Pan-Mitogenomics Approach Discovers Diversity and Dynamism in the Prominent Brown Rot Fungal Pathogens

Gozde Yildiz1 and Hilal Ozkilinc1,2*

1 School of Graduate Studies, MSc Program in Biomolecular Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey; 2 Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Monilinia fructicola and Monilinia laxa species are the most destructive and economically devastating fungal plant pathogens causing brown rot disease on stone and pome fruits worldwide. Mitochondrial genomes (mitogenomes) play critical roles influencing the mechanisms and directions of the evolution of fungal pathogens. The pan-mitogenomics approach predicts core and accessory regions of the mitochondrial genomes and explains the gain or loss of variation within and between species. The present study is a fungal pan-mitogenome of M. fructicola (N = 8) and M. laxa (N = 8) species. The completely sequenced and annotated mitogenomes showed high variability in size within and between the species. The mitogenomes of M. laxa were larger, ranging from 178,351 to 179,780bp, than the mitogenomes of M. fructicola, ranging from 158,607 to 167,838bp. However, size variation within the species showed that M. fructicola isolates were more variable in the size range than M. laxa isolates. All the mitogenomes included conserved mitochondrial genes, as well as variable regions including different mobile introns encoding homing endonucleases or maturase, non-coding introns, and repetitive elements. The linear model analysis supported the hypothesis that the mitogenome size expansion is due to presence of variable (accessory) regions. Gene synteny was mostly conserved among all samples, with the exception for order of the rps3 in the mitogenome of one isolate. The mitogenomes presented AT richness; however, A/T and G/C skew varied among the mitochondrial genes. The purifying selection was detected in almost all the protein-coding genes (PCGs) between the species. However, cytochrome b was the only gene showing a positive selection signal among the total samples. Combined datasets of amino acid sequences of 14 core mitochondrial PCGs and rps3 obtained from this study together with published mitochondrial genome sequences from some other species from Heliotales were used to infer a maximum likelihood (ML) phylogenetic tree. ML tree indicated that both Monilinia species highly diverged from each other as well as some other fungal species from the same order. Mitogenomes harbor much information about the evolution of fungal plant pathogens, which could be useful to predict pathogenic life strategies.

Keywords: pan-mitogenomics, mitogenome, evolution, Monilinia species, brown rot
INTRODUCTION

Fungi are one of the most remarkable and diverse kingdom, with approximately 720,256 species compared with other eukaryotic organisms worldwide (Blackwell, 2011; Badotti et al., 2017). Fungal genomics exhibit important data for studies of adaptive behavior and evolutionary research due to their highly dynamic and fast-evolving features. High-throughput sequencing technologies have allowed for sequencing the tremendous number of fungal nuclear genomes across many species, with a total of 2,5997. However, relatively limited data is available for whole mitochondrial genomes. For instance, only 793 mitochondrial genomes have been announced by NCBI Organelle Genome Resources2. Mitochondrial genomes present valuable information to explain both adaptive traits and the evolution of pathogens. Fungal mitochondrial genes can be targeted for plant disease management (Medina et al., 2020) and provide specific markers for population studies, as well as species diagnosis (Santamaria et al., 2009). Furthermore, mitogenome data contribute to expand information of fungal phylogenetics (Chen et al., 2019; Nie et al., 2019; Li Q. et al., 2020). Fungal mitochondrial genomes consist of highly conserved proteins and RNA encoding genes related to respiration and translation processes (Aguilera et al., 2014; Franco et al., 2017). Moreover, the presence of mitochondrial-encoded ribosomal protein genes, such as rps3, and its homologs (var1 and SS) differs among fungal groups, and these genes may have been transferred to the nuclear genomes in different eukaryotic species (Bullerwell et al., 2000; Smits et al., 2007; Sethuraman et al., 2009; Korovesi et al., 2018; Yildiz and Ozkilinc, 2020). Furthermore, copy number, gene duplications, gain/loss of introns, and transposable and repetitive elements are the main factors causing mitogenome variations (Basse, 2010; Aguilera et al., 2014). Because of these factors, mitogenome sizes may vary within and among fungal taxonomic groups (Burger et al., 2003). Recent studies showed that homing endonucleases, such as GIY-YIG and LAGLIDADG families, play a significant role in shaping fungal genome structure and contribute to variations within and between species (Sandor et al., 2018; Kolesnikova et al., 2019; Yildiz and Ozkilinc, 2020).

