UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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# Análises genômicas, evolutivas e funcionais de genes da terpeno sintase em *Psidium cattleyanum* Sabine.

# Genomic, Evolutionary, and Functional Analyses of Terpene Synthase Genes in *Psidium cattleyanum* Sabine.

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Porto Alegre

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Genética e Biologia Molecular; Thesis presented to the Institute of Genetics and Molecular Biology at the Federal University of Rio Grande do Sul as part of requirements for obtaining a PhD in genetics and molecular biology.

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## SUMMARY

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**CHAPTER II: Development of Genome wide functional markers in *Psidium cattleyanum* Sabine (Myrtaceae), a neotropical polyploid complex.**

**CHAPTER III : Genomic characterization, molecular cloning and expression analysis of two terpene synthases from *Psidium cattleyanum* (Myrtaceae).**

# LIST OF ABBREVIATIONS

**Overview**

cTP – chloroplast transit peptide
DMAPP – dimethylallyl pyrophosphate
MEP –2C-methyl-D-erythritol-4-phosphate
MVA – mevalonic acid
TPS – terpene synthase
WGD – whole-genome duplication
EO – essential oil
CT – chemotype

**Chapter I**

CDS – chrysanthemyl diphosphate synthase
CDP – chrysanthemyl diphosphate
CPPs – ent-copalyl diphosphate synthase
CPS – copalyl diphosphate synthases
DGE – differential gene expression
diTPS – diterpene synthases
FC – fold-change
FDR – false discovery rate
FDS – farnesyl diphosphate synthase genes
FPKM - fragments per kilobase of transcript per million fragments mapped
FPP – farnesyl diphosphate
GFPP – geranylfarnesyl diphosphate
GGPP– geranylgeranyl diphosphate
GPP – geranyl diphosphate
IBs – inclusion bodies
IDI – isopentenyl diphosphate isomerase
IP – isopentenyl monophosphate
IPP – isopentenyl diphosphate
KS – kaurene synthases
NNPP – nerilneril diphosphate
NPP – neril diphosphate
PT – prenyl transferase

**Chapter II**

SNP – Single Nucleotide Polimorphism
PCA – principal component analysis
bp – base pair
DNA – DNA deoxyribonucleic acid
RNA – ribonucleic acid
MAF – minor allele frequency
GO – Gene Ontology
IBD – Identity by Descent

**Chapter III**

ORF – open reading frame
CDS – coding sequences
Tm – melting temperature
Res – restriction enzymes sites
IMAC immobilized metal affinity chromatography
IPTG isopropil-p-D-tiogalactosido

GC–MS gas chromatography–mass spectrometry
SDS-PAGE – sodium dodecyl sulfate-polyacrylamide electrophoresis
I – induced
NI – non induced
MW – molecular weight
ladder – L
quantitative trait loci – QTLs

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## RESUMO

*Psidium cattleyanum* Sabine, comumente conhecido como araçá, é uma frutífera neotropical com notável capacidade de adaptação à diversos ambientes. A espécie é dividida em dois morfotipos (indivíduos com variações morfológicas e/ou cromáticas). Um deles apresenta coloração dos frutos vermelha quando em estado de maturação, enquanto o outro morfotipo exibe frutos de coloração amarela. A espécie também destaca-se por apresentar um complexo poliplóide, caracterizado por indivíduos contendo números variados de conjuntos cromossômicos (2n=3x=33 a 2n=12x=132). Além da variabilidade em seu conteúdo de DNA, e sua morfologia, apresenta variação na constituição de metabolitos secundários. Estudos anteriores detectaram diferenças no perfil de óleo entre o araçá amarelo e o vermelho e isso pode ser devido a diferentes técnicas de isolamento, ou ao local de coleta. No óleo essencial do araçá, os terpenóides podem ser produzidos pelo metabolismo primário e desempenham papéis essenciais no crescimento, e desenvolvimento da planta. Estes metabólitos quando produzidos pelo metabolismo secundário, medeiam interações com polinizadores e fungos, defendem a planta contra patógenos e herbívoros, protegem do estresse térmico, desempenhando importantes papéis ecológicos na interação planta-ambiente. Devido à sua importância econômica, foram realizados grandes esforços para investigar os mecanismos moleculares que determinam a diversidade estrutural dos genes da terpeno sintase (TPS) em espécies de frutos capsulares secos da família Myrtaceae, que apresentam versatilidade funcional e concentrações elevadas de terpenos foliares. Estes genes sintetizam enzimas que catalisam a condensação, ciclização, ou rearranjos, convertendo os precursores geranil difosfato (GPP), neril difosfato (NPP) ou farnesil difosfato (FPP) em mono (C10), sesqui- (C15), di- (C20), tri-(C30), tetra-(C40) e politerpenoides (com mais de 40 átomos de carbono). Nas espécies frutíferas neotropicais a influência genética na produção de óleos essenciais é desconhecida. Assim, a presente tese tem o objetivo de investigar as diferenças na via de biossíntese dos terpenoides entre os morfotipos vermelho e amarelo, bem como a sua biossíntese em indivíduos com variação no conteúdo de DNA, visando contribuir para a compreensão da diversificação e adaptação desta planta que caracteriza-se por expandir se rapidamente ambientes naturais, encontrada nos tropicos e subtropicos, considerada invasora em algumas regiões. No primeiro capítulo, identificamos e caracterizamos a família de genes terpeno sintase (TPS) e exploramos o perfil do óleo essencial do araçá vermelho e amarelo. Os genes da subfamília TPS-b1 destacam-se por serem responsáveis pela biossíntese de monoterpenos, compostos majoritários encontrados no perfil do óleo de ambos os morfotipos. Notavelmente, o óleo essencial do araçá vermelho foi dominado por 1,8-cineol e linalol, enquanto o araçá amarelo teve uma maior proporção de α-pineno. Foram realizadas análises de expressão gênica, que indicam padrões de expressão distintos, sugerindo regulação genética influenciando o conteúdo de óleo essencial. E por fim, análises filogenéticas e de seleção foram realizadas entre espécies de Myrtaceae, a fim de elucidar os mecanismos que explicam a variabilidade química dos compostos do óleo essencial, juntamente com a evolução da tribo Myrteae. No segundo capítulo, exploramos marcadores de polimorfismos de nucleotídeo único (SNP) em *P. cattleyanum*. As análises de ontologia genêtica das sequências deridadas de DarTseq (STAGs) ancoradas em transcritos revelaram variantes genéticas em vias enriquecidas associadas à biosíntese de terpenos, indicando a potencial influência regulatória dessas vias sobre características adaptativas no araçá. Além disso, foi possível obter um conjunto de marcadores conservados, polimórficos e funcionais para a espécie. No terceiro capítulo, iniciamos estudos de clonagem e ensaio enzimático em genes-chave da via de biossíntese dos terpenos. Os resultados desta pesquisa oferecem informações sobre a organização genética do metabolismo dos terpenos no araçá e possíveis papeis em mecanismos de adaptação nessa espécie frutífera neotropical, contribuindo para aplicações de conservação e melhoramento molecular, especialmente frente as mudanças climáticas.

