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Genome-Wide Identification and Analysis of DOF Gene Family in Eugenia uniflora L. (Myrtaceae)

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Abstract: Eugenia uniflora is a Brazilian native plant species with great ecological and economic importance. It is distributed throughout the Atlantic forest, where two distinct populations show local adaptation to the contrasting conditions of restinga and riparian forest. Among various TFs described in plants, the DOF TF family has been reported to affect flowering and vascular development, making them promising candidates for characterization in E. uniflora. In this study, 28 DOF genes were identified by a genome-wide analysis, of which 20 were grouped into 11 MCOGs by Bayesian phylogeny, suggesting a shared functionality between members. Based on RNA-seq experiments, we have detected eight drought responsive genes, and SNPs identification revealed population unique polymorphisms, implying a role in local adaptation mechanisms. Finally, analysis of conserved motifs through MEME revealed 15 different protein motifs, and a promoter region analysis returned 40 enriched TF binding motifs, both reporting novel biological functions circa the DOF gene family. In general, the DOF family is found to be conserved both in sequence and expression. Furthermore, this study contributes to both DOF literature and the genetic exploration of native species, elucidating their genetic potential and bringing to light new research topics, paving the way to future studies.

Keywords: DOF; transcription factor; phylogeny; molecular evolution; native species; neotropics; adaptation; diversity

1. Introduction

The Atlantic Forest (AF) is a South American biome estimated to host over 15,000 different plant species [1], most of them belonging to the Myrtaceae botanical family [2], which includes the Eugenia, Psidium, Syzigium and Eucalyptus genera. One of its representatives, Eugenia uniflora L., also known as “Brazilian cherry tree”, is a model plant for genetic and biodiversity studies [3,4]. It is a small- to medium-sized woody tree reaching up to 12 m under optimal environmental conditions. Blooming mainly in spring, it gives rise to small, white flowers, which can undergo cross-pollination or self-fertilization, yielding ripe fruits of yellow, red and black colors. The leaves and fruits of E. uniflora are rich in vitamin A, B and C, lycopene, calcium, iron, phosphorus, anthocyanins and flavonols. The extracts of these tissues are widely used in folk medicine and have many biological activities described in the literature [5–9]. Eugenia uniflora presents local adaptation to different ecoregions with contrasting characteristics within the AF (Figure 1C) [10]. It presents itself as a shrub in the Brazilian Restinga (RE), coastal location of sandy and saline terrain, strong insolation and intense gusts of wind (Figure 1A), while growing to be 5 m tall in the Riparian Forest (RF), characterized by closed, humid and tropical vegetation (Figure 1B). This disparity between populations was also confirmed at the genetic level, where individuals from the RE had much smaller genetic variation compared to RF individuals, possibly due to a recent population expansion of individuals adapted to the conditions of the RE [11].
DNA-binding with one finger (DOF) proteins are part of a transcription factor (TF) family particular to the Viridiplantae clade [12]. They have been identified in numerous species, including monocots (rice, maize and sorghum) [13–15], eudicots (arabidopsis, cotton and tomato) [16–18], ferns (Selaginella moellendorffii) [12], mosses, (Physcomitrella patens) [19], and algae (Chlorella vulgaris and Chlamydomonas reinhardtii [12,20]). These proteins are mainly expressed in vascular tissues, and DOF over-expression studies have reported higher fruit yields, making them compelling candidates for characterization studies [21]. DOF TFs have a highly variable number of representatives, ranging from 1 in C. reinhardtii to more than 100 in polyploid species, such as cotton [12,17]. This diversity is the result of multiple duplication events at the genetic and genomic levels [17]. Sequences range from 200 to 500 amino-acids and are characterized by a C2C2 zinc-finger DNA-binding domain [22], that specifically binds to 5’-(A/T)AAAG-3’ motifs [23] at the gene promoter region, and at least one other domain for protein interaction. They have already been described as regulators of several physiological functions, such as flowering [24], lipid metabolism [20], starch synthesis [25], regulation of development in response to abiotic stresses [26,27], acclimation to cold [28] and others [29–31].

As aforementioned, E. uniflora individuals exhibit contrasting phenological characteristics among their populations (RE and RF), some of which are maintained even when greenhouse-grown (e.g., different flowering periods). Thus, the characterization of DOF TFs (described as actuators in flowering and abiotic stresses), and their comparison of different populations is important both from an ecological and economical points of view (conservation, gene flow between populations and, since this species is cultivated, a phenological standardization is essential). The objective of the present study was to carry out a genomic characterization of the DOF gene family in E. uniflora, and as result, we have identified 28 DOF members in E. uniflora’s genome, further classified into 11 Majorly Conserved Orthologous Groups (MCOGs) by a phylogenetic approach. These MCOGs returned new phylogenetic groups not yet described, and brought to light the confounding DOF classification system used in the current literature. As for E. uniflora populations, DOF
genes appear to be conserved both in sequence and expression profiles, serving as actuators in local adaptation.