Fungal mitogenomes have been evaluated for genomic features (Li Q. et al., 2020), comparative mitogenomics (McCarthy and Fitzpatrick, 2019), and pan-mitogenomics (Brankovics et al., 2018). The pan-genomic approaches, using a comparative genomics-based methodology to identify the core and accessory genomes or genomic regions, were applied on bacterial genomes at first (Tettelin et al., 2008). Core genomes tend to be conserved among strains, such as many housekeeping genes involved in translation, metabolism, and oligopeptide metabolism (McCarthy and Fitzpatrick, 2019). However, the accessory genome includes dispensable, variable, and “unessential regions,” which may not be present in all strains or isolates within a clade (Torres et al., 2020). Thus, genome content can vary in distinct populations of a single fungal species, and the inventory of the variation at the genomic level in different isolates is crucial to characterize the complete set of accessory genes (Stajich, 2017). Two-speed genome evolution is referred to indicate compartmentalization of the fungal genomes as shared and slowly evolving regions as well as variable and fast-evolving regions (Dong et al., 2015; Bertazzoni et al., 2018; Torres et al., 2020). Pangenomics approach has recently resolved different fungal nuclear genomes (Kelly and Ward, 2018; McCarthy and Fitzpatrick, 2019; Badet et al., 2020); however, only few studies focused on mitochondrial genomes. For instance, sequencing of mitogenomes of Aspergillus and Penicillium species were analyzed by presenting core and accessory genes through comparative mitogenome analyses (Joardar et al., 2012). The mitogenome of phytopathogenic fungus Fusarium graminearum was analyzed considering the pan-mitochondrial genomics concept (Brankovics et al., 2018).

Monilinia species include phytopathogenic fungi that belong to the Ascomycota division. They cause brown rot disease on many stone and pome fruits, which results in severe economic losses around the world. Monilinia laxa, Monilinia fructicola, and Monilinia fructigena are the prevalent pathogenic species of the Monilinia genus causing this disease (Holb, 2006, 2008). The complete mitogenome of M. laxa was characterized by our previous study for the first time and presented higher content of mobile introns in comparison to some of the other phytopathogenic species from closely related genera (Yildiz and Ozkilinc, 2020). Thus, we expected high mitochondrial diversity within this pathogenic species and its relative, causing the same disease. This study aimed to uncover mitochondrial variations, by pan-mitogenomic approach, of the 16 mitogenomes from the two prominent and most abundant species (M. fructicola and M. laxa) that are known to cause brown rot disease. Mitogenomes were annotated as well as phylogenetics, and evolutionary selections were evaluated based on the protein-coding regions of the mitogenomes. This provides an essential foundation for future studies on population genetics, taxonomy, and crop protection strategies from the perspective of mitogenomics.

MATERIALS AND METHODS

Fungal Isolates and DNA Extraction

Isolates of Monilinia species were selected from the collection of Dr. Ozkilinc. Original isolates were long term stored at −20°C on Whatman filter papers no 1. Isolates were obtained from infected peach fruits from different orchards in six cities of Turkey (Ozkilinc et al., 2020). Sixteen isolates from a large collection of Monilinia samples were selected. The list of selected fungal pathogens of M. fructicola and M. laxa species is represented in Table 1. Selected isolates were grown from their original stored cultures on potato dextrose agar media at 23°C in the dark. Mycelia were transferred to potato dextrose broth and incubated at room temperature on a rotary shaker at 150 rpm for 5–7 days for genomic DNA isolation (Yildiz and Ozkilinc, 2020). Total DNA extractions were carried out using a commercial kit for fungi/yeast genomic DNA isolation (Norgen Cat. 27300, Canada), following the manufacturer’s protocol. Concentration and purity of DNAs were assessed with a spectrophotometer

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1https://www.ncbi.nlm.nih.gov/genome
2https://www.ncbi.nlm.nih.gov/genome/organelle/
Genome Sequences and de novo Assembly

The whole-genome sequence libraries of the 16 *Monilinia* spp. isolates were constructed using Illumina platform with TruSeq Nano kit to acquire paired-end 2 × 151 bp with 350-bp insert size, provided by Macrogen Inc., Next-Generation Sequencing Service, Geumcheon-gu, Seoul, South Korea.

Identification of Repetitive Sequences

The repetitive elements in the mitogenomes were determined by using Tandem Repeats Finder (Benson, 1999). The repetitive sequences and their motifs were compared within and between the species.

Pan-Mitogenomics Analysis to Predict Conserved and Variable Regions Within Species

The percentages of the conserved and variable regions of the mitogenomes within each species were determined using bioinformatics tools Spine and AGEnt (Ozer et al., 2014). Additionally, R programming language with a deviance function from the stats package (R Core Team, 2013) was applied to interpret the statistical analysis of whether the intragenic intron sizes have contributed to differentiation in mitogenome sizes within the species. Thus, a linear correlation between the mitochondrial genome size (as the dependent variable) and intron length (as the independent variable) was tested based on the null hypothesis of positive correlation expectation.