## ABSTRACT

*Psidium cattleyanum* Sabine, commonly known as araçá, is a neotropical fruit tree with a remarkable ability to adapt to different environments. The species is divided into two morphotypes (individuals with morphological and/or colour variations). One of them has red fruit colour when ripe, while the other morphotype has yellow fruit. The species also stands out for having a polyploid complex, characterised by individuals containing varying numbers of chromosome sets ($2n=3x=33$ to $2n=12x=132$). In addition to the variability in its DNA content and morphology, it also shows variation in the constitution of secondary metabolites. Previous studies have detected differences in the oil profile between yellow and red araçá and this may be due to different isolation techniques or the collection site. In the essential oil of araçá, terpenoids can be produced by primary metabolism and play essential roles in the growth and development of the plant. When produced by the secondary metabolism, these metabolites mediate interactions with pollinators and fungi, defend the plant against pathogens and herbivores, protect it from heat stress and play important ecological roles in plant-environment interaction. Due to their economic importance, great efforts have been made to investigate the molecular mechanisms that determine the structural diversity of terpene synthase (TPS) genes in dried capsular fruit species of the Myrtaceae family, which show functional versatility and high concentrations of leaf terpenes. These genes synthesize enzymes that catalyse condensation, cyclisation or rearrangements, converting the precursors geranyl diphosphate (GPP), neryl diphosphate (NPP) or farnesyl diphosphate (FPP) into mono- (C10), sesqui- (C15), di- (C20), tri- (C30), tetra- (C40) and polyterpenoids (with more than 40 carbon atoms). In Neotropical fruit species, the genetic influence on the production of essential oils is unknown. The aim of this thesis is to investigate the differences in the terpenoid biosynthesis pathway between the red and yellow morphotypes, as well as their biosynthesis in individuals with variations in DNA content, to understanding the diversification and adaptation of this plant, which is characterized by rapidly expanding natural environments, found in the tropics and subtropics, and considered invasive in some regions. In the first chapter, we identified and characterized the terpene synthase (TPS) gene family and explored the essential oil profile of red and yellow araçá. The TPS-b1 subfamily genes stand out for being responsible for the biosynthesis of monoterpenes, the majority compounds found in the oil profile of both morphotypes. Notably, the essential oil of red araçá was dominated by 1,8-cineol and linalool, while yellow araçá had a higher proportion of α-pinene. Gene expression analyses were carried

out, indicating distinct expression patterns, suggesting genetic regulation influencing essential oil content. Finally, phylogenetic and selection analyses were carried out among Myrtaceae species to elucidate the mechanisms that explain the chemical variability of essential oil compounds, along with the evolution of the Myrteae tribe. In the second chapter, we explore single nucleotide polymorphism (SNP) markers in *P. cattleyanum.* Gene ontology analyses of DarTseq-derived sequences (STAGs) highlighted genetic variants associated with transcripts in pathways involved with terpene biosynthesis, underscoring their regulatory influence on adaptative traits of the species. In addition, we provide a collection of conserved, polymorphic, and functional markers specific for the genus. In the third chapter, we initiate cloning and enzyme assay studies on key genes in the terpene biosynthesis pathway. The findings of this research provide information on the genetic organization of terpene metabolism in araçá and possible roles in adaptation mechanisms in this neotropical fruit species, contributing to conservation and molecular breeding applications, especially in the context of climate change.

## 1. OVERVIEW

The chemical composition of a plant's essential oil is a complex mixture of volatile, lipophilic, soluble in organic solvents, and fragrant compounds obtained from aromatic plants (Yang et al., 2020). They are characterized by their low molecular weight and hydrophobic nature (Sell et al., 2020). The constituents of essential oils are mainly of terpenoid origin and vary continuously over a wide absolute range (from a small proportion to almost all of the extractable oil) (Gershenzon et al., 2007). These terpenes include monoterpenes and sesquiterpenes in the form of hydrocarbons and their oxygenated derivatives (alcohols, aldehydes, ketones, esters, ethers, peroxides, and phenols). In some cases, other compounds such as phenylpropanoids, fatty acids, and their esters, and, more rarely, nitrogen- and sulfur-containing compounds are also present (Zuzart et al., 2015; Alves-Silva, 2022).

The essential oil composition and content in plants depend on several factors, such as sexual, seasonal, ontogenetic, ecological, and environmental properties (Khan et al., 2023). Also, we know that this variation is controlled on a genetic level. Genetic variations may result in the expression of different metabolic pathways, and, consequently, quantitative and qualitative variations in essential oil composition may occur. This indicates that individuals of the same botanical species, with the same genome and phenotype, may differ in chemical composition or chemical group (chemotype, CT), and thus biological activities (Zuzart et al., 2015; Benomari et al., 2023).

The Myrtaceae family is known for storing high amounts of volatile terpenes with great CT variation and industrial interest (Külheim et al., 2015; Webb et al., 2014; Padovan et al., 2014).

As secondary metabolites, these molecules regulate ecological interactions between plants and their environments, affecting a plant's susceptibility to specialised herbivores and pathogens (Santos Pereira et al., 2018; Unsicker et al., 2009), or its tolerance to adverse abiotic environments (Suni et al., 2008; Vickers et al., 2009). Especially in neotropical species with fleshy berries, they also act to attract pollinators and seed frugivores (Nevo et al., 2020).