2. Materials and Methods

2.1. Identification of DOF Genes in Eugenia uniflora

To identify DOF genes in *E. uniflora*, all DOF gene sequences from its closest phylogenetic species—*Eucalyptus grandis*—were used as query for BLASTp [32] search against the contigs of the *E. uniflora* genome (SUB10728242). Hits obtained, with an e-value below $1.00^{-5}$, were validated by manual filtering using transcript visualization and ORF prediction with Unipro UGENE [33], *ab initio* gene prediction with FGENESH [34], and visualization of hits of RNA-seq experiment alignment files with TABLET [35]. All sequences obtained were compared with the databases available at NCBI (ncbi.nlm.nih.gov, accessed on 29 October 2022) and Phytozome (phytozome-next.jgi.doe.gov, accessed on 29 October 2022) by BLASTp to confirm their existence.

2.2. In Silico Predictions

The Expasy server’s ProtParam online tool (web.expasy.org/protparam, accessed on 29 October 2022) was used to compute the chemical characteristics of *E. uniflora*. The subcellular location prediction was performed on the DeepLoc1.0 server [36]. The gene structure was manually assembled in Adobe Illustrator CC (adobe.com/products/illustrator.html, accessed on 29 October 2022). The MEME tool from the MEME-suite [37] was used to identify conserved protein motifs, 15 motifs between 6 and 100 amino-acids with one or no occurrence were predicted from all sequences present in the phylogenetic analysis.

2.3. Alignment and Phylogeny Reconstruction

Sequences of DOF genes were gathered from 16 species that have already been phylogenetically characterized. Included species in this study are: *Arabidopsis thaliana* [16], *Cajanus cajan* [38], *Camellia sinensis* [39], *Citrus sinensis* [40], *Cucumis sativus* [41], *Daucus carota* [42], *Eucalyptus grandis* [43], *Manihot esculenta* [44], *Medicago truncatula* [45], *Musa acuminata* [46], *Oryza sativa* [13], *Populus trichocarpa* [47], *Selaginella moellendorffii* [12], *Solanum lycopersicum* [18], *Vitis vinifera* [48] and *Zea mays* [14]. The alignment used in the phylogeny used the protein sequences of the DOF genes and was generated by the MAFFT [49] tool. Not all sequences were kept for future analysis (Supplementary File S1). MEGAX [50] was used to visualize and remove sequences without the presence of the DOF domain. Only regions referring to the DOF domain and adjacent regions with a possible phylogenetic signal were considered for alignment. Columns with high representation of gaps were excluded from the final alignment. Gaps were converted to missing data. The most suitable evolutionary model for the resulting alignment was predicted by ModelFinder, included in the IQTree [51] package. The phylogenetic tree was constructed by Bayesian analysis in BEAST [52] using 50,000,000 generations, while discarding 10% of the most discrepant trees. The FigTree program (tree.bio.ed.ac.uk/software/figtree, accessed on 29 October 2022) was used to visualize and manipulate the trees.

2.4. Differential Expression Analysis, SNP Identification and Promoter Motif Enrichment Analysis

The raw transcriptome datasets of *E. uniflora* leaves were recovered from the NCBI Sequence Read Archive at Bioproject PRJNA784246 (Turchetto-Zolet et al. unpublished). Drought experiment reads of plants in natura, controlled conditions, and under drought treatment were aligned against *E. uniflora* genome contigs using the STAR software package [53]. SNP identification was performed using Picard (broadinstitute.github.io/picard, accessed on 29 October 2022) followed by the GATK best practice workflow for variant identification (gatk.broadinstitute.org/hc/en-us, accessed on 29 October 2022). Differential expression analysis was performed using DESeq2 package [54] and promoter region analysis was performed on the NewPlace database [55]. Heatmap plots were assembled.
from Fragments per kilobase per million (FPKM) values—differential expression analysis (Figure 6)—and raw number of motifs found in the 2 kbp upstream region of DOF genes starting from the coding sequence (CDS)—promoter motif analysis (Figure 7). Differentially expressed genes were selected according to their fold-change (FC) values > 2. Only motifs that presented more than 8 copies were included in the promoter analysis heatmap. Both axes were grouped by the minimum distances of their profiles using the UPGMA algorithm. The Python data visualization toolkit Matplotlib [56] and Adobe Illustrator CC illustration were used to generate the figures.

3. Results

3.1. Identification and Classification of E. uniflora DOF Genes

A total of 32 genes were identified in E. uniflora, of which 28 remained after manual filtering steps (Table 1). Proteins cover sizes between 186 and 507 residues (g8059 and 2186), with most proteins being between 250 and 350 residues. The majority of proteins had their isoelectric point (pI) above 7.0, with the exception of 8 proteins (g1532, g2186, g3994, g5186, g8476, g15014, g23438, g28923). All proteins were predicted to be located in the cell nucleus, as to be expected from typical transcription factors.