Estimation of Codon Usage and Evolutionary Selection Patterns in Mitogenomes of *Monilinia* Species

The non-synonymous (Ka) and synonymous substitution rates (Ks) were calculated for all PCGs by using DnaSP v6.10.01 (Rozas et al., 2017). Since all the coding regions were almost the same within the species, the evolution rate was estimated on the total data set from both species. The strength of selection was inferred by considering that if the calculated ratio is equal to, greater than, or less than 1 indicates neutral evolution, positive (diversifying) selection, or purifying (negative) selection, respectively. Ka/Ks values for all protein-coding regions were visualized with ggplot in R programming language (R Core Team, 2013). The Relative Synonymous Codon Usage (RSCU) was obtained using MEGA 7 software (Kumar et al., 2016) and determined for all coding
regions. Furthermore, the nucleotide frequency of occurrence in each protein-coding gene (including the full length of the exons and introns) as well as in genes related to the ribosome (rnl and rns) was assessed for A/T and G/C asymmetry by using the following formulas:

$$\text{AT skew} = (A - T)/(A + T); \text{GC skew} = (G - C)/(G + C)$$

**Phylogenetic Analysis Based on Amino Acid Sequences of Mitochondrial Protein-Coding Genes**

Amino acid translation of the PCGs in the mitochondrial genomes of *M. fructicola* and *M. laxa* isolates were obtained based on the mitochondrial translation code data four using the Geneious 9.1.8 program (Kearse et al., 2012). The phylogenetic tree was constructed using a concatenated amino acid matrix of the 14 core mitochondrial genes and ribosomal protein of *M. fructicola* and *M. laxa* isolates. To strengthen the evolutionary relationships between our data and other genera of the Helotiales, amino acid sequences of each of the PCGs and ribosomal protein were included from published mitochondrial genome data. Additional datasets were obtained from NCBI GenBank under the following accession numbers; KC832409.1 (*Botrytinia fuckeliana*), KJ434027.1 (*Sclerotinia borealis*), KT283062.1 (*Sclerotinia sclerotiorum*), KF169905.1 (*Glarea lozoyensis*), NC_015789.1 (*Phialophora subalpina*), KF650572.1 (*Rhynchosporium agropyri*), KF650575.1 (*Rhynchosporium secalis*), KF650573.1 (*Rhynchosporium commune*), and KF650574.1 (*Rhynchosporium orthosporum*). The multiple protein sequences were concatenated by using the Geneious 9.1.8 program (Kearse et al., 2012) and aligned by ClustalW using MEGA software version 7 (Kumar et al., 2016). The maximum likelihood (ML) tree was constructed using RAxMLGUI v2.0 (Silvestro and Michalak, 2012) with 1,000 bootstrap replicates under BLOSUM62 substitutional matrix. The phylogenetic tree was visualized by FigTree v1.4. program (Rambaut, 2012) and rooted at the midpoint.

**RESULTS**

**Sequence Features of Mitogenomes of *Monilinia* Species**

Most of the mitogenomes were extracted as one contig. However, the mitogenomes of five isolates (Yolkenari-1, Yildirim-2–10th, Ti-B3-A3-2, BG-B1-A17, and SC-B2-A4) were represented by four contigs. These contigs were mapped by using the other completed mitogenomes as reference. Statistics of QUAST reports for the assembled mitogenomes of 16 isolates of *Monilinia* species are provided in Table 2. After mapping, the mitogenome sizes ranged from 158,607 to 167,838 bp for *M. fructicola* and from 178,351 to 179,780 bp for *M. laxa*. Intraspecific length variations within isolates of *M. fructicola* were larger than the variations observed for the *M. laxa* isolates. An isolate of *M. laxa* (isolate code is MM-B2-A2) had the largest mitogenome size with 179,780 bp, while the isolate (coded as T-B1-A5) from *M. fructicola* species had the smallest mitogenome size with 158,607 bp. Total GC% content of all mitogenomes ranged between 30.0 and 31.1%.

**Annotated Mitogenomes of Brown Rot Fungal Pathogens**

The annotated mitogenome, with circular structure, of *M. fructicola* (BG-B1-A4) was chosen as representative sequence and submitted for the first time to the NCBI GenBank (accession number MT005827) (Figure 1 and Supplementary Table 1).