The remarkable terpenoid diversity among plants can be controlled by terpene synthases (TPS) enzymes, which catalyze terpenoid biosynthesis. The genes encoding these enzymes are believed to evolve by tandem duplication and specialization, and throughout their evolutionary history, selective pressures have given rise to a highly expanded and diverged TPS gene family in the plant kingdom (Chen et al., 2011).

Despite the molecular mechanisms and biosynthetic pathways of terpenoid synthesis have been comprehensively elucidated (Vranová et al., 2013; Tholl, 2015), the genetic basis of CT pattern, its segregation in wild populations and the evolutionary scenario that gave rise to this diversity of molecules remains elusive in most cases. Obtaining direct empirical data in species of the *Psidium* genus is a great challenge because the diploid state is documented in a low percentage of species (Marques et al., 2016). Furthermore, in some species, such as *Psidium cattleyanum*, only polyploid records are known.

Considering that measuring the extent of how polyploidy can affect the biosynthesis of the essential oil, the diversification and ecology can be even more complicated, because *P. cattleyanum* exhibits polyploid records ranging from 2n = 22 = 2x to 132 = 12x (Costa and Forni-Martins, 2006; Éder and Silva et al., 2007; Marques et al., 2016; Tuler et al., 2019; Machado et al., 2020). The explanation lies in the number of loci and alleles affecting the trait, the degree to which these loci interact (e.g. epistasis with competition for a common precursor molecule), the mode of gene action (allelic interaction), whether there is pleiotropy (e.g. a multi-product TPS), and the degree to which a phenotype interacts with the environment. In other words, the trait's genetic architecture (Soares et al., 2021).

For example, suppose only one loci control CT and it is a diploid plant, it has alleles (Aa/A1A2) at each locus. In that case, there are three possible genotype combinations in a bialelic segregation, and four possible genotype combinations in a multiallelic segregation.  This scenario could be complicated if we have hexaploid, for example, because there are 20 gametes, seven possible genotypes (biallelic segregation), or 400 possible genotype combinations (multialelic segregation). Hence, the question arises: what are the resulting phenotypes observed in araça morphotypes, and what are the genetic factors that contribute to regulating and maintaining this CT diversity (Soares et al., 2021).

In addition, each TPS has a specific product profile that it will produce, given the abundance of a precursor. With two enzymes competing for the precursor, predicting the final profile is even more challenging (Vattekkatte et al., 2020). Another relevant question to be unravelled is the pattern of terpenoid composition. Could it be shaped by differential selection for chemistry in different environments and have an adaptive value, or is this arrangement the product of a sequence of chance events dispersing polymorphisms with no adaptive value?

In this thesis, I present three manuscripts that contribute to advancing studies on the biosynthesis pathway of essential oils in *P. cattleyanum*. In order to unravel the factors controlling morphotypes on a molecular level, in chapter I, the TPS gene family and their organization were identified. Genomic regions associated with changes in terpenoid composition were explored, revealing candidate genes and their connections with gene expression profiles. Also, the phylogenetic and positive selection analysis was used to speculate upon evolutionary scenarios to explain the CT distribution.

In the second chapter, we try to study the effects of polymorphism and DNA variation in the terpene biosynthesis pathway and terpene yield. The SNP in functional genes, especially for exonic non-synonymous mutations, could be used to develop potential molecular markers for functional gene mapping and genetic improvement for terpene biosynthesis.

In the third chapter, we commence the functional characterization of key genes involved in terpene biosynthesis. Functional studies involving the characterization of TPS genes and their possible regulation in different morphotypes can help elucidate the patterns found in this thesis.

## 2. GENERAL INTRODUCTION AND LITERATURE REVIEW

### 2.1 *Psidium cattleyanum*: a polyploid complex

*Psidium* is a neotropical genus of the Myrtaceae family with about 95 species (WCSP, 2023). It is a monophyletic genus with a rapid diversification rate (Vasconcelos et al. 2017). There are around 60 species in Brazil, distributed in various vegetation formations (Tuler et al., 2023). *Psidium* species are recent polyploids due to the relatively recent radiation of the tribe around

9.9–20.8 million years ago, and it stands out as a key characteristic for the wide geographical distribution and success in colonizing diverse environments (Costa & Forni-Martins, 2006; Marques et al. 2016; Vasconcelos et al. 2017; Tuler et al. 2019; Machado et al. 2020).

Since adverse environmental conditions favor the meiotic irregularities and the union of unreduced gametes (Mason et al., 2015), the frequency of polyploids likely increases in these areas (Fox et al. 2020). This is the case of *P. cattleyanum,* an edible fruit plant that occurs in the Atlantic Forest from the northeast of Brazil (Bahia) to the north of Uruguay (Endringer et al., 2023). It was introduced in several parts of the world and has become a serious invader, suppressing native vegetation and causing ecological disturbance (Mbobo et al., 2022; Tassin et al., 2006; Enoki et al., 2017).

Chromosome numbers of 2n = 33, 44, 46, 48, 55, 58, 66, 77, 82, 88, 99, 110, and 132 have already been reported, originating from the basic chromosomal number of x = 11 (Marques et al., 2016; Costa and Forni-Martins 2006; Atchison 1947; Hirano & Nakazone, 1969; Singhal et al. 1984; Raseira & Raseira, 1996; Costa, 2009; Vázques, 2014; Souza et al., 2014; Éder e Silva et al., 2007; Marques et al., 2016; Souza-Pérez and Speroni 2017; Tuler et al., 2019; Machado et al., 2020; Machado et al., 2021). In this species, the high ploidy (11x and 12x) were associated with tolerance to higher solar incidence, temperature, and less precipitation, occupying a distinct ecological niche (Machado et al., 2022). The ripe fruits of *P. cattleyanum* species present red and yellow epicarps, divided into two morphotypes: the red guava, and yellow guava (Chalannavar et al., 2013). Previous studies have failed to associate certain ploidy levels with one of the two fruit colors (Machado et al., 2020).

The reproductive strategies of *P. cattleyanum* include both autogamy and allogamy, with a preference for allogamy (Oliveira et al., 2020), and exhibits characteristics of apomixis (Souza-Pérez et al., 2021). The presence of several reproductive strategies generates a great diversity of ploidy levels, characterizing it as a polyploid complex and could potentially explain the success of *P. cattleyanum* to colonize different habitats (Machado et al. 2020).