Table 1. Physicochemical properties and predicted subcellular location of Eugenia uniflora DOF proteins. Protein size is in amino-acid residues (Aa), molecular weight in kilo Daltons (kDa), and isoelectric point (pI) in pH.

| Protein | Length (Aa) | Weight (kDa) | pI | Subcellular Location |
|---------|-------------|--------------|----|---------------------|
| g1433   | 276         | 29,822       | 8.85 | Nucleus             |
| g1532   | 331         | 36,445       | 6.52 | Nucleus             |
| g2006   | 307         | 33,047       | 8.79 | Nucleus             |
| g2186   | 507         | 55,049       | 6.10 | Nucleus             |
| g3898   | 332         | 35,571       | 9.19 | Nucleus             |
| g3994   | 340         | 36,687       | 6.72 | Nucleus             |
| g4036   | 193         | 21,308       | 7.55 | Nucleus             |
| g5186   | 306         | 32,971       | 6.74 | Nucleus             |
| g5301   | 243         | 26,111       | 8.54 | Nucleus             |
| g6300   | 381         | 41,256       | 8.70 | Nucleus             |
| g6740   | 259         | 26,047       | 8.45 | Nucleus             |
| g8059   | 306         | 32,971       | 6.74 | Nucleus             |
| g8476   | 295         | 31,875       | 5.87 | Nucleus             |
| g10059  | 491         | 52,622       | 8.37 | Nucleus             |
| g13301  | 360         | 38,370       | 9.07 | Nucleus             |
| g14258  | 370         | 38,939       | 8.82 | Nucleus             |
| g15014  | 325         | 34,705       | 6.51 | Nucleus             |
| g16418  | 362         | 38,399       | 9.59 | Nucleus             |
| g17019  | 458         | 49,638       | 6.87 | Nucleus             |
| g21423  | 258         | 27,408       | 9.55 | Nucleus             |
| g23438  | 467         | 50,378       | 5.23 | Nucleus             |
| g25319  | 308         | 32,858       | 8.50 | Nucleus             |
| g25339  | 303         | 33,578       | 8.45 | Nucleus             |
| g25726  | 253         | 25,782       | 9.13 | Nucleus             |
| g28923  | 269         | 29,061       | 5.05 | Nucleus             |
| g32088  | 447         | 48,074       | 8.89 | Nucleus             |
| g32327  | 291         | 31,451       | 9.76 | Nucleus             |

A total of 647 sequences covering the 16 species studied were recovered from the different databases used in each study, of which 32 sequences were removed for various reasons (Supplementary File S1)—including the lack of the DOF domain—leaving 615 sequences for further analyses. The total size of the alignment was manually curated to include only 61 sites, covering the DOF domain and adjacent regions with a possible phy-
logenetic signal. The tree generated by Bayesian statistics (Figure 2) returned 12 different MCOGs with high support—Posterior Probability (PP) > 0.94—and representativeness of sequences from different species.

Figure 2. Phylogenetic tree of 615 DOF amino-acid sequences. Conserved clades with species representativity are colored, together with their branches. Outer circle colors denote sequences derived from monocots (dark grey), eudicots (light grey), and Selaginella moellendorffii (white). Black dots indicate *Eugenia uniflora* sequences.

Among the recovered groups, the largest was subdivided into two groups (Group I, representing the Cycling DOF Factors (CDF) subfamily of DOF genes, and II). Group II, even though paraphyletic, was considered to be just one group. The reasoning was based on species representativity (each of its divisions represents only monocot or eudicot species) and a PP equal to 1 on the branch that refers to Groups I and II. Groups VI and XII also presented PP equal to 1. Group III is another group with only monocot species. In general, it was not possible to determine the evolutionary relationships between MCOGs, given by the low support values between the groups, with the exception of Groups I and II. Of the 28 genes of *E. uniflora*, 20 were grouped into 11 MCOGs (Groups I–II and IV–XII), with 5 sequences present in the CDF group, while 8 were not grouped (g25726, g6872, g5186, g1532, g14258, g32327, g3898 and g16418). Among the sequences present in the conserved groups, g2186, g6740, g15014, g6300, g25319 and g8476 did not have their closest ortholog being an *Eucalyptus grandis* gene. Regarding the ungrouped sequences, g32327 and g16418 also did not cluster with *Eucalyptus grandis*. 
3.2. Gene Structure and Domain Conservation of E. uniflora DOF Genes

Among the 28 genes identified in E. uniflora, none had more than 1 intron in their gene structure, while 13 had no introns at all (g1433, g4036, g5301, g6740, g8059, g8476, g13301, g14258, g15014, g25339, g25726, g28923, g32327) (Supplementary Figure S1). The CDF subfamily returned the largest intron sizes, with a mean of nearly 1000 nucleotides. Some sequences had exons with only a few dozen nucleotides, mainly in the N-terminal region, with the exception of the g1532 sequence, which had a small exon in its C-terminal region. In general, the different MCOGs found seem to be represented in the genetic structure of their representative sequences.

Of the 15 protein motifs identified in the protein sequences of DOF genes (Figures 3 and 4), sizes vary, from small motifs of 11 amino-acids (motifs 2, 9, 10 and 11) up to motifs with 46 amino-acids (motif 1).

Figure 3. Conserved protein motifs and SNPs from Eugenia uniflora DOF genes. The phylogenetic relationships between DOF genes are shown on the left. Colored boxes represent protein motifs. Black lines represent protein sequences without annotated motifs. Triangles indicate identified SNPs locations. On the right, a comparison of SNPs between the two populations is shown. Filled slices represent homozigote polymorphisms and outlined slices heterozigotes ones.

