotation was conducted for 16 isolates that exhibited different propiconazole sensitivity (4 highly sensitive and 4 sensitive per species) utilizing draft genome assemblies generated by SPADES [46]. The same custom libraries and parameters, initially developed for the reference-based genome assemblies, were used for these analysis. The output file was filtered to select only interspersed repeats by removing "Simple repeats" and "Low Complexity" matches and was further processed with the Perl script "One code to find them all" [51].

Investigating the potential role of transposable elements in DMI-propiconazole resistance

The presence/absence of transposable elements (TEs) around the propiconazole target *CYP51* and non-target

ABC transporter genes were manually checked for all 16 isolates that exhibited different levels of sensitivity to propiconazole (4 highly sensitive and 4 sensitive per species) to investigate their potential role in DMI group fungicide propiconazole resistance. *CYP51* and *ABC* genes were obtained from NCBI (with GenBank Accession Numbers MT724702.1 (*M. fructicola*, *CYP51* gene); LT615209 (*M. laxa*, *CYP51* gene); AY077839.1 (*M. fructicola*, *ABC* transporter gene) and mapped to reference genomes to determine their scaffold and location. Since the ABC transporter gene is not annotated for *M. laxa*, the sequence from *M. fructicola* was used for mapping on *M. laxa*. The presence/absence of TEs at/or in the neighboring regions of *CYP51* and ABC transporter genes were then manually inspected on *de novo* genome assemblies using the outputs of "onecodetofindthemall" Perl script for each isolate separately.

Experimental evolution design

An experimental evolution was planned as directed evolution for propiconazole resistance adaptation. Propiconazole-susceptible isolate of *M. fructicola* (BG-B1-A17) was selected as it is a well-characterized isolate in our lab for experimental evolution application. This isolate was chosen not only to examine whether observed TE differences and other genetic variations are replicable under fungicide-induced selection but also to benchmark the feasibility of applying experimental evolution to study resistance mechanisms. This isolate was exposed to propiconazole at discriminative dose (0.22 ug/mL) for 10 transfer cycles. Each culture developed from a transfer was considered a "generation" in this study. For each transfer/generation, a 1 cm diameter mycelium from the outer edge was transferred every 10 days to a new PDA plate with and without propiconazole at the discriminative dose with three biological replicates for both control and fungicide-amended groups. The in vitro fungicide response phenotype was monitored across both control and propiconazole exposed generations. The fungicide response of the 10th generations was evaluated based on mycelial growth in propiconazole-amended media relative to the control across replicates according to the in vitro fungicide calculations given above. The 10th generation of the isolate exposed to directed evolution by the fungicide treatment was collectively named the evolved isolate, while those from the first original isolate of the isolate stored at -20 °C was named the ancestral isolate. While pre-existing variation may contribute to the design, the experimental design was intended to mimic natural selection under fungicide pressure, making the observed resistance an expected outcome. At the 10th generation, a single hyphal tip from evolved isolate was transferred onto Whatman No. 1 filter paper for preservation. These preserved strains were stored at a temperature of -20˚C, ensuring its long-term viability for future investigations and reference. Our experimental evolution design was also presented visually in Fig. 1.

Whole genome sequencing and data processing for the evolved and ancestral isolates

Total genomic DNA was extracted from ancestral and evolved isolates using the Norgen Plant/Fungi DNA isolation kit (Norgen, Canada) from mycelium grown in 50 mL of potato dextrose broth (PDB) in a rotary shaker at 150 rpm at room temperature, following the manufacturer's protocol. The DNA concentrations were determined using a Qubit v. 3.0 fluorometer (Thermo Fisher Scientific, U.S.A.). Sequencing was performed by Macrogen Inc., Sequencing Service (South Korea) using the Illumina TruSeq Nano Library construction kit with a 350 bp insert size, followed by Illumina NovaSeq 6000 platform with 2 × 150 bp paired-end sequencing. Removal of low-quality reads and adapters for Illumina reads was accomplished using Trimmomatic version 0.36 [52] with the following parameter settings: "ILLUMINACLIP: TruseqHT.fa:2:30:10 LEADING:10 TRAILING:10; SLIDINGWINDOW:5:20; MINLEN:151". Quality statistics of the fastq files were assessed using [53].

Transposable element content dynamics and the investigation of transposable element DMI-propiconazole resistance for ancestral and evolved isolates

De novo genome assembly, transposable element identification, classification, and annotation were conducted, to elucidate the dynamics of transposable element (TE) content in the ancestral and evolved BG-B1-A17 isolate.

Subsequent to the analyses mentioned above, an examination was performed to assess the presence or absence of transposable elements (TEs) within the genome sequences of both ancestral and evolved isolate, specifically targeting the propiconazole-targeted *CYP51* gene and the non-target ABC transporter genes. The identical approaches and pipelines were employed as described above related section.