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**Table 2** QUAST report for the 16 mitogenomes of *Monilinia* spp.

| Sample ID | Contigs | Largest contig | Total length | N50* | N75 | L50** | L75 | GC% | AT% | Species |
|-----------|---------|----------------|--------------|------|-----|-------|-----|-----|-----|---------|
| MM-B2-A2  | 1       | 178431         | 178431       | 178358 | 178358 | 178358 | 178358 | 1 | 1 | 30.09 | 69.91  |
| 2B1-A5    | 1       | 178431         | 178431       | 178358 | 178358 | 178358 | 178358 | 1 | 1 | 30.04 | 69.96  |
| MT-B1-A3-1| 1       | 178421         | 178421       | 178358 | 178358 | 178358 | 178358 | 1 | 1 | 30.05 | 69.95  |
| Yildirim-1 | 1       | 178358         | 178358       | 178358 | 178358 | 178358 | 178358 | 1 | 1 | 30.06 | 69.94  |
| Ni-B3-A2  | 1       | 178357         | 178357       | 178357 | 178357 | 178357 | 178357 | 1 | 1 | 30.06 | 69.94  |
| MM-B4-A4  | 1       | 178353         | 178353       | 178353 | 178353 | 178353 | 178353 | 1 | 1 | 30.05 | 69.95  |
| T-B1-A4-2 | 1       | 178351         | 178351       | 178351 | 178351 | 178351 | 178351 | 1 | 1 | 30.05 | 69.95  |
| Ti-B3-A2  | 1       | 178351         | 178351       | 178351 | 178351 | 178351 | 178351 | 1 | 1 | 30.05 | 69.95  |
| B5-A4     | 1       | 159666         | 159666       | 159666 | 159666 | 159666 | 159666 | 1 | 1 | 30.95 | 69.05  |
| BG-B1-A4  | 1       | 159664         | 159664       | 159664 | 159664 | 159664 | 159664 | 1 | 1 | 30.94 | 69.06  |
| T-B1-A5   | 1       | 158607         | 158607       | 158607 | 158607 | 158607 | 158607 | 1 | 1 | 30.95 | 69.05  |
| Yolkenari-1| 1       | 69540          | 49868        | 59243  | 59243  | 59243  | 59243  | 2 | 2 | 31.19 | 68.81  |
| Ti-B3-A3-2| 1       | 66346          | 46965        | 59242  | 59242  | 59242  | 59242  | 2 | 2 | 31.21 | 68.79  |
| Yildirim-2-10th| 4   | 66346          | 46965        | 59242  | 59242  | 59242  | 59242  | 2 | 2 | 31.21 | 68.79  |
| BG-B1-A17 | 4       | 64885          | 42596        | 56701  | 56701  | 56701  | 56701  | 2 | 2 | 31.05 | 68.95  |
| SC-B2-A4  | 4       | 64884          | 42592        | 56701  | 56701  | 56701  | 56701  | 2 | 2 | 31.05 | 68.95  |

* N50: length is at least half (50%) of the total base content of the assembly.
** L50: is the number of contigs equal to or longer than the N50 length.
FIGURE 1 | (A) Annotated reference circular mitogenomes of M. fructicola, showing core conserved protein-coding genes (blue) with introns-encoded homing endonucleases (pink and turquoise), reverse transcriptase (black), orf (orange), two ribosomal subunits (green), rps3 (yellow), introns (gray), and tRNAs (red). (B) Length variation of 14 protein-coding genes, two ribosomal subunits, and rps3 among 16 mitogenomes of Monilinia pathogens.

Besides, all the mitogenomes were submitted to NCBI GenBank and the accession numbers were provided in the “Data Availability Statement” section at the end of the paper. On the other hand, the complete mitogenome of the isolate Ni-B3-A2 of M. laxa species was submitted to the NCBI GenBank with accession number MN881998 by our previous study (Yildiz and Ozkilinc, 2020). Mitogenomes of the 16 isolates of Monilinia species had the 14 PCGs responsible for mitochondrial oxidative phosphorylation system (OXPHOS) and ATP synthesis, including cytochrome c oxidase subunits 1, 2, and 3 (cox1-3), NADH dehydrogenase subunits 1–6 and 4L (nad1-6 and nad4L), ATP synthase subunits 6, 8, and 9 (atp6-8-9), and cytochrome b (cob or cytb). Furthermore, two ribosomal RNA genes for large and small subunits (rnl and rns), 32 transfer RNA (tRNAs), and a gene-encoding ribosomal protein S3 (rps3) were annotated. A set of 32 tRNAs of Monilinia isolates encoded for 20 essential amino acids were involved in the mitochondrial protein synthesis (Figure 1). However, alanine (Ala) and cysteine (Cys) amino acids were absent in the mitochondrial tRNAs of M. fructicola and M. laxa, respectively. Moreover, for both species, some of the tRNA genes encoding different anticodons corresponded to the same amino acids. For instance, four copies of arginine amino acid, which were encoded by TCT and TCG anticodons (Supplementary Table 2). The AT-rich content for tRNA codons was observed in both species (Supplementary Table 2). The mitogenomes of M. fructicola and M. laxa also contained some unidentified open reading frames, represented as ORFs encoding hypothetical proteins, and those ORFs were conserved within the species.