Machado et al. (2022) provided insights about polyploid speciation at the intraspecific level in *P. cattelyanum*, observing low gene flow in 12 natural populations accessed by microsatellite markers, high rates of differentiation between populations/cytotypes (different ploidy levels) and high niche divergence. They also pointed out that in *P. cattleyanum*, the lower rates of genetic diversity in high ploidy cytotypes may be related to the species' asexual reproduction characteristics, geographic isolation, and intraspecific divergence of environmental niche to expand the geographical range.

## 2.2 Plant scent and terpene biosynthesis in the Myrtaceae family

Terpenoids belong to a diverse group of natural chemicals. They are among the most versatile group of plant natural products, having more than 80,000 different chemicals with a variety of structural types (Christianson et al., 2017). Most terpene synthase enzymes catalyze the conversion of some common substrates into diverse terpene structures found in plant essential oils, mainly through reaction mechanisms involving the formation of intermediate carbocations, cyclizations, hydride shifts, skeleton rearrangements, and different termination steps (Jiang et al., 2019; Christianson et al., 2006). The products of these enzymes can further be modified by oxygenation through the action of cytochrome P450 monooxygenases or methylation by methyltransferases to form additional compounds (Figure 1; Fähnrich et al., 2011).

The biosynthesis of terpenes starts with two five-carbon molecules in their skeleton: the allylic dimethylallyl diphosphate (DMAPP) and its isomer homoallylic isopentenyl diphosphate (IPP) (Figure 1). These building blocks are sequentially assembled to produce linear chains of varying lengths, to form geranyl diphosphate (GPP), the precursor of monoterpenes (C10), trans-geranylgeranyl diphosphate (GGPP), the precursor of diterpenes and tetraterpenes (C20;C40) such as carotenoids, and geranylfarnesyl diphosphate (GFPP, C25) the precursor for sesterterpenoids biosynthesis (Nagegowda et al., 2020). In plants, these precursors are formed in the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids (Figure 1; Rudolf et al., 2020). On the other hand, the mevalonic acid (MVA) pathway is responsible for producing trans-farnesyl diphosphate (FPP), used in the synthesis of sesquiterpenes (C15) and triterpenes (C30), in the cytosol and for terpenoid biosynthesis in mitochondria, e.g. ubiquinones, polyprenols (Figure 1; Vranová et al., 2013). Studies have shown that cross-talk between these pathways makes terpenoids with isoprene units of mixed origins and substrate specificity even broader (Pazouki & Niinemets, 2016; Jia et al., 2016; Nagegowda et al., 2020).

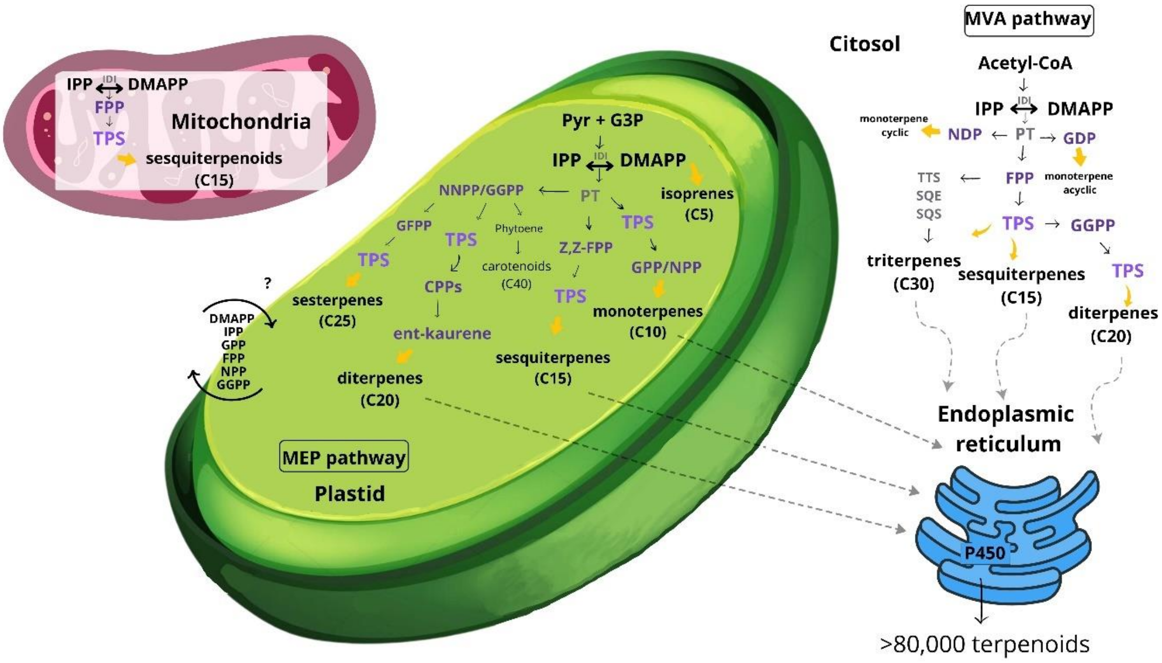

Figure 1. General scheme of terpene biosynthesis pathways in plants. All terpenoids are derived from two isomeric five-carbon precursors, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP). Condensation of IPP and DMAPP produces prenyl diphosphate precursors highlighted in blue (FPP farnesyl diphosphate, GPP geranyl diphosphate, NPP neril diphosphate, GFPP geranylfarnesyl diphosphate, NNPP nerilneril diphosphate, GGPP geranylgeranyl diphosphate). The TPS genes encoding the enzymes are highlighted in purple, and the main products formed are in bold. IDI isopentenyl diphosphate isomerase, PT prenyl transferase, IP isopentenyl monophosphate, CPPs ent-copalyl diphosphate (CPP) synthases.

Much of the current research has focused on identifying genes encoding terpene synthase (TPS) enzymes in plants, which can control the chemodiversity of terpenes and are responsible for the unique composition of each taxon (Karunanithi et al., 2019; Zhou and Pichersky, 2020). Advances in research include studies involving the identification and classification of TPS genes in bacteria, fungi, non-vascular land plants (liverworts), bryophytes, vascular plants, including gymnosperms and angiosperms, insects, but no TPS genes were detected in algae and vertebrate animals (Jiang et al., 2019; Zarley et al., 2023).