The vast majority of proteins had 3 or 4 motifs found in their sequence, with the exception of CDF proteins (g2186, g10059, g17019, g23438, g32088), which had up to 11 motifs found. Motifs 3, 9, 10 and 11 are typical motifs present in CDF proteins and have already been characterized as responsible for protein-protein interaction with other participants in the flowering regulation process, such as FLAVIN-BINDING KELCH REPEAT.
F BOX PROTEIN (FKF1), TOPLESS (TPL) and GIGANTEA (GI). Motifs 5, 6, 13 and 14 were also found exclusively in the CDF proteins and in more than one representative, although their functions are unknown. Motif 12 appears to be conserved in sequences belonging to Group XI, also with unknown function. Apart from motif 15, all the other motifs showed considerable conservation of their residues.

| Motif | Motif Logo | Motif Consensus Sequence |
|-------|------------|--------------------------|
| 1.    | ![Motif Logo 1](image1.png) | PEAQLKCRCSITNKFGYNVYSLQPR1FCXCTYREWTEKGGTLR |
| 2.    | ![Motif Logo 2](image2.png) | NYPVGGGRK |
| 3.    | ![Motif Logo 3](image3.png) | VPKTLR1DDC6AAKSS1W3T |
| 4.    | ![Motif Logo 4](image4.png) | ZIPQSMARARLAKI |
| 5.    | ![Motif Logo 5](image5.png) | DEKVNAQTSVP10ANP |
| 6.    | ![Motif Logo 6](image6.png) | KNTGTVLPDDAPLEASMASYSLNAPR |
| 7.    | ![Motif Logo 7](image7.png) | MVSSPR1LPNW |
| 8.    | ![Motif Logo 8](image8.png) | GSNVNYTLLASSLSSINQDLIIWKLIQQHSLA |
| 9.    | ![Motif Logo 9](image9.png) | KDP4KLFGKTBLP |
| 10.   | ![Motif Logo 10](image10.png) | AALSBNQOE |
| 11.   | ![Motif Logo 11](image11.png) | NSIPGKUSKD |
| 12.   | ![Motif Logo 12](image12.png) | HEQQDNLALP |
| 13.   | ![Motif Logo 13](image13.png) | KNSASHYKTELEAAGQARDFAHGTH |
| 14.   | ![Motif Logo 14](image14.png) | YPNNSAPPFAFPG |
| 15.   | ![Motif Logo 15](image15.png) | SNNENIQQORNE |

Figure 4. Conserved protein motifs identified in *Eugenia uniflora* DOF genes. Motifs are ordered based on false discovery rate, from lowest to highest.

3.3. *E. uniflora* Populations and Their DOF Gene Arsenals

A total of 30 high quality SNPs were identified from expression data of the two populations (Figures 3 and 5), but with only 9 SNPs resulting in non-synonymous mutations. Of the 28 genes, two (g2186 and g15014) had 6 SNPs each, the highest number identified among all sequences. SNPs were identified in four of the five genes representing Group I. Seventeen SNPs are specific to only one of the populations (12 from the RF population and 5 from the RE population). Almost two thirds (18) of the identified SNPs are found in the third position of the codons referring to the reading phase of the DOF genes, all being synonymous mutations, except for the second SNP of the g17019 gene. Of the remaining 13 SNPs, 5 are located in the regions of the motifs found (g21423, g6300, g2186, g17019; 1/2). Three SNPs are exclusive to RF individuals (g32327, g21423, g15014, 1) and 1 to RE individuals (g2186).

Regarding the differential expression experiment, of the 28 genes, 4 (g6740, g8476, g23438 and g28923) were not expressed in at least one analyzed condition studied, and 4 genes (g1433, g4036, g8476 and g23438) did not have their expression altered between the two populations or between treatments (Figure 6A). Of the 24 differentially expressed genes (DEGs) in at least one of the conditions, 8 have a clear drought responsive profile, 5 of which are upregulated (g3893, g3994, g5186, g6300, g13301) and 3 are downregulated (g2186, g5301, g25726). Genes g5301, g13301 and g25726 are the three most differentially
expressed, with FC values around 4.9, 4.7 and 6.9 respectively. Overall, control conditions (RF<sub>C</sub> and RE<sub>C</sub>) and the in natura RF (RF<sub>N</sub>) have little variance amongst each other, also true for both RF and RE drought stress conditions (RF<sub>S</sub> and RE<sub>S</sub>) (Figure 6B). The highest number of DEGs was found when comparing RE<sub>S</sub> with RF<sub>N</sub> (19).