The largest gene sizes among the mitochondrial PCGs in the isolates of M. fructicola were detected in cytochrome c oxidase (cox) subunits 1,2,3 and cytochrome b (cytb), ranging from 23.8 to 11 kb (Figure 1). The large-sized PCGs of M. laxa isolates were cox1,2,3, cytb, and nad5 genes, ranging from 15.3 to 11 kb. Gene
lengths were similar within the species but varied between the species. However, \textit{atp8} and \textit{atp9} genes showed the same size for all the 16, regardless of the species (Figure 1 and Supplementary Table 1). An unknown sequence with 1,219-bp length was detected within the \textit{rnl} gene, only in the mitogenomes of the isolates of \textit{M. fructicola}. This unknown sequence was not matched with any sequence in the NCBI gene bank. Furthermore, another duplication of this unknown sequence was detected within the \textit{rnl} of the isolate named Ti-B3-A2, which has one of the largest mitogenome among all. On the other hand, these sequences were not present in any of the isolates of \textit{M. laxa}.

\section*{Skewness and Codon Usage Analysis}

Nucleotide contents of the 16 mitogenomes were represented according to their AT and GC skew values (Figure 2). Many of the PCGs showed negative AT skews for both species, except for the genes of \textit{atp6}, \textit{cox1}, \textit{cox2}, and \textit{cox3}, which exhibited a positive AT skew (Figure 2). The AT skew of \textit{nad4l} varied among species as positive and negative asymmetry for \textit{M. fructicola} and \textit{M. laxa}, respectively. The GC skews of the core PCGs for 16 mitogenomes showed positive asymmetry except for \textit{atp8} and \textit{nad3}, which exhibited negative GC skews. Moreover, the GC skew of \textit{nad6} was positive for the isolates of \textit{M. fructicola} but negative for the isolates of \textit{M. laxa}. In addition, genes encoding ribosomal subunits (\textit{rnl}, \textit{rns}) and ribosomal protein (\textit{rps3}) showed positive AT and GC skewness in all the mitogenomes of \textit{Monilinia} samples (Figure 2).

Codon usage analysis for the 14 mitochondrial PCGs and \textit{rps3} indicated that the most frequently used codons are as shown in the Supplementary Table 3. Codon usage patterns were quite similar between the 16 mitogenomes. The total number of codons was the highest for Leucine (Leu), Isoleucine (Ile), Lysine (Lys), and Phenylalanine (Phe) amino acids in mitogenomes of \textit{Monilinia} species (Supplementary Table 3).

\section*{Comparison of Gene Arrangements of the Annotated Mitogenomes}

The Mauve alignment reflected a conserved synteny among the 16 mitogenomes, which were divided into 11 homologous regions and represented with different colored synteny blocks (Figure 3). Gene order in the mitogenomes of \textit{M. fructicola} isolates 3 was conserved with the exception for the order of the \textit{rps3} gene, which

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Graphical illustration showing the (A) AT- and (B) GC-skew in the mitochondrial genes of the 16 isolates of Monilinia spp.}
\end{figure}
differed in the isolate coded T-B1-A5 (Figure 3). Rps3 was found as a free-standing gene in the mitogenomes analyzed. Gene order in the mitogenomes of M. laxa isolates was conserved within the species (Figure 3).

Repetitive Sequences in the Mitogenomes of the Isolates of Monilinia species

Different repetitions were detected within the two species (Table 3). Isolates of M. fructicola presented two sequence motifs, which were (TAC)$_{29}$ and (TC)$_{18}$ located in the intergenic regions (Table 3). Among the eight mitogenomes of M. fructicola, 33–37 repeats were detected, and these repetitions covered 1.23–1.41% of the total mitogenome sizes. All the mitogenomes of M. laxa represented repetitive sequences in 59–60 bp in length comprising 1.60–1.69% of the total mitogenomes. The most longest repeats of more than 10 bp were (AT)$_{17}$ in the mitogenome of M. laxa was detected previously (Yildiz and Ozkilinc, 2020). This repetition was found in the seven other isolates of the species. (AT)$_{17}$ sequences were within an intron of the cytb gene, as reported previously (Yildiz and Ozkilinc, 2020).