CYP51 gene expressions in the Propiconazole Resistant Evolved isolate and in the isolates with different Propiconazole susceptibility levels

To assess the potential impact of the identified nested transposable element, as explained in the Results, on the expression level of the *CYP51* gene, we conducted expression analysis on four *M. fructicola* isolates. This included two isolates from the experimental evolution set: the ancestral isolate lacking the TE and the evolved isolate containing the TE. Additionally, we examined two other isolates from the fungicide sensivity assay dataset: BG-B3-A1, a highly sensitive isolate lacking the TE, and YK-1, a less sensitive isolate harboring the TE upstream

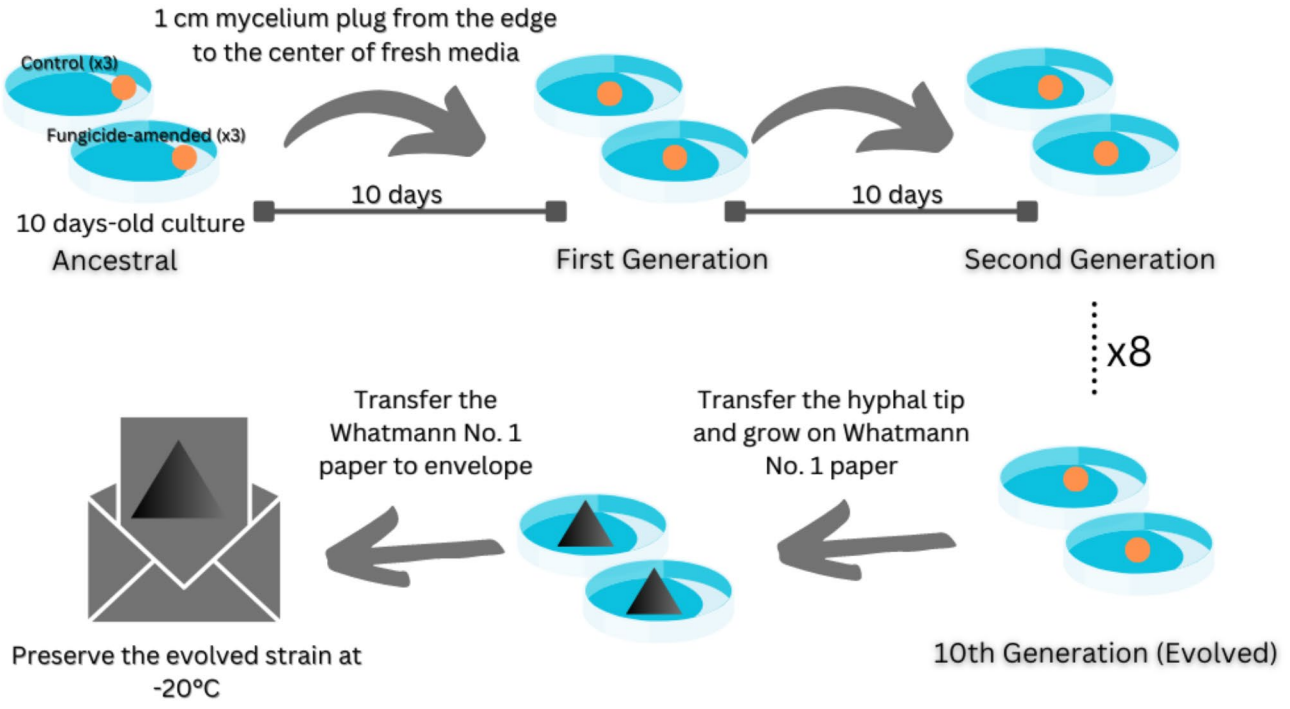

Fig. 1 Illustration of the experimental evolution assay used to investigate fungicide resistance in *Monilinia fructicola* and *Monilinia laxa* isolates. The figure demonstrates the successive generations of the isolates exposed to propiconazole at the discriminative dose, with hyphal transfer to new Petri dishes and preservation of the evolved strain at -20 degrees Celsius at the 10th generation

of the *CYP51* gene. Overall, two of the isolates lacked the nested transposable element at the upstream region of the *CYP51* gene, while the remaining two isolates harbored this element. Mycelial plugs were obtained from 10-day-old PDA cultures of isolates and transferred to fresh 50 mL of PDB in a rotary shaker at 150 rpm. For RNA isolation, mycelia grown in PDB for 7 days were subjected to propiconazole at the pre-defined discriminative dose for *M. fructicola* (0.22 ug/mL) for 1 h and collected and ground in liquid nitrogen using a mortar and pestle for each sample seperately. The frozen tissues were then homogenized in TRizol reagent and centrifuged at 14,000 rpm for 1 min to separate the cell debris. Phase separation was achieved using chloroform. The samples were precipitated in isopropanol at 80 °C for 24 h, washed with pure ethanol, and diluted in RNAse-free water. RNA quantification was performed using the Qubit v. 3.0 fluorometer (Thermo Fisher Scientific, U.S.A.). Subsequently, cDNA was synthesized from 15 µg of total RNA using the GoScript Reverse Transcription System (Promega, USA) following the manufacturer's instructions. The qRT-PCR was carried out using the GoTaq qPCR Master Mix (Promega, USA) in accordance with the manufacturer's instructions. *RealCYPF2/R2* primer pair, developed by [24], were used to target the *CYP51* gene in our selected isolates. For normalization, *Beta-Mfactin-F/R* primers designed by [54] were used to target the beta-actin gene. The experiment was conducted with three biological replicates and three technical replicates in each qRT-PCR run. Relative *CYP51* gene expression levels were determined using the comparative CT method ($2^{-\Delta\Delta CT}$) [55]. To assess the impact of the nested transposable element (TE) in the upstream region of the *CYP51* gene, isolates lacking the TE and exhibiting extreme sensitivity to propiconazole were utilized as controls.