| Gene  | CDS Position | NT Mutation | Codon Position | AA Mutation | Δ | D | RE | RF |
|-------|--------------|-------------|----------------|-------------|---|---|-----|-----|
| g2006 | 375          | C → T       | 3rd            | P → P       |   |   |     | ●   |
| g2186 | 282          | C → T       | 3rd            | G → G       |   |   |     | ●   |
|       | 564          | T → C       | 3rd            | N → N       |   |   |     | ●   |
|       | 1180         | G → T       | 1st            | A → S       | ● |   |     | ●   |
|       | 1230         | C → T       | 3rd            | I → I       |   |   |     | ●   |
|       | 1281         | T → G       | 3rd            | R → R       |   |   |     | ●   |
|       | 1448         | T → C       | 2nd            | V → A       | ● | ● |     | ●   |
| g6300 | 250          | A → C       | 1st            | A → S       | ● | ● |     | ●   |
|       | 354          | C → T       | 3rd            | P → P       |   |   |     | ●   |
|       | 724          | A → G       | 1st            | I → V       | ● |   |     | ●   |
| g15014| 49           | A → G       | 1st            | T → A       | ● |   |     | ●   |
|       | 54           | C → T       | 3rd            | A → A       |   |   |     | ●   |
|       | 108          | C → G       | 3rd            | G → G       |   |   |     | ●   |
|       | 141          | G → C       | 3rd            | P → P       |   |   |     | ●   |
|       | 603          | G → A       | 3rd            | T → T       |   |   |     | ●   |
|       | 888          | C → A       | 3rd            | P → P       |   |   |     | ●   |
| g17019| 1235         | A → G       | 2nd            | K → R       | ● | ● |     | ●   |
|       | 1254         | C → G       | 3rd            | D → E       | ● | ● |     | ●   |
| g21423| 210          | T → C       | 3rd            | T → T       |   |   |     | ●   |
|       | 655          | C → G       | 1st            | Q → E       |   |   |     | ●   |
|       | 689          | G → C       | 2nd            | G → A       |   |   |     | ●   |
|       | 751          | G → A       | 1st            | G → R       | ● | ● |     | ●   |
| g23438| 77           | G → C       | 2nd            | G → A       |   |   |     | ●   |
|       | 423          | T → C       | 3rd            | C → C       |   |   |     | ●   |
|       | 870          | G → A       | 3rd            | E → E       |   |   |     | ●   |
|       | 1209         | T → C       | 3rd            | C → C       |   |   |     | ●   |
| g32088| 939          | A → G       | 3rd            | P → P       |   |   |     | ●   |
|       | 1104         | C → G       | 3rd            | G → G       |   |   |     | ●   |
|       | 1200         | C → T       | 3rd            | S → S       |   |   |     | ●   |
| g32327| 357          | A → T       | 2nd            | Q → L       | ● | ● |     | ●   |

**Figure 5.** Characteristics of the 30 identified DOF SNPs in *Eugenia uniflora*. For every SNP, the gene, CDS mutation position, nucleotide mutation, codon position, and amino-acid changes are shown. The last four columns indicate, in order, a non-synonymous mutation (Δ), a mutation inside a motif (D), RE specificity, and RF specificity.
Figure 6. Differential gene expression analysis of *Eugenia uniflora* DOF genes. (A) Each gene phylogenetic groups is indicated on its left. Ungrouped genes are indicated by a crossed-out “O”. Asterisks represent statistically significant FCs, while red lines represent no expression. Each column refers to a sample condition: Riparian Forest in natura (RF\textsubscript{N}), RF Control (RF\textsubscript{C}), RF Stressed (RF\textsubscript{S}), Restinga (RE\textsubscript{N}), RE\textsubscript{C}, and RE\textsubscript{S}. Each gene has six comparisons on every column, exemplified by the scheme over the FC color gradient. (B) DEGs comparison amongst every condition. Thicker lines represent more DEGs.

3.4. Promoter Region Analysis

All *Eugenia uniflora* DOF genes had their upstream 2 kbp, starting from the CDS region, used for promoter analysis with the exception of two genes: g32088 and g32327, which were recovered only 1327 and 138 bp respectively due to their close location to the 5' region of the genome contigs.

A total of 290 unique motifs were identified, of which 40 were left after filtration steps (Figure 7). The number of copies per motif varies from 0 to 55 copies, the case of the CTRMCAMV35S motif. Among the motifs found in the promoter region of DOF genes, sequences related elements of the transcription machinery, such as TATABox and CAATBox, were enriched. Recognition sequences of different transcription factors were
also well represented, some already mentioned in both in silico and in vivo studies of the promoter region (WRKY, ABRE, MYB, BELL) [30,41,47,57–59], with the vast majority yet to be confirmed (CAMTA3, BBR/BPC, SURE). Among the tissue-specific-expression elements found, primary and secondary root, vascular tissues, leaves, seeds, flowers and pollen are already mentioned in the literature [60], however we also identified a nodule specific motif. As for the biological function, three different motifs for light induction were found (PRECONSCRHSP70A, INRNTPSADB and GT1CONSENSUS). Other motifs include responses to sulfur, calcium, copper, chlorophyll precursors, etiolation, drought, salinity, pathogen induction, and vernalization. Interestingly, the motif that was the most represented over all DOF genes was not related to classical transcriptional machinery, such as CAATBox, CCAATBox or TATABox, but the DOF recognition motif itself.

Figure 7. Motif enrichment analysis of the promoter region of Eugenia uniflora DOF genes. Rows represent DOF genes while columns are motifs found. Each gene has its phylogenetic group represented on its left. Crossed-out “O”s represent ungrouped genes. Both genes and motifs were clustered using their count profiles, denoted by the cladograms on the bottom and right.

4. Discussion

4.1. Phylogenetic Relationships Reveal DOF Genes Acting in Similar Pathways

Twenty eight DOF genes were identified in E. uniflora with their physicochemical characteristics shown in Table 1. Their genetic structure was reconstructed and, overall, sequences shared a similar structure among orthologous groups identified in the phylogeny (Figures S1 and 2). Motifs 1 and 2, referring to the DOF domain, are present in all sequences; why they appear separately is not well understood, but it is a recurrent phenomenon in the prediction of conserved DOF sequence motifs through MEME [61,62]. From the reconstruction of phylogenetic relationships, eight E. uniflora DOF genes were not grouped into orthologous groups. However, the other 20 genes are present in clades with high support (PP > 0.94) and their functions could be inferred (Figure 2). The composition of the other groups vary greatly in the literature. Some are preserved, such as the case of Group VI [16,63], and the vast majority end up being subdivisions of larger groups, such as...
Groups X, XI (the second largest group) and XII [12,63]. These inconsistencies arise due to many methodological choices (e.g., the inclusion of only a few species, utilization of less refined phylogenetic methods neighbor-joining, and labelling of poorly supported groups, PP < 80), stating a need for more evolutionary focused studies circa the DOF TF family.