Terpene synthase genes have two functional domains: the N-terminal (containing the β and γ domains, with Pfam ID PF01397) and the C-terminal (or α domain, with Pfam ID PF03936). Based on these domains, genes can be full-length, containing both domains in the gene and protein sequence, or partial, containing only one of the two domains (Finn et al., 2010; Zhou and Pichersky, 2020; Chen et al., 2021). In plants, the loss of one of the domains often occurs in many species, likely triggered by partial duplication mechanisms. The functionality of these TPSs with a single domain is still unknown (Jiang et al., 2019).

Based on the reaction mechanism and the products formed, TPS enzymes can be classified into two classes. Class I enzymes active sites, which are located in the C-terminal region, are characterized by highly conserved aspartate-rich DDxxD and “NSE/DTE” motifs found within an “α-domain” (Figure 2). These two motifs flank the entrance to the active site and are responsible for assisting in the positioning of the diphosphate substrate, coordinating divalent ions and water molecules, as well as stabilizing the active site (Christianson et al., 2006). In contrast, the active site of Class II enzymes presents a “DxDD” motif, located in the N-terminal region between a pair of alphahelical double-barrel domains, “β-domain” and “γ-domain” (Yang et al., 2020; Zhou and Pichersky, 2020; Jia et al., 2022; Figure 2). The N-terminal domain also can have a conserved motif RRX8W (R, arginine; W, tryptophan; X, alternate amino acid), which acts in initiating the cyclization or isomerization reaction (Williams et al., 1998) or in stabilizing the protein through electrostatic interactions (Hyatt et al., 2007).

According to their phylogenetic relationships, the TPS gene family is divided into seven major clades (or subfamilies), designated TPS-a through TPS-g (Chen et al., 2011). Involved in the synthesis of primary metabolites, and belonging to the ancestral clade, the TPS-c subfamily conserved in land plants, is characterized by the "DXDD" motif and encodes copalyl diphosphate synthases (CPS), kaurene synthases (KS) related to the production of ent-kaurene, a precursor of gibberellins, and other diterpene synthases (diTPS), precursors of diterpenes. There are also the TPS-e/f subfamilies, grouped into a single clade and conserved in vascular plants, which encode both the "DXDD" and "DDXXD" motifs, encodes copalyl diphosphate/kaurene synthases, which are critical enzymes for the production of gibberellic acid. Lastly, TPS-h is found only in *Sellaginella moellendorffii*, which synthesizes diterpenes and TPS-d subfamily specific to gymnosperms and *Selaginella* spp., and it produces mono-, sesqui-, and diterpenes (Chen et al., 2011; Külheim, 2015).

The angiosperm TPS involved in the secondary metabolism of plants are encoded by multiple-gene copies that arose by duplication and then provided the basis for diversification (Figure 2). It includes TPS-a, which encodes only sesquiterpenes found in eudicots and monocots. There is also the TPS-b subfamily, which produces monoterpenes and is subdivided into TPS-b1, which produces cyclic monoterpenes, and TPS-b2, which produces isoprenes (C5) and ocimenes (acyclic monoterpenes). Lastly, the TPS-g subfamily acts in the biosynthesis of cyclic/acyclic monoterpenes, sesqui-, and diterpenes. The TPS-g subfamily is closely related to TPS-b but lacks the conserved R(R)X8W motif in its encoded proteins (Chen et al., 2011; Külheim, 2015).

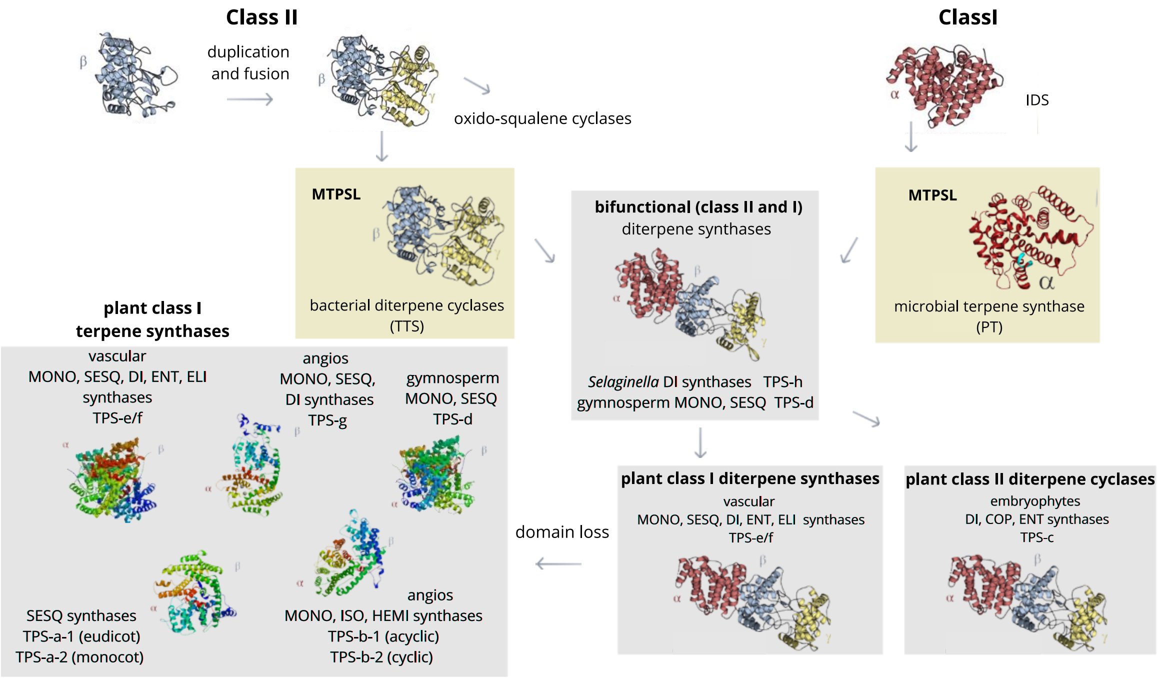
Class II
duplication and fusion
β
γ
oxido-squalene cyclases
ClassI
α
IDS
MTPSL
bacterial diterpene cyclases (TTS)
bifunctional (class II and I) diterpene synthases
*Selaginella* DI synthases TPS-h
gymnosperm MONO, SESQ TPS-d
MTPSL
microbial terpene synthase (PT)
plant class I terpene synthases
vascular
MONO, SESQ, DI, ENT, ELI synthases
TPS-e/f
angios
MONO, SESQ, DI synthases
TPS-g
gymnosperm
MONO, SESQ
TPS-d
SESQ synthases
TPS-a-1 (eudicot)
TPS-a-2 (monocot)
angios
MONO, ISO, HEMI synthases
TPS-b-1 (acyclic)
TPS-b-2 (cyclic)
domain loss
plant class I diterpene synthases
vascular
MONO, SESQ, DI, ENT, ELI synthases
TPS-e/f
plant class II diterpene cyclases
embryophytes
DI, COP, ENT synthases
TPS-c