Results

Propiconazole sensitivity of the *Monilinia* populations

The IC_{50} values for a geographically representative population of *M. fructicola* isolates ranged from 0.15 to 0.29, while the range for *M. laxa* isolates was between 0.10 and 0.19 (Supplementary Table S1). The representative group for each species was formed by selecting isolates from each sampled city to ensure geographic representation of the population. The mean IC_{50} values, which served as discriminatory doses, were 0.22 and 0.15 ug/ml for *M. fructicola* and *M. laxa*, respectively. These doses were then applied to the entire collection of each species.

A total of 129 *Monilinia* isolates obtained from peach fruits in Türkiye were assessed for sensitivity to propiconazole, and the results showed that over 90% of the isolates were sensitive, with an RG (Relative Growth) value < 50 for both *M. fructicola* and *M. laxa*. The RG values for *M. fructicola* ranged from 3.33 to 69.61, and from 5 to 33.33 for *M. laxa*, with median values of 24.67 and 25.85, respectively. Isolates with RG values above the

Table 1 Relative growth values and resistance levels of selected isolates based on in vitro mycelial growth inhibition assay against propiconazole for both species. (HS: highly sensitive; S: sensitive)

Species	Isolate Code	Relative Growth	Resistance Level
M. fructicola	BG-B3-A1	3.33	HS
	SC-B2-A3	12.27	HS
	SC-B2-A4	18.94	HS
	B5-A4	21.81	HS
	T-B1-A5	28.00	S
	YK-1	29.13	S
	BG-B1-A8	39.59	S
	BO-B3-A1	47.67	S
M. laxa	2B1-A2-2	5.00	HS
	Ni-B3-A2	18.46	HS
	MM-B4-A4	20.90	HS
	MT-B1-A3-1	21.73	HS
	T-B1-A4-2	28.33	S
	MM-B2-A2	30.47	S
	2B1-A5	32.25	S
	MM-B4-A3	33.33	S

median were classified as "Sensitive," while those below the median were classified as "Highly Sensitive." Based on this classification, 50 *M. fructicola* isolates were "Highly Sensitive," and 58 were "Sensitive," while the sensitivity levels of *M. laxa* isolates were equally distributed, with 10 "Sensitive" and 10 "Highly Sensitive" isolates. The RG values and resistance levels (RL) for the entire collection is presented in Supplementary Table S2.

To ensure equal representation of resistance status in downstream genomic analyses for both *M. fructicola* and *M. laxa*, eight isolates per species were selected based on their RG value and resistance level. Specifically, four isolates with high RG values and a "Sensitive" resistance status, and four isolates with low RG values and a "Highly Sensitive" resistance status were chosen for each species. The RG values and resistance level status of the selected isolates were presented in Table 1.

Transposable element content and their dynamics in *Monilinia* species

Based on the reference genomes, total transposable element (TE) length for *M. fructicola* and *M. laxa* was 3.94 Mb (8.97% of the genome) and 4.24 Mb (9.92% of the genome), respectively. A total of 15,327 and 10,710 TE elements were identified for *M. fructicola* (isolate Ti-B3-A3-2)and *M. laxa* (isolate Yıldırım-1), respectively. Class I elements were more abundant in comparison to Class II elements for both species (Table 2). While these summary statistics provide a general overview of the TE composition, a large portion of elements remained unclassified.

The majority of the TE content was composed of LTR retrotransposons, with *Bel-Pao*, *Ty1-Copia*, *Gypsy-Dýrs1*, and retrotransposon families being the most prevalent. For both species *Gypsy* was the most abundant family among the LTRs, followed by *Copia* and *LINE L2-CR1-Rex* families, for *M. fructicola* (Supplementary Table S3) and *M. laxa* (Supplementary Table S4). Class II elements were present at low levels for both species, with hobo-Activator being the most common family of Class II/DNA transposons. Additionally, the number of *SINE* elements was the lowest of them all, with only 30 and 35 elements found in *M. fructicola* and *M. laxa*, respectively, accounting for 0.004% and 0.005% of the genome. The details about transposable element (TE) contents and their extents for *M. fructicola* and *M. laxa* were shown in Supplementary Table S3 and Supplementary Table S4, respectively.

The TE content within and between *M. fructicola* and *M. laxa* species exhibited high similarity, with an average of 9.29% (15809 elements) and 8.59% (10401 elements) of the genomes comprising TEs, respectively. In addition to TE identification in reference genomes, TEs were identified among all studied isolates (Table 3), though only the total number of Retrotransposons and DNA Transposons are shown in this table. TE contents within the species were similar to those seen in their reference genomes and showed intraspecies similarity.