Five genes (g2186, g10059, g17019, g23438 and g32088) were grouped together with Group II, referring to the CDF subfamily of DOF genes, which represents the average number of CDF genes found by species, with some exceptions [64]. CDFs are DOF genes that act in the repression of CONSTANS (CO) and FLOWERING LOCUS T (FT) and thus in flowering inhibition. They have also been characterized to respond to abiotic stresses [65]. Their protein interactions and regulation networks, such as ubiquitination by the GI/FKF1 complex, are well explored in the literature [66]. CDF grouped genes of E. uniflora also contain domains 3, 9, 10 and 11, for interaction with FKF1, TPL and GI respectively, with only the function of motif 11 yet to be discovered [64,67–69], corroborating with their possible role as flowering regulators (Figures 3 and 4). Interestingly, 3 other motifs with no described function (5, 6 and 13) also appear conserved in E. uniflora CDF genes. The same three motifs are organized in such a way that there are 4 represented combinations among the 5 CDF genes, (No domains, 6, 6 + 13, and 5 + 6 + 13). This may be a diversification of CDF genes to interact with other proteins and act in other signaling pathways that are not flowering, as all have been described as pleiotropic genes in CO and FT inhibition [24].

Group I holds genes orthologous to CDF genes, but with very different functionalities. In past phylogenetic studies, Groups I and II were considered as a single group [13,16], but recently, phylogenies with higher sequence numbers present these groups separately, even if in lower support clades [19,63]. In this study, Group I was found with a PP equal to 1, indicating that these genes, despite having a probable common ancestor with the CDF genes, are not part of the same functional group. The g8059 gene was grouped into Group I, together with sequences from A. thaliana, such as AtDOF1.5 (AT1G29160/COG1) and AtDOF2.3 (AT2G34140/CDF4), the only ones experimentally characterized between eudicots and monocots. COG1 and CDF4 are transcription factors involved in the inhibition of phytochrome response pathways, both phytochrome A and B (phyA and pyhB), and the transduction of these signals to hormonal pathways, such as the synthesis of gibberellic acid (GA), abscisic acid (ABA) and brassion steroids (BRs) [70–73]. Motif 9, referring to the TPL corepressor protein binding domain, was also identified as conserved in Group I sequences, probably acting as a key domain for the action of its repressor activities. The presence of this domain in both Group I and Group II genes is further evidence of their likely common ancestry. The most likely hypothesis is the formation of Group I from gene duplications in the ancestors of these sequences and subsequent loss of its C-terminal region by negative or neutral selection, thus maintaining the TPL-binding domain. As with Eucalyptus grandis, M. truncatula and S. moellendorfii, only one sequence was identified in Group I, indicating a convoluted evolutionary history, since other species usually present two, possibly marked by pseudogenization and gene duplication events.

Group III, also reconstituted by other phylogenies (d3) [13], presents only monocot sequences, which may represent an evolutionary novelty exclusive to this clade. Although one M. acuminata sequence is present, this clade might be an exclusivity to Poales, after all, the genes of M. acuminata tend to be grouped in a greater number of sequences. A phylogeny with a greater number of species representing monocots would be ideal for a proper investigation. Only the rice OsDOF18 gene (LOC_Os08g38220) was characterized in this group, acting on the inhibition of genes of the jasmonic acid (JA) biosynthesis pathway and chlorophyll degradation in leaves and on inducing the expression of ammonia transporters in roots [31,74]. Although they seem to be two uncorrelated pathways, studies evaluating soil nitrogen availability and JA biosynthesis found changes in the expression of the same genes that OsDOF18 regulates [75]. On the other hand, Group IV did not present any sequence of monocots, only eudicots and the lycophyte S. moellendorfii, indicating a possible loss of this MCOG in cereals. The gene g6740 was grouped with the sequence DOF5.8 (AT5G66940), characterized by the expression in procambium and pro vascular cells, related
to the presence of auxin in the plant, since DOF5.8 is regulated by MONOPTEROS [76,77]. DOF5.8 also regulates the expression of ANAC089, an auxin-responsive factor responsible for controlling responses to abiotic stresses such as salinity [78]. Group V has two E. uniflora, g4036 and g5031, grouped with two other A. thaliana genes, AtDOF1.7 (AT1G51700/ADOF1) and AtDOF3.1 (AT3G21270/ADOF2). Although there is not much information about the function of these two genes in specific characterization studies, multi omics-data have returned both genes as differentially overexpressed in the overexpression of factors involved in cell division and vascular tissue development (TDIF-PXY) [79]. The same study also detected genes regulated by ADOF1 (LBD4, PHB, PHV and REV) and by ADOF2 (REV) from a Y1H analysis. Apart from the LBD4 gene, the genes regulated by ADOFs belong to the HD Zip III family, responsible for mediating auxin responses, formation of leaf axes and maintenance of the shoot apical meristem. Furthermore, ADOF1 was found to be overexpressed in roots of plant subjected to salinity stress [80]. Peculiarly, another study identified ADOF1 mRNA to be expressed mainly in the aerial tissue, later mobilized to the root system [81].