Introns in the Mitogenomes of Monilinia Pathogens

Introns distributing within genic and intergenic regions, as well as mobile intron groups, were detected in all mitogenomes obtained from different isolates of two pathogenic species. The intron content of different genes varied in both species. In M. fructicola, the number of introns for each gene was Cox1 with 13 introns, cox2 with five introns, cox3 with seven introns, cytb with seven introns, nad2 and nad4 with one intron, nad5 with two introns, atp6 with two introns, and large and small ribosomal subunit with four introns. On the other hand, nad1, nad3, nad4l, atp8, and atp9 were found as intronless genes. Besides, some of the mitogenomes showed intron expansions within nad2 and nad5. The isolates Ti-B3-A2, Ti-B3-A3-2, and Yolkenari-1 presented an additional intron (total 1,459 bp in size) in the nad2 gene in comparison to the other samples. The nad5 gene in the mitogenomes of the two isolates (Ti-B3-A2 and Yolkenari-1) included two extra introns (total 3,568 bp in size) to compare other isolates of the same species (Figure 4).

For all the eight mitogenomes of M. laxa, a total of 34 intron locations were found in cox1 gene with six introns, cox2 gene with seven introns, cox3 gene with four introns, cytb gene with
several introns, nad5 gene with five introns, nad1 gene with one intron, as well as the nad2, nad4, atp6, and rnl genes with one intron. On the other hand, nad3, nad4l, atp8, atp9, and rns did not contain any intron. Furthermore, MM-B2-A4 had one extra intron within the rns gene encoding a small ribosomal subunit. The total number of introns were greater in the mitogenomes of M. fructicola than in the mitogenomes of M. laxa; however, the total intron lengths were smaller in the mitogenomes of M. fructicola (Figure 4). Introns in the mitogenomes of M. fructicola isolates covered 42.2% of the whole mitogenome, and within the non-coding regions (28%) and within coding regions (14.2%). The introns in mitogenomes of M. laxa isolates covered 38.9% of the whole mitogenome and were within the non-coding region (16.3%), and within coding sequences (22.6%).

For M. fructicola isolates, the most intronic sequence carrying gene was the cytB with 72.8%, and intron rich genes followed by cox1 (66.5%), cox2 (61.5%), and cox3 (53%). The most intronic content was found in the cox2 gene with 65.4% and followed by nad1 (65.2%), cytB (62.1%), and cox3 (53%) for M. laxa isolates. Besides, group I and group II mobile introns were detected in the mitogenomes of M. fructicola (Figure 1). Different LAGDIDAGD and GIY-YIG elements encoding homing endonucleases were detected within genic and intergenic regions of the mitogenomes of M. fructicola. Each of these elements from group I was represented as a single copy. Representation of group I mobile introns was given in detail for one isolate of M. laxa in our previous study (Yildiz and Ozkilinc, 2020). Group I mobile introns encoding homing endonucleases were found as approximately 18.1% of the whole mitogenome of M. fructicola and 35.4% of the whole mt-genome of M. laxa. Moreover, three different sequences were annotated as group II introns in the mitogenomes of M. fructicola. These sequences were the same and located in the same positions within all mitogenomes of the isolates of M. fructicola. These sequences were annotated as encoding reverse transcriptase/maturase (Figure 1). Group II introns were not detected within the mitogenomes of M. laxa.

**Pan-Mitogenomics**

The core mitochondrial genes included 14 PCGs, rns, rnl, and rps3. Except for rnl, rns, nad2, and nad5, all core genes were found fully conserved within species. Genic and intergenic introns, mobile introns (group I and group II), and repetitive sequences considered accessory regions of mitogenomes. However, many of these accessory elements were conserved within the species. The conserved regions formed a large portion of the mitogenome (ranging between 94 and 98%), while the variable regions covered 1.19–5.6% of the whole mitogenomes of the M. fructicola isolates. The most accessory-rich mitogenomes were detected in the isolates coded Ti-B3-A2 and Yolkenari-1 within M. fructicola (Figure 5). The whole mitogenomes of the isolates of M. laxa isolated within the species with the exception that one isolate (called MM-B4-A2) carried 0.7% of the total genome as a variable, which was not shared with any other isolate within this species (Figure 5).

According to the linear model test, intragenic introns had a significant effect ($P < 0.0001$ for both species) on mitogenome length variation. $R^2$ values explaining dependent (mitogenome length) and independent (intron length) variables were found as 0.9851 and 0.995 for M. fructicola and M. laxa, respectively.

**Evolutionary Selection on Mitochondrial Genes of Monilinia Species**

The evolutionary rates among the 16 mitogenomes from the two species showed that most of the genes were under negative (purifying selection) (Figure 6). The cytB gene indicated neutral selection (Figure 6). The cytb gene was under diversifying or positive selection, and the remaining genes were under purifying selection (Figure 6).

**Maximum Likelihood Analysis of Mitochondrial Protein-Coding Genes of Monilinia spp. and Other Genera From the Heliotales**

The ML tree was obtained for the amino acid sequences of 14 PCGs and rps3 of the M. fructicola, M. laxa, as well as some other species from Heliotales (Figure 7). Since all the amino acid sequences were conserved within the species, isolates were clustered together for each Monilinia species. However,
**FIGURE 4** | Intron size variations among 16 isolates of *Monilinia* spp.