Furthermore, we produced divergence landscapes of TEs based on the reference genomes for both *M. fructicola* and *M. laxa* (Fig. 2). These landscapes reflect the amount of divergence of each TE copy, assuming neutral evolution upon insertion into the genome. As older copies accumulate more substitutions, this approach allows for the estimation of time since duplication. Thus, these

Table 2 Classification, number, length, and relative representation of transposable elements in reference genomes of *Monilinia fructicola* (isolate Ti-B3-A3-2) and *Monilinia laxa* (isolate Yıldırım-1)

Class	M. fructicola			M. laxa		
	Number of Elements	Length Occupied (bp)	% of the Genome	Number of Elements	Length Occupied (bp)	% of the Genome
Class I (Retrotransposons)	4642	647,936	1.47	4627	2,288,342	5.35
Class II (DNA Transposons)	3508	247,489	0.56	2676	159,784	0.38
Unknown	7177	3,065,317	6.96	3407	1,807,607	4.22
TOTAL	**15,327**	**3,948,425**	**8.97**	**10,710**	**4,244,622**	**9.92**

Table 3 Transposable element content in selected *Monilinia fructicola* and *Monilinia laxa* isolates based on the number and percentage of the genome occupied

Species	Isolate Code	Retrotransposon		DNA Transposon			
		Number of Elements	Percent of Genome	Number of Elements	Percent of Genome	Total Number of Elements	Percent of Genome
M. fructicola	B5-A4	4744	1.40	3424	0.53	15,657	9.20
	BG-B1-A8	4778	1.59	3347	0.52	16,119	9.67
	BG-B3-A1	4768	1.45	3353	0.54	15,624	9.09
	BO-B3-A1	4747	1.56	3366	0.54	15,684	9.28
	SC-B2-A3	5044	1.70	3386	0.52	16,099	9.45
	SC-B2-A4	4741	1.52	3318	0.51	15,833	9.31
	TB1-A5	4816	1.55	3353	0.52	15,819	9.33
	YK-1	4810	1.50	3443	0.55	15,637	9.02
	Average	**4806**	**1.53**	**3373**	**0.52**	**15,809**	**9.29**
	2B1-A5	4569	4.62	2507	0.35	10,412	8.88
	2B1-A2-2	4512	4.48	2492	0.35	10,311	8.51
M. laxa	MM-B2-A2	4532	4.25	2548	0.36	10,394	8.56
	MM-B4-A3	4504	4.41	2524	0.35	10,341	8.67
	MM-B4-A4	4546	4.39	2588	0.36	10,457	8.58
	MT-B1-A3-1	4519	4.35	2556	0.36	10,389	8.53
	NI-B3-A2	4557	4.29	2553	0.36	10,442	8.50
	TB1-A4-2	4604	4.32	2545	0.36	10,469	8.54
	Average	**4542**	**4.38**	**2539**	**0.35**	**10,401**	**8.59**

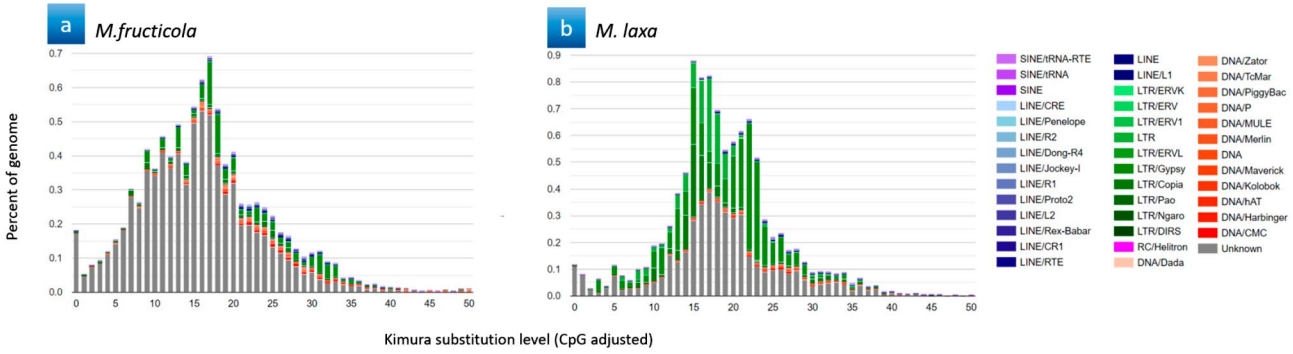

Fig. 2 Repeat landscape plots and TE burst events in (**a**) *M. fructicola* (isolate Ti-B3-A3-2) and (**b**) *M. laxa* (isolate Yıldırım-1). The sequence divergence of each transposable element (TE) copy from the consensus sequence is quantified using Kimura-2 distance. TE insertions positioned towards the left indicate more recent insertion events

TE landscapes offer insight into the sequence divergence of each detected TE, their inferred ancestral copy, and the evolutionary history of TE insertions within these species. For both species, the majority of TE burst events were found to be old, with Kimura-2 distance values between 10 and 30, and recent insertions with Kimura-2 distance < 10 were relatively low. However, *M. fructicola* showed relatively younger TE burst events, and the TE insertion events appeared more dynamic compared to *M. laxa* based on the distribution of the Kimura-2 distances (Fig. 2). *LTR* elements predominantly comprised the majority of TE burst events. Furthermore, based on out manual investigations of the distances, recent insertions, i.e., TE copies with zero divergences, were found to be composed of *LTR-Copia* and *Pao*, *LINE-Jockey* elements for both species. The oldest insertions into the genomes were found to be from the Class I/*SINE* and Class II/*Tc-Mariner* elements for both species. It should be noted that identifying specific TE families for both *M. fructicola* and *M. laxa* remains challenging due to the limited number of known reference genomes, which makes accurate classification of TEs more difficult.