Figure 2. Evolutionary origin, function, and taxon distribution of plant terpene synthases. The ancestral TPS gene may have originated through fusion or horizontal gene transfer (HGT) of a class I bacterial α and class II γβ-domain proteins. Fusion of the ancestral monofunctional genes will have given rise to bifunctional TPS containing three helical domains (αβγ). Duplication and subsequent loss of activity in the α- and βγ-domains, respectively, lead to the emergence of monofunctional plant class I and class II. Through further loss of the γ-domain and various neo-functionalizations and specializations, the large classes of βα-domain class mono- and sesqui-TPSs will have arisen. Legend: COP - Copalyl diphosphate synthase; DI - Diterpene synthase; ENT - Ent-kaurene synthase; ELI - Elinalool synthase; HEM - Hemiterpene; ISP - Isoprene synthase; MONO - Monoterpene synthase; SESQ - Sesquiterpene synthase.

For most dicots and monocots, the TPS-a subfamily is the largest group in the TPS gene family, with some exceptions, such as *Carica papaya* and *Citrus clementina*, which lost the TPS-a subfamily during the long evolutionary period. The genome of *Arabidopsis thaliana* has 33 complete TPS genes (Tholl & Lee, 2011), while economically important plants such as grape (*Vitis vinifera*), tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), apple (*Malus domestica*), and carrot (*Daucus carota*) have been found to have 57, 33, 40, 44, and 65 TPS genes, respectively (Martin et al., 2010; Falara et al., 2011; Chen et al., 2020; Nieuwenhuizen et l., 2013; Keilwagen et al., 2017).

Among the eudicots, species of the Myrtaceae family from the Australian continent, belonging to the Eucalypteae tribe, with dry capsule fruits, stand out for having the largest number of TPS genes. This group of plants includes *Eucalyptus grandis* with 70 complete genes, *Eucalyptus globulus* with 69 complete genes, and *Corymbia citriodora* with 89 complete genes. In the three Eucalyptus species, approximately 46% of the genes are TPS-a, about 34% are TPS-b, and ~10% are TPS-g (Myburg et al., 2014; Butler et al., 2018). *Eucalyptus grandis* seems to have a gene duplication rate 3-5 times higher than other eudicot species, such as *Arabidopsis thaliana* and *Populus trichocarpa* (Myburg et al., 2014), a factor that may have contributed to the expansion of TPS genes in eucalyptus.

Another Australian Myrtaceae species, Mānuka (kahikātoa) (*Leptospermum scoparium*), naturally found in New Zealand, has 49 TPS genes (23 TPS-a, 10 TPS-b1, 6 TPS-b2, 1 TPS-c, 5 TPS-e/f, 3 TPS-g) (Thrimawithana et al., 2019). In *Melaleuca alternifolia*, which diverged from the Eucalyptus genus about 68 million years ago, only 37 TPS genes were identified, with the TPS-b1 subfamily proportionally larger compared to other species of the family (Calvert et al., 2018; Thornhill et al., 2015).

So far, there are no studies with genomes of species from the Myrteae tribe, with about 2500 species and 51 genera (Vasconcelos et al., 2017). There is a wide diversity of terpenes in Myrtaceae leaves, with α-Pinene and 1,8-cineole being the most common compounds found in the species. Since many samples contain high levels of α-Pinene, this compound is likely the ancestral leaf CT of all Myrtaceae. In contrast, leaf 1,8-cineole appears to be a defining feature of only some tribes. The reaction cascade leading to these two compounds includes the same carbocation intermediate (Kampranis et al., 2019), suggesting that only a small change in the amino acid sequence of an enzyme could allow significant production of both compounds (Padovan et al., 2014).

Transcriptomic studies involving the characterization of terpene synthase genes in the Myrteae tribe have only been performed in *Eugenia uniflora*, with the identification of four unigenes (Guzman et al., 2014), and *Rhodomyrtus tomentos*a, a perennial medicinal plant widely used in China, with identification of 138 candidate unigenes involved in terpene biosynthesis.

Studies that include representative genera of this tribe can help understand the patterns of foliar terpene diversity in Myrtaceae (He et al., 2018).

The leaf profile of terpenes presents striking variations between different cultivars or morphotypes within a single species, or between different species and populations (Köllner et al., 2004; Souza et al. 2017). The main factors related to this differential modulation are individual genetic variability derived from different genes or allelic variations in a locus and differential expression (Padovan et al., 2010; Padovan et al., 2012; Külheim et al., 2015; Webb, et al. 2014), with the latter being more complex and difficult to study as it involves genetic and epigenetic mechanisms that vary not only between individuals but also between developmental stages, cell types, and are strongly influenced by the environment (Bustos-Segura et al., 2017). Additional factors that could be involved include the stability of proteins involved in the pathway, differential enzymatic kinetics, post-translational regulation, and the transport system of terpenes. Terpenes are actively transported between cells, especially in species with specialized storage and secretion structures (Lange et al., 2019).

Consequently, quantitative and qualitative variations in essential oil composition may occur. When significant differences are found, an intraspecific category (CT) is defined (Zuzarte et al., 2015). The identification of genes related to secondary metabolism, along with specific taxonomic distribution patterns in lineages, becomes essential for studying the evolutionary dynamics in such a short time of divergence among Myrtaceae, providing indications of the success of distribution and adaptation of these species (de Oliveira Bünger et al., 2016).