**FIGURE 5** | Predicted percentage of conserved and variable regions of the mitogenomes within each species (A) *M. fructicola* and (B) *M. laxa.*
**DISCUSSION**

In this study, the complete mitogenomes of *M. fructicola* and *M. laxa* isolates were evaluated in-depth to understand variations within and between the species utilizing the pan-mitogenomic approach. The sizes of the 16 mitogenomes varied from 158,607 to 179,780 bp. The length of fungal mitogenomes is highly variable among the fungal species. It can range from 30 kb for the yeast *Candida parapsilosis* (Nosek et al., 2004) to 235 kb for *Rhizoctonia solani* (Losada et al., 2014). Based on the published data, mitogenomes of *Monilinia* pathogens seem to be quite large compared with other fungal mitogenomes. *S. borealis*, *S. sclerotiorum*, and *Botrytis cinerea*, closely related genera to *Monilinia*, presented mitogenomes of be 203, 128, and 82 kb in size, respectively (Mardanov et al., 2014). Mitogenome size differences are mainly related to the number and size of introns and repetitive elements that constitute the accessory part of the mitogenomes. Moreover, a variable number of genes of tRNAs, as well as loss/gain of some genes, such as *nad* and *atp* subunits, affect the mitogenome sizes (Aguileta et al., 2014; Franco et al., 2017; Li X. et al., 2020; Yildiz and Ozkilinc, 2020). In our study, the main factors that determined the differences in size of mitogenomes were introns, mobile intron groups, and repetitive sequences. Moreover, an unknown sequence and its duplicate was detected within the *rnl* gene within the isolates of *M. fructicola* and were not found in the mitogenomes of isolates of *M. laxa*. The origin and/or the duplication of this unknown sequence could be due to the mobility of mobile introns. Since this sequence was found within an rRNA-encoding gene, a group I intron-encoding RNA working as a ribozyme could be the source for this sequence. This further indicates that dynamism has been shaping the evolution and structure of the fungal mitogenomes continuously.

Fungal mitogenomes are known to have a high AT content, as confirmed in this study as well as in previous studies (Torriani et al., 2014; Franco et al., 2017). The high GC content of genomes was reported to affect the genome to evolve the advantage to maintain DNA stability in the high temperature, UV exposure, and fungicides (Musto et al., 2006; Marsolier-Kergoat, 2013). Furthermore, GC content has an important effect on evolutionary selection, recombination, gene conversion, and recombination in fungal plant pathogens (Stukenbrock and Dutheil, 2018). These considerations can be extended to mitogenomes as well.

Fungal mitogenomes have clustered with many tRNA genes with the different anticodons, indicating a strong preference for A or T, in the third position of codons. This strong preference using A/T has been defined in other species as wobble pairing and codon usage bias (Novoa and de Pouplana, 2012; Wei et al., 2014). However, having the decoders or iso-acceptors may cause mischarging (Pan, 2018), but this situation was not discussed or shown in any fungal mitochondrial genome.

Repetitive elements were 1.23–1.69% in the mitogenomes assessed in this study. The total number of these elements was
found greater and more diverse in *M. laxa* than in *M. fructicola*. Expansion of repetitive elements may have caused replication slippage and the correction of mitochondrial replication process together with proofreading efficiency may differ among the species. It is known that repeat-rich areas evolve more rapidly than other genomic regions (Raffaele and Kamoun, 2012; Dong et al., 2015). If the repeat-rich regions locate within genic regions, changes in these elements may indicate evolutionary selections of certain traits such as resistance or host adaptation (Raffaele et al., 2010; Nardi et al., 2012). Furthermore, exploring repetitive elements in mitogenomes could be highly useful in population genetics analyses, and they could have an important role for the dynamic structure of mitogenomes. Besides, mitochondrial repetitive elements can be useful molecular markers to study population structures.

Core PCGs related to mitochondrial OXPHOS and ATP synthesis are usually essential for the organisms’ life and, thus, highly conserved within the mitogenomes. However, accessory regions could affect some traits, such as pathogenesis and virulence reference (Tettelin et al., 2008), and not be crucial for the survival of the organism. We observed a negative correlation between mitogenome size and virulence degree for these isolates (unpublished data by HÖ). It is well known that accessory regions in the nuclear genomes may change to adapt to evolutionary processes among the fungal isolates of the same species (Plissonneau et al., 2018; Badet and Croll, 2020). However, the effects of accessory regions of mitogenomes on different fungal traits have not been clarified. Mobile introns are another primary source of the size difference for mitogenomes that are highly found in both *Monilinia* species. Our previous study showed that *M. laxa* was the most intron-rich species compared with closely related species from other genera (Yildiz and Ozkilinc, 2020). These mobile introns included many different elements from group I in both species as well as group II introns in *M. fructicola*. Relatedness and phylogenetic signals of these elements within and between *Monilinia* species is another question that requires further investigations. Besides, these elements may have a contribution to certain traits such as virulence or fungicide resistance, and our ongoing studies have been directed to answer those questions.