Transposable element Dynamics through Experimental Evolution

During the course of experimental evolution, we investigated the dynamics of transposable elements (TEs) in *M. fructicola*. The ancestral isolate, BG-B1-A17, which was subjected to the DMI fungicide propiconazole at a

Table 4 Transposable element (TE) content changes during experimental evolution: comparing the observed changes in TE content from the ancestral isolate to the evolved isolate. Categorization of TE content based on TE class and order, including count of each TE category. Positive values indicate an increase, while negative values indicate a decrease in TE count

TE Class	TE Order	Count in Ancestral Isolate	Count in Evolved Isolate	Change in Count
Class I	SINE	43	35	-8
	Penelope	283	282	-1
	LINEs	1967	1954	-13
	LTRs	2806	2813	+7
Class II	Hobo-Activator	479	477	-2
	Tc1-IS630-Pogo	400	388	-12
	PiggyBac	48	44	-4
	Tourist/Harbinger	106	121	+15
	Other (Mirage, P-element, Transib)	61	63	+2
Rolling Circles		247	256	+9
Unclassified		7521	7715	+194
Total		13,961	14,148	+187

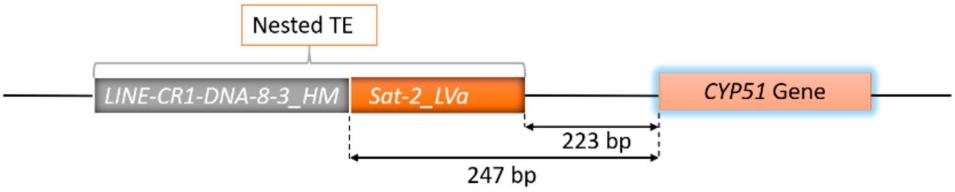

Fig. 3 Illustration of the discovered nested transposable elements at the upstream region of the *CYP51* gene of less sensitive *M. fructicola* isolates. The nested transposable elements, *LINE-CR1-DNA-8-3_HM* (Class II) and *Sat-2-LVa* (Class I), are depicted in relation to the upstream region of the *CYP51* gene. The distances of the nested transposable elements from the upstream of the *CYP51* gene are indicated

discriminative dose for 10 generations, exhibited notable changes in TE content.

Overall, the total interspersed repeats showed a slight increase, with 13,961 TEs in the ancestral isolate compared to 14,148 TEs in the evolved strain. When considering the proportion of TE content relative to the ancestral genome, we observed a TE expansion of 0.18%, representing a net 101,860 bp increase in the evolved strain. This expansion was accompanied by the total net change of 187 TE elements with addition of 227 TEs and removal of 40 TEs including both known and potentially novel TE classes.

Upon further classification analysis, we observed interesting changes in specific TE classes. While the number of unclassified elements increased by 194, indicating potential diversification or acquisition of novel TE sequences, the majority of these changes occurred in elements whose functions and classifications remain unknown. Furthermore, compared to the ancestral isolate, a total of 15 retroelements and 1 DNA transposon reduction were detected in the evolved isolate, while a total of 9 increased in rolling circle elements. Although the evaluation of whether these elements are autonomous and active was not conducted, the changes observed in TE content still point to a successful experimental evolution, reflecting the dynamic nature of TEs in response to fungicide exposure. The changes in TE contents, which occurred as a result of the experimental evolution, were presented in detailed, together with the TE categories, in Table 4.

Detected Nested-TE associated with DMI-Propiconazole Resistance response for *M. Fructicola*

TE investigations near the *CYP51* gene revealed a TE insertion at the upstream region of the *CYP51* gene in three out of eight *M. fructicola* isolates. Among these, two isolates (T-B1-A5 and YK-1), which were the least sensitive to propiconazole, had a nested TE consisting of Class I/Sat-2_LVa and Class II/LINE-CR1-DNA-8-3_HM elements located 223 bp upstream of the *CYP51*gene (Fig. 3). In contrast, no TE was found at or near the *CYP51* gene in any *M. laxa* isolates. Nested TE occur when a TE is inserted into a pre-existing TE within the genome [56].

The presence or absence of the TE was not consistently distributed, as it was not exclusively present in all four less sensitive isolates or absent in all four highly sensitive isolates. The same nested TE was detected in one of the highly sensitive *M. fructicola* isolate (SC-B2-A4) in addition to being found in two of the least sensitive isolates (T-B1-A5 and YK-1). Moreover, this TE was absent in a sensitive *M. fructicola* isolate (BO-B3-A1). To predict the order of insertions for the nested TE, we manually investigated the Kimura-2 distances of