### 2.3 Natural selection favoring advantageous traits and molecular or functional specificity

In natural populations, beneficial mutations frequently emerge in a population, increasing its frequency until fixation. The consecutive fixation of beneficial alleles enhances the overall fitness of the population. As a result of a challenge imposed by the environment, this change may be adaptive and positively selected, ensuring the persistence of this trait in the population through its descendants (Barghi et al., 2020).

However, once this occurs, the direction of selection dynamics shifts to maintain the fitness advantage conferred on the altered characteristic. This leads to the exclusion of other alterations unless they are evolutionarily neutral or advantageous. Consequently, positive selection generally transforms into purifying (negative) selection. When different selective regimes favor contrasting phenotypes, and exchanges between fitness components can maintain genetic variation if gains in one aspect of fitness are balanced by losses in others. These distinct forms of selection imprint their signatures on the genome and can be detected with statistical tools of molecular population genetics (Kroymann, 2011).

Different approaches at the genome-wide level are commonly used to identify footprints of selection (Biswas and Akey, 2006, Oleksyk et al., 2010). Examples where positive selection is one of the evolutionary forces driving the diversification of plant secondary metabolism, include the sesquiterpene TPS1 ortholog in rice (OryzaTPS1). Some amino acid residues within or around the active site cavity have been altered, with probable impacts on fitness, leading to changes in terpene emission profiles and exhibiting promiscuity of the product attributed to its intermediate carbocation reaction mechanisms. Therefore, changes in rice sesquiterpene emission profiles caused by functional divergence of OryzaTPS1 may reflect the challenges imposed by the environment, changes in spectra of herbivorous insects, and their natural enemies present in different species' environments (Chen et al., 2014).

Another case involves chrysanthemyl diphosphate synthase (CDS) which catalyzes the formation of chrysanthemyl diphosphate (CDP) and plays a crucial role in defence against insects and herbivores, being the most widely used plant-derived pesticide (Liu et al., 2012). CDS enzyme is involved in producing pyrethrins and irregular monoterpenes, resulting from the duplication of farnesyl diphosphate synthase (FDS) genes. FDS and its products are found in prokaryotic and eukaryotic organisms, with copy numbers varying from one in grapes to five in rice. In contrast, CDS products are only present in *Anthemideae*.

Another example of how positive selection contributes to the acquisition of beneficial mutations during the evolutionary process involving TPS is found in triterpenoid genes, where positively selected representative sites located in the catalytic region. These mutational patterns contribute to functional changes in proteins and diversification of the farnesyl pyrophosphate synthase (FPS) genes among land plants, likely enhancing enzyme activity in the triterpenoid biosynthesis pathway when plants adapt to terrestrial environments. Selection events in coding sequences can indicate how the diversity of FPS genes among plants can be attributed to functional divergence due to plant adaptation to their niche environments (Qian et al., 2017).

Future studies aiming to estimate the extent to which adaptive changes are reflected in the genome will need to incorporate comparisons of paralogous sequences, given the importance of gene duplications for the diversification of plant secondary metabolism (Kroymann, 2011).

Moreover, most work primarily focuses on "key" sites that determine variability in a single biosynthetic step. However, a challenging question for the future is how entire new biochemical pathways arise in plant secondary metabolism (Kroymann, 2011).

## 2.4 Molecular Cloning and Heterologous Expression of Terpenoid Synthase Genes

As complete genomes have been sequenced, the identification of TPS genes has been conducted by different research groups for various species. Sequence comparison using phylogeny in the TPS gene family may allow placement of a new sequence into one of the six subfamilies and give clues to the type of terpene they produced (C10, C15, or C20). But the fact that the sequences encode functionally identical or highly similar enzymes, combined to the fact that the enzymes are multi-product, does not allow the precise characterization of the function (Keeling et al., 2011). The utilization of heterologous expression makes it possible to obtain active recombinant proteins from cloned synthases, and to produce high yields of pure proteins, enabling the identification of the enzymatic products of the enzymatic reaction formed (Ma et al., 2021).

Together with single-product enzymes, molecular cloning and functional expression have yielded insights about multi-product enzymes, such as δ-selinene synthase and γ-humulene synthase from *Abies grandis*, with respectively, 34 and 52 different sesquiterpenes, whereas a third synthase produces only (E)-a-bisabolene (Steele et al., 1998). Until now, in the Myrtaceae family, the structures of only a few terpene synthase genes have been described using this technology. The research is still limited to a few economically important species of dried capsular fruits species.

Functional expression in *Backhousia citriodora (*BcLS gene) in *E. coli* yielding an active enzyme capable of catalyzing the conversion of geranyl diphosphate to linalool and to lesser amounts of cyclic monoterpenes (Sugiura et al., 2011). Several functionally characterized genes in Eucalyptus spp. include isoprene synthases in *Eucalyptus globulus* (Sharkey et al., 2013), a bicyclogermacrene synthase, an isoledene synthase, and γ-terpinene synthase from *Eucalyptus grandis* (Külheim et al., 2015), as well as pinene and cineole synthases from *Eucalyptus polybractea* (Goodger et al., 2021; Kainer et al., 2019). These enzymes are all multi-product, producing between 5 and 15 terpenes.

Genes from other species have also been cloned, including isoprene synthases from *Metrosideros polymorpha* (Yeom et al., 2018) and a nerolidol, 1,8-cineole, β-ocimene, β-caryophyllene, and two viridiflorol synthases in *Melaleuca quinquenervia* (Hsieh et al., 2021). Lastly, sabinene hydrate, terpinolene, 1,8-cineole, isoprene and linalool from *Melaleuca alternifolia* (Shelton et al., 2004; Padovan et al., 2014; Sharkey et al., 2013).

Only one fleshy fruit from the Myrteae tribe, *Rhodomyrtus tomentosa,* originating from South–East Asia, has been characterized for TPS (He et al., 2018). Their study reveals enzyme activity *in vitro* with RtTPS1-4 mainly producing (+)-α-pinene and (+)-β-pinene, with GPP, while RtTPS1 and RtTPS3 are also active with FPP, producing β-caryophyllene, along with a smaller amount of α-humulene. Thus, it demonstrates the need for functional characterization of TPS genes in neotropical Myrtaceous fruit-bearing plants.