Pan-mitogenomics approach identified that the core and accessory compartmentalizations occurred within *M. fructicola* species. Even though isolates of *M. laxa* carried many different introns, mobile elements, and repetitive sequences considered as an accessory part of the genomes, these sequences were mainly conserved among the mitogenomes within this species.
Only one isolate of *M. laxa* diverged from the other *M. laxa* isolates with a unique region that was represented as a variable part for the mitogenomes of *M. laxa*. This approach indicates that introns, mobile groups, and repetition patterns are highly conserved and stable within the mitogenomes of *M. laxa*. In contrast, the mitogenomes of *M. fructicola* showed variability and dynamism within the species. This could be related to possible recombination and/or selection pressures on mitogenomes of *M. fructicola*.

A/T and G/C skewness varied among the PCGs as well as between the species for some genes. Interestingly, genes related to ribosomal RNA and protein synthesis were in positive G/C and A/T skewness, indicating the richness of G and A over C and T, respectively. GC compositions were also interpreted as related to transcription start sites in plants and fungi (Fujimori et al., 2005). The different skewness along the regions may also indicate diverse selection pressures on two species. This study indicated that most of the coding genes have been evolving under strong purifying selection between the species according to the Ka/Ks ratios. Diversely, cytB was under a positive selection signal between the species. CytB gene is one of the target regions of respiratory inhibitors that have been intensively used against fungal plant pathogens. Positive selection signals on this gene could be related to different adaptation responses of the species against fungicide selection pressure, which will be discussed in our further study for these pathogens.

Amino acid sequences of the core mitochondrial PCGs and rps3 were fully conserved within *M. fructicola* and *M. laxa* species. Combined protein-coding data set based on ML phylogenetic indicated that these two species are highly diverged from each other as well as from other fungal species from the Heliotales group. However, since one of the main variation contributors is the mobile introns, phylogenetic effects of these elements would be interesting. Mobile introns may shape evolutionary relationships differently in comparison with core PCGs as presented by Megarioti and Kouvelis (2020). Besides, mobile introns may reveal evolving lineages within each fungal species, and this will be investigated in a further study.

Considerable mitogenomic variations were observed within and between these two important pathogenic species within the *Monilinia* genus. Fungal mitochondrial genomes are still waiting for many hidden information on fungal traits and evolution. Pangenomic approach is successfully applicable for fungal mitogenomes due to relatively expanded accessory regions, as shown in this study. Since these organisms are important plant pathogens worldwide, resolving their mitogenomes may suggest new disease management strategies and predictions in evolutionary trajectories of the pathogens and the disease.

**DATA AVAILABILITY STATEMENT**

The first mitogenome of *M. fructicola* was submitted in this study can be found in GenBank (Accession number MT005827). Besides, the mitogenome of *M. laxa* used in this study, was submitted in our previous research and can be found in GenBank (Accession number MN881998.1). All the mitogenomes was submitted to GenBank with following accession numbers for each isolate used in this study: MW794295 for isolate B5-A4, MW794296 for isolate BG-B1-A17, MW794297 for isolate SC-B2-A4, MW794298 for T-B1-A5, MW794299 for isolate Ti-B3-A2, MW794300 for isolate Ti-B3-A3-2, MW794301 for isolate Yolkenari-1, MW794302 for isolate Yildirim-2-10th, MW794303 for isolate Yildirim-1, MW794304 for isolate T-B1-A4-2, MW794305 for isolate MT-B1-A3-1, MW794306 for isolate MM-B4-A4, MW794307 for isolate MM-B2-A2, and MW794308 for isolate 2B1-A5.

**AUTHOR CONTRIBUTIONS**

GY performed laboratory work and all the data analyses. HO designed the research, recommended, and directed the data analysis methods, and controlled all steps of the study. Both authors confirmed and discussed the results and wrote the article together.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.647989/full#supplementary-material

**Supplementary Table 1** | Characterization of annotated mitochondrial genes in *Monilinia fructicola*-Isolate BG-B1-A4, accession number: MT005827.

**Supplementary Table 2** | Set of 32 tRNAs encoding different anticodons in the mitogenomes of *Monilinia fructicola* and *Monilinia laxa* species.

**Supplementary Table 3** | Dataset of the relative synonymous codon usage (RSCU) and the total number of usages for each of the codons in the 14 mitochondrial PCGs, and rps3.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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