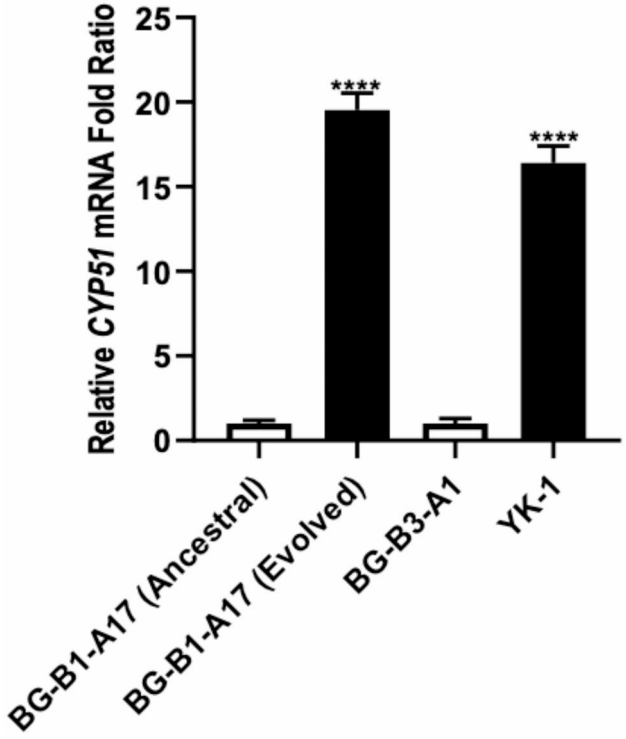

Fig. 4 Relative expression levels of the *CYP51* gene in isolates with the nested transposable element (TE) at the upstream region (BG-B1-A17 (Evolved) and YK-1) compared to isolates lacking the TE (BG-B1-A17 (Ancestral) and BG-B3-A1) used as controls. The significance of the p-value is denoted on the histogram ($P < 0.0001$)

LINE-CR1-DNA-8-3_HM and *Sat-2_LVa* elements. Class II/*LINE-CR1-DNA-8-3_HM* showed a relatively lower Kimura-2 distance to its consensus sequence (19.98) compared to the Class I/*Sat-2_LVa* element [23], suggesting that the *LINE* element was inserted into the other element later. These values were derived directly from the TE-specific Kimura-2 distances, which are calculated as part of he general Kimura-2 distance landscape for the reference genome. Other highly sensitive *M. fructicola* isolates (B5-A4, BG-B3-A1, and SC-B2-A3) did not have a TE present at/near the *CYP51* gene. Furthermore, through investigations of the experimental evolution isolates, it was observed that the ancestral isolate of *M. fructicola* lacked the transposable element (TE) identified near the *CYP51* gene, whereas the evolved isolate of *M. fructicola* carried this TE insertion. This nested insertion, consisting of two TEs, occurred consistently with no isolates showing only a single TE insertion. Additionally, no TE was detected at or near the ABC transporter gene for both species.

The Effect of the Nested-TE on *CYP51* gene expression

The gene expression analysis revealed significant differences in the expression levels of the *CYP51* gene associated with the presence of the nested transposable element. Comparing isolates, the BG-B1-A17 (evolved) isolate exhibited a remarkable 19.54-fold increase in *CYP51* gene expression compared to the TE-lacking BG-B1-A17 (ancestral) isolate. Similarly, the YK-1 isolate demonstrated a substantial 16.41-fold increase in *CYP51* gene expression compared to the TE-lacking BG-B3-A1 isolate. The differences in fold changes of expression levels were assessed using one-way ANOVA, and statistically significant differences ($P < 0.0001$) were observed both between the BG-B1-A17 ancestral and evolved isolates, as well as between the BG-B3-A1 and YK-1 isolates in terms of relative expression values. Across the experimental evolution isolates and independent in vitro assays, the presence of the nested TE consistently correlated with elevated *CYP51* expression when compared to isolates lacking the TE. This substantial level of change provides strong support for a potential regulatory role of this nested TE. Nevertheless, additional functional studies are required to establish causation conclusively. The corresponding results have been visually depicted in Fig. 4, illustrating the statistically significant differences in fold changes of expression levels between the studied isolates.

Discussion

For decades, fungicides have been a crucial tool in controlling plant diseases caused by fungal pathogens. To develop effective disease management strategies, it is essential to have knowledge of the resistance status of fungal populations against fungicides in view of evolution. While studies have been mainly focused on genome-wide SNPs to elucidate the genetic mechanisms of fungicide resistance so far, more research is needed to fully understand the complexity of this phenomenon. The determination of TE contents in fungal genomes, which stand out with their highly dynamic structures, illuminates many adaptation responses. For the first time, we have defined the sensitivity level of our population, consisting of 109 *M. fructicola* and 20 *M. laxa* isolates from five different regions in Türkiye, against the DMI group fungicide propiconazole. Our novel method, developed previously [22], was used to determine the sensitivity level of the population, which was found to be sensitive to propiconazole. In addition to determining the in vitro sensitivity levels of the population and genome-wide variant characterization, we examined the potential role of transposable elements at or near selected genes in DMI resistance for both species and for isolates with different sensitivity levels.