Group VI has one E. uniflora gene (g13301) and no A. thaliana sequences, the same case for Group VIII with the g25339 gene. The only characterized gene in Group VI is OsDOF28 (LOC_Os03g55610), responsible for root apical meristem cell division by inhibition of OsACS1, responsible for ethylene biosynthesis [26]. In contrast to the promotion of cell division in the root of Group VI, Group VII groups genes g15014 and AtDOF5.4 (AT5G60850), the later functionally characterized as an inhibitor of cell cycle progression in callous cells by repression of expansins, cyclins, kinase-dependent cyclins and xyloglucan endo-transglycosylation enzymes [82]. It has also been shown to be induced by ABA and responsible for inhibiting the growth of secondary roots by regulating RSL280. Group IX has genes g6300 and AtDOF1.4 (AT1G28310), however no sequences are characterized. Another gene related to vascular development is AtDOF5.6 (AT5G62940/HCA2) present in Group X, together with the sequence g3994. The generation of dominant mutants for the HCA2 led to malformation of the interfascicular cambium and periclinal divisions of its cells [83].

Group XI has five A. thaliana genes AtDOF1.8 (AT1G64620), AtDOF2.5 (AT2G46590/DAG2), AthDOF3.7 (AT3G61850/DAG1), AtDOF4.1 (AT4G00940/ITD1) and AtDOF4.6 (AT4G24060) grouped with four genes from E. uniflora (g1433, g2006, g21423 and g25319). DAG1 and DAG2 were characterized in mutants with altered germination times. Mutants for DAG1 show a lower need for incidence of red light for germination, while mutants for DAG2 demonstrate the opposite [84]. Phytochrome Interacting Factor3-Like 5 (PIL5), responsible for inhibiting the germination process, is degraded via phyB-mediated ubiquitination and forwarding to the 26S proteasome. PIL5 induces the expression of members of the DELLA family, such as GA Insensitive (GAI) and Repressor of ga1-3 (RGA), inhibitors of the GA synthesis pathway and essential for the maintenance of endogenous levels of ABA [85,86]. The DAG1 gene has been shown to be related to the expression of PIL5, suggesting an induction of DAG1 by PIL5 [87]. It is known that the interaction between DAG1 and GAI is necessary for the repression of AIGA3ox1, a gene of the GA biosynthesis pathway [88]. DAG1 has also been shown to inhibit the expression of CYP707A2, the ABA catabolic pathway gene, demonstrating similarity with DELLA proteins functions [89]. On the other hand, DAG2 is inhibited by the expression of PIL5 and DAG1. As for the genes regulated by DAG2, RGA appears to be negatively regulated, while the GA biosynthesis genes: GA3ox1 and GA3ox2 are shown to be induced, however it is not known whether this interaction is direct or not [90]. In general, it is proposed that the DAG1 and DAG2 genes act in the inhibition of germination by GA catalysis and inhibition of ABA catalysis. As for the ITD1 gene, its function is not known, but its expression pattern shows localization in the cells of the cortex and endoderm of the root [91]. Furthermore, it has an intercellular traffic domain that overlaps with the DOF domain, allowing its dispersion from the expression region to nearby tissues by diffusion via plasmodesmata, a fundamental characteristic for the performance of some DOF genes involved in the development of vascular tissues [92].
All sequences present in Group XI showed domain 12. The AtDOF1.8 and AtDOF4.6 genes are not characterized. Group XII has two A. thaliana genes: AtDOF1.2 (AT1G21340) and AtDOF3.5 (AT3G52440) together with E. uniflora genes (g8476 and g28923).

4.2. DOF Genes as Drivers for Local Adaptation

Monitoring greenhouse-grown E. uniflora plants, individuals coming from the RE showed a shorter flowering time when compared to individuals coming from the RF. In order to search for genetic variants linked to E. uniflora populations and that are also related to alterations in the functionality of DOF genes, 30 SNPs were identified, of which 2 population specific markers resulted in non-synonymous mutations (Figure 5). The g21423;4 SNP, exclusive to RF individuals, is located in motif 15, a highly variable region between the DOF sequences and most likely does not cause a functional change in the context of the g21423 gene protein, belonging to Group XI of genes related to germination. In similar terms, the g2186;6 SNP, even being present in a CDF protein, occurs in a motif not yet characterized, and in a variable region within this motif. Both g17019 SNPs were obtained within the FKF1 binding domain and correspond to conserved amino-acid changes within the motif, however they are not unique to any population. The SNP g17019;1 was found only in heterozygosity, which may suggest a greater importance of conservation of the first lysine residue of this motif when compared to aspartic acid residues, but even if the binding function to FKF1 is compromised, the overlapping function of CDF genes would probably be enough to cover early flowering phenotypes. At first, the greenhouse observations cannot be explained by genetic variants present in DOF gene sequences. Perhaps a search for variants in regulatory regions such as the promoter will result in findings, but with a lack of motif characterization, further studies become difficult.