A comprehensive bioinformatics pipeline was utilized to investigate the TE content and dynamics in the two important fungal phytopathogens of *Monilinia* species. A combinational approach was employed for TE detection/annotation in the genomes of *M. fructicola* and

M. laxa. Initially, a *de novo* library was constructed for the reference assemblies using RepeatModeler version 2.0.2 [47]. RepeatModeler's RECON [48] utilizes whole-genome alignments to detect and classify repeats *de novo*, while RepeatScout [49] in RepeatModeler utilizes a k-mer based approach to identify repetitive sequences in the genome. Since neither approach requires a library, novel repeats are also discovered through RepeatModeler [47]. Subsequently, RepeatClassifier, a program implemented in RepeatModeler, is used to classify the elements in a custom library that combines the *de novo* repeat library and Repbase library. This custom library is then subjected to RepeatMasker version 4.1.2 (Smit, et al. 2015), which uses a library of previously characterized repetitive elements in a similarity-based manner. Despite RepeatClassifier's use of an extensive homology-based approach, a large proportion of the identified elements remained unclassified (7177 and 3407 elements for *M. fructicola* and *M. laxa* reference genomes, respectively), possibly due to insufficient representation of fungal transposable elements in the RepBase library used. On the other hand, it is also possible that the unclassified TEs in both *M. fructicola* and *M. laxa* are novel repetitive sequences. While there are several approaches available for the manual curation and classification of TEs, these methods require a high level of expertise in TE biology. Despite this, over 15 TE families, representing approximately 9% of the genome, were successfully curated for both species. Class I/Retrotransposons, which replicate via a copy-paste mechanism, are considered major contributors to repetitive sequences across the tree of life, and in both *M. fructicola* and *M. laxa*, these elements (especially *LTR* elements) comprise the largest fraction of the genome. In other fungal pathogens, Class I elements have been associated with genome size expansion. For instance, the genome of *Blumeria graminis*, which mainly consists of *LTR* elements, is four times larger than an average ascomycete genome [57], while two *LTR* families make up 29% of the *Phytophthora infestans* genome, a representative oomycete [58]. Additionally, Kimura-2 distances of TEs in the reference genomes of both species revealed that most insertions/proliferations are relatively old, and the number of recent insertions is relatively low. Consistent with the literature, almost all TE burst events mainly consist of *LTR* elements. However, the exact role of LTRs in evolutionary processes remains uncertain due to the complexity of their dynamics and interactions with host genomes. Further investigations are needed to better understand their contribution to future evolutionary processes in both species. After determining the genome structures of the two important fungal phytopathogens of *Monilinia* species in detail [41], the TE information we obtained with this study allowed us to explain DMI-Propiconazole responses.

Beyond the agricultural assessment of propiconazole use specifically, the finding of pathogen populations as sensitive to a particular fungicide and our knowledge on the genome structures and TE contents of these pathogens has opened a very useful door for digging for fungicide resistance adaptation. Thus, an experimental evolution approach was designed to direct propiconazole resistance adaptation for the first time. The development and application of this method allowed us to demonstrate the feasibility of applying experimental evolution to study genome-level changes under fungicide pressure. While additional biological replicates and a broader analysis of isolates would strengthen the findings, this study showcases the utility of experimental evolution for observing genome changes, particularly as a complement to standing genetic variation with directed evolution under fungicide selection. Experimental evolution provides a powerful tool for studying evolutionary processes and adaptations in real-time. By subjecting *M. fructicola* to controlled selection pressures, we were able to observe and analyze the changes in their phenotypic and genotypic characteristics over successive generations. The results obtained through our experimental evolution approach demonstrated the efficacy of the method in generating phenotypic variations related to sensitivity to DMI fungicides. Specifically, we were able to derive an evolved strain from an ancestral form, representing different levels of sensitivity. This outcome serves as a proof-of-concept for the feasibility of using experimental evolution to study fungicide resistance in *Monilinia* species. The successful application of experimental evolution in this study opens up new avenues for future investigations in the field of fungicide resistance. Other researchers and scientists can adopt and employ this method to study the evolution of resistance in *Monilinia* species or other mycelial fungi.

The dynamics of TE content observed during the experimental evolution of *M. fructicola* provide valuable insights into the genetic changes and adaptive mechanisms underlying the response to fungicide pressure. The overall increase in total interspersed repeats and the expansion of TE content in the evolved strain suggest the potential role of TEs in promoting genomic plasticity and facilitating adaptation processes. The additional 101,860 bp of TE sequences and the net increase of 187 TEs in the evolved strain indicate a significant TE expansion during the experimental evolution. Interestingly, the changes in specific TE classes revealed contrasting patterns. While the number of unclassified elements increased, potentially suggesting the acquisition or diversification of novel TE sequences, there was a decrease in retroelements, DNA transposons, and an increase in rolling circle elements. The content change in retroelements, DNA transposons, and rolling circle elements suggest

potential genetic rearrangements, deletions, or inactivation events during the experimental evolution. However, as this study focused on changes in TE numbers, we did not analyze these structural changes in detail. Future studies incorporating structural variant analyses could provide deeper insights into the mechanisms driving these changes. Such changes in TE composition and content may reflect the genomic responses to selective pressures and the fine-tuning of TE-mediated processes in *M. fructicola*. The observed dynamics of TEs during experimental evolution, including their increase, decrease, and potential de-repression, align with previous findings that highlight the capacity of host organisms to regulate and modulate TE activity. Despite the host's mechanisms of inactivation and repression, studies have demonstrated the potential for TE de-repression, suggesting that these elements can still play a significant role in shaping the genetic and adaptive landscape of fungal pathogens [59]. Moreover, this TE dynamics during experimental evolution support the notion that TEs play a dynamic and active role in shaping fungal genomes under environmental stresses [59]. The acquisition and loss of TE sequences can contribute to genomic diversity and facilitate rapid adaptation to changing conditions. The TE-mediated genomic changes observed in the evolved strain may have functional consequences, potentially impacting gene regulation, expression, or other important biological processes.