In order to understand the possible modulations of DOF genes, a gene expression analysis of plants under drought conditions was performed (Figure 6). DOF genes with the highest differential expression between control and drought-exposed conditions were genes g5301, g13301 and g25726. Gene g25726 was grouped with the Egr.F00039 gene from Eucalyptus grandis (PP = 1), its closest ortholog and without known function. These genes may be a novelty exclusive to the Myrtaceae family, considering that both sequences were not present in conserved clades. The gene g13301, belonging to Group VI, was overexpressed under conditions exposed to drought. Ethylene, even being a phytohormone related to stress signals and orchestration of adaptive responses, is an auxin antagonist and its presence in the root stops the growth of its cells. In the context of physiological responses to drought, a stressful condition leads to a search for water by the roots. Inhibition of ethylene synthesis and promotion of apical root cell division in search for water sources may be one of the reasons for g13301 differential expression, if its function is similar to its ortholog (OsDOF28). The third differentially expressed gene was g5301, representative of Group V. According to the previous gene, cell division of meristematic cells present in the root must be followed by mechanisms for differentiation and maturation, such as regulation of vascular development genes. Interestingly, its g4036 ortholog was not differentially expressed, which may be due to a mechanism analogous to the specificity of the AtDOF1.7 salinity response [80]. Although some sequences were not detected in leaf tissues, in general, CDF genes appear to be repressed in the drought treatment. Other members of conserved groups, such as g25339 (Group VIII), g15014 (Group VII), g8059 (Group I), g6300 (Group IX) and g3994 (Group X) also showed changes in their expression by drought treatment. As for differences between populations in their natural environment, only genes g10059 (Group II) and g13301 (Group IV) showed differential expression. Possibly related to abiotic stresses of the RE environment, but with so many possibilities (salinity, drought, insolation) it is difficult to correlate with a specific response. Individuals from E. uniflora populations do not have alterations in the expression of DOF genes when greenhouse-grown, and based on their overall expression profiles, DOF genes seem to act as conserved tools to combat possible stresses present in the environment,
rather than specialized pathways that confer an evolutionary advantage to individuals in a population.

The promoter region analysis (Figure 7) returned a total of 40 motifs described in the literature, with 9 repeats or more. The motif patterns found for each gene were grouped by clustering and the conserved orthologous groups were not recovered, indicating a high diversification of the promoter regions and their binding factors. The clustering of motif profiles returned two main groups: a group of 5 motifs with high representation in all DOF sequences (DOFCOREZM, CACTFPCCA1, GT1CONSENSUS, CAATBOX and ARRIAT), with an average of 20 or more repetitions, and another group representing the other 35 reasons and high variability. Among the most represented motifs, the presence of the DOFCOREZM and ARRIAT motifs indicate a high self-regulation of DOF genes, and a regulation by cytokinin-responsive factors, such as ARRI. The CACTFPCCA1 motif, specific to leaf mesopholic cells, and the GT1CONSENSUS motif, light signal transduction pathway regulation—such as phyA—indicate a wide expression of DOF genes in leaves. Finally, the CAATBOX motif is related to the recruitment of other transcription factors to the promoter region. Motifs for specific factors, such as MYC and WRKY genes, were highly represented, as were root and pollen tissue specific motifs. Some motifs have multiple copies in only a few genes, such as the BBR/BPC binding motif in the g4036 and g6300 gene, as well as the copper-response motifs present in the g25339 and g28923 genes.

5. Conclusions

In conclusion, 28 DOF genes were identified in E. uniflora, of which 20 were grouped into 11 phylogenetically conserved groups with a high probability for conservation of functionality. The eight remaining genes require a more robust phylogenetic analysis for proper grouping, yielding higher branch supports. The addition of more sequences would also be of interest for explaining MCOGs evolutionary relationships, since our results did not manage to do so. The analysis of conserved motifs corroborated with the data presented in the phylogeny, identifying new DOF protein motifs. It would be compelling to functionally characterize these same motifs and genes, possibly revealing new protein interactions and functionalities. With the differential expression analysis, we found eight drought-responsive DOF genes with little expression changes among populations. Hence appearing to be well-preserved tools that E. uniflora populations use to respond to environmental stresses. Future studies utilizing other tissues (e.g., roots and fruits) would also be of interest for providing a full picture of the local adaptation process. Finally, a comprehensive analysis of the promoter region revealed the diversity of regulatory regions of E. uniflora DOF genes, including previously unreported motifs, such as nodule and pollen specific which could be of interest for gene reported and promoter engineering studies. Overall, this work identified new DOF genes and brought to light aspects of their evolutionary history not yet addressed, presenting new avenues of research for the analysis of this family of genes in future analyzes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13122235/s1, Figure S1: E. uniflora DOF genes genetic structure.

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**Abbreviations**

The following abbreviations are used in this manuscript:

- **AA**: Amino-acid
- **AF**: Atlantic Forest
- **CDS**: Coding sequence
- **DEG**: Differentially expressed genes
- **DOF**: DNA-binding with one-finger
- **CDF**: Cycling-dof-factor
- **FC**: Fold-change
- **PP**: Posterior probability
- **RE**: Restinga
- **RF**: Riparian Forest
- **TF**: Transcription Factor
- **MCOG**: Majorly Conserved Orthologous Group

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