Moreover, we explored the effect of the certain nested TE in DMI resistance. Interestingly, through our investigation, we detected a nested TE in the upstream region of the *CYP51* gene in two *M. fructicola* isolates displaying a less sensitive phenotype, while it was absent in the remaining six isolates. Furthermore, the examination of experimental evolution isolates supported this finding with a noteworthy distinction between the ancestral isolate, which lacked the identified TE, and the evolved isolate, which exhibited the presence of this TE insertion. These findings suggest a potential association between the presence of the nested TE and reduced sensitivity to DMI fungicides in *M. fructicola*. Based on these findings, we investigated the impact of a nested transposable element (TE) on the expression levels of the *CYP51* gene in *M. fructicola* isolates and our results revealed a significant difference in *CYP51* gene expression between TE-containing and TE-lacking isolates. Specifically, the TE presence was associated with a substantial increase in *CYP51* gene expression, suggesting a potential role of this element in modulating gene expression and potentially contributing to fungicide resistance. The identification of this nested TE in relation to DMI resistance adds valuable insights to our understanding of the molecular mechanisms underlying fungicide resistance in *Monilinia* species. The findings of our study align with previous research indicating the influence of genetic elements upstream of the *CYP51* gene on resistance. Notably, the "Mona" element has been associated with increased *CYP51* gene expression and resistance to DMI group fungicides in *M. fructicola* [26]. It is worth mentioning that the "Mona" element has also been found in sensitive *M. fructicola* isolates, indicating the complexity of the resistance mechanisms in this species [25]. Also, no Mona element was found in any of the genomes we studied, nor was it detected when we scanned the entire population for this element (data not shown). It is possible that population structure differences (fungicide history, standing or *de novo* variations, or different TE contents) may trigger different resistance responses at the population level within the species against to the fungicide having the same mode of action. Considering this expectation for the different responses to the same fungicide even at the same species level, it is not surprising that our newly discovered element is not found in *M. laxa*, or that the resistance response has not been as elucidated as in *M. fructicola*.

We proved that a nested-TE has a direct effect on propiconazole adaptation in *M. fructicola*, as demonstrated by the TE insertion observed in our evolved lab-isolate and the differential expression levels of the *CYP51* gene in TE-containing and TE-lacking isolates. However, it is important to acknowledge that gene expression changes alone may not fully explain resistance phenotypes, as resistance is often a complex trait involving multiple loci and mechanisms. Future studies should aim to unravel the precise regulatory mechanisms by which the nested TE influences *CYP51* gene expression. Investigating the interactions between the TE and other regulatory elements in the upstream region of the *CYP51* gene could provide valuable insights into the functional consequences of TE presence. Additionally, genome-wide investigations would be beneficial to comprehensively explore the fungicide resistance mechanisms in *Monilinia* species.

Conclusion

Overall, these findings contribute to our understanding of the complex mechanisms underlying fungicide resistance in *Monilinia* species. Further investigations are warranted to elucidate the precise regulatory mechanisms and to comprehensively explore the resistance mechanisms from a genome-wide perspective. Such knowledge will aid in the development of effective strategies to manage fungicide resistance and ensure sustainable disease control in agricultural systems. In addition, we also introduced and demonstrated the utility of experimental evolution as a valuable approach for studying fungicide resistance in *Monilinia* species. By showcasing the effectiveness of this method and its potential for

future investigations, we hope to inspire further research in the field and facilitate advancements in our understanding of the evolution and management of fungicide resistance.

Abbreviations

ABC	ATP-binding cassette
CT	Cycle threshold
CYP51	Cytochrome P450 51
DMI	Demethylation inhibitor
IC_{50}	Inhibitory concentration 50%
LINE	Long interspersed nuclear element
LTR	Long terminal repeat
MBC	Methyl benzimidazole carbamate
MOA	Mode of action
RG	Relative growth
TE	Transposable element

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions
M.R.D. conducted the laboratory experiments, performed data analyses, and drafted the manuscript with guidance from H.O. H.O. supervised the research, provided recommendations for data analysis, and made substantial contributions to all aspects of the manuscript. All authors critically reviewed, discussed, and approved the final version of the manuscript.

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Data availability
All data generated and utilized in this study have been deposited in NCBI GenBank with the BioProject accession number PRJNA846280. The whole genome sequencing data for the nine M. fructicola isolates used in this study are available in NCBI GenBank under the SRA accession numbers SRS13297834, SRS13297843, SRS13297853, SRS13297830, SRS13297847, SRS13297850, SRS13297832, SRS13297851, and SRS13297852, accompanied by their respective isolate names. Similarly, the whole genome sequencing data for the nine M. laxa isolates employed in this study can be found in NCBI GenBank under the SRA accession numbers SRS13297846, SRS13297836, SRS13297837, SRS13297838, SRS13297840, SRS13297841, SRS13297843, SRS13297844, and SRS13297845, along with their isolate names.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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