Terpenoid synthases are operationally soluble enzymes localized to the cytosol (sesquiterpene synthases) or plastids (monoterpene synthases and diterpene synthases), and they can be expressed functionally in prokaryotic, eukaryotic hosts and even plant cells (Ma et al., 2021). *E. coli and S. cerevisiae* are representative prokaryotic and eukaryotic expression systems that have been employed to examine the biological function of putative TPS genes from diverse organisms (Wang et al., 2021).

Finally, along with advancement of next-generation sequencing technologies, the information about functional TPS genes from fungi, plant, and even invertebrates is expanding, which provides valuable genetic components for enhancing production yields or diversifying the structural repertoire of terpenoids with promising bioactivity. Also, heterologous biosynthesis of natural products is one of the most active areas of research, which significantly reduces natural poverty, environmental pollution, and economic costs (Cravens et al., 2019; Zhu et al., 2021).

### 2.5 Effects of ploidy increase and later neofunctionalization of TPS genes

In general, it was observed an increase in essential oil yield in plant species due to the larger genome, evidencing the impact of the genomic changes (2n chromosome number and 2C nuclear value) in the secondary metabolism, which is a trait of ecological and economic importance. Experimentally, the tetraploid induction (2n = 4x = 72 chromosomes) in *Lippia integrifolia* (family Verbenaceae) increases the essential oil yield compared to diploids (2n = 2x = 36 chromosomes), in addition to larger leaves and trichomes, structures related to essential oil yield (Iannicelli et al., 2016). Additionally, Silva et al. (2023) showed essential oil yield increased in relation to 2C value and to GC% in *Psidium* species. Notably, *Psidium* *guajava* (diploid) possesses two and *Psidium guineense* (tetraploid) four copies of the one specific TPS gene, as well as eight and sixteen copies respectively of the conserved regions that occur in eight TPS genes.

In Myrtaceae family, the chemical variability of essential oils from diploid and autotetraploid germplasms (autotetraploid A and B) of *Eucalyptus benthamii* was characterized, showing monoterpenes such as 1,8-cineol, limonene, α-terpineol, and α-terpinyl acetate, which were not found previously in diploid germplasms. They also tested their larvicidal and allelopathic effects. Exploring the context of chemical diversity with bioassays, it was found that diploid germplasms were twice more lethal against *Aedes aegypti* larvae than autotetraploids. Despite the low toxicity of autotetraploid EOs against *A. aegypti* larvae, 1,8-cineole and α-terpinyl-acetate, present in autotetraploids EOs, were linked as biomarkers to myrtle rust disease (da Silva et al., 2021).

Qualitative differences in EOs were also described in other plants, such as *Trachyspermum ammi* L., in which the EOs extracted from diploid accessions did not present α-terpineol. In *C. limon* autotetraploids produced β-bisabolene, but this compound was absent in diploid EOs. The changes in EO composition are possibly associated with new gene expression profiles and enzyme activities, which modulate the biosynthesis of secondary metabolites (da Silva et al., 2021).

Mehari (et al., 2023) identified 84 and 86 TPS genes in two tetraploid cotton species *Gossypium hirsutum* and *Gossypium barbadense*, higher than in their ancestors, the diploids, *Gossypium arboreum* and *Gossypium raimondii,* with 70 and 64 TPS genes, respectively. They observed a total of 45 tandem duplicates, 147 segmental duplicates, and 539 whole-genome duplications (WGDs) during the expansion of the TPS gene family. Furthermore, all angiosperm lineages show vestiges of past rounds of WGD, and the consequent gene duplication. In addition, polyploids suffer changes in genomic structure and epigenetic remodeling after WGD events. Changes in scent patterns have been shown in some orchid polyploids. Such changes in floral traits could impact pollinator attraction and lead to differentiation in the pollinator spectrum, causing the isolation of diploids and polyploids and facilitating polyploid establishment (Picazo-Aragonés et al., 2020).

## 3. OBJECTIVES

### 3.1. GENERAL OBJECTIVES

Comprehensively investigate the terpene biosynthesis in *P. cattleyanum,* verifying the genetic contribution to the chemical diversity observed in different morphotypes.

### 3.2 SPECIFIC OBJECTIVES

- Describing terpene profiles in leaves *P. cattleyanum*, both red and yellow morphotypes.
- Predicting terpene synthase (TPS) genes based on sequence similarity and classifying them at the gene subfamily level in *P. cattleyanum.*
- Reconstruct the phylogenetic relationships of the TPS genes detected in species of the Myrtaceae family.
- Identify candidate regions showing signatures of positive selection in the genome of two morphotyes of *P. cattleyanum* at the TPS genes to help define the most interesting candidate regions that potentially contribute to the chemical diversity observed in these morphotypes.
- Quantify the abundance of transcripts in *P. cattleyanum* leaves and associate the differential expression of terpene synthase genes with the different CTs found in the essential oils of this species.
- Identify the genomic positions of a Single Nucleotide Polymorphisms (SNP) associated with expression regulation in *P. cattleyanum* and hold a functional significance for the natural individuals.
- Demonstrated polymorphisms situated within the coding region of TPS genes, which have the potential to impact protein function, in gene expression, thereby potentially affecting enzymatic activity, either by suppressing or enhancing it.
- Identify candidate genes for heterologous expression. To optimize the cloning, expression, purification and enzymatic characterization of recombinant TPS involved in the terpene biosynthesis in *P. cattleyanum*.

## 4. THESIS ORGANIZATION

The results obtained in this work will be presented in three chapters:

Chapter I: we conducted genome-wide identification, evolutionary and expression analyses of the terpene synthase gene (TPS) family in *P. cattleyanum* red guava, and yellow guava morphotypes. This work resulted in an article entitled "Genome-wide identification, expression profile and evolutionary relationships of TPS genes in the neotropical fruit tree species *Psidium cattleyanum*", published in the journal Scientific Reports.

Chapter II: we conducted a comparative transcriptomics-based analysis focusing on SNP markers associated with different DNA content. Therefore, we provided potential functional SNPs, elements and pathways associated. This article will be submitted in the journal Botanical Journal of the Linnean Society.

Chapter III: presents the preliminary results of the study obtained during a sandwich doctorate at Cardiff University - Wales, under the supervision of Prof Dr Hilary Rogers. It describes the initial characterization of the TPS genes responsible for the major compounds found in the oil of the two morphotypes. This is an article in preparation that is still in the experimental and data analysis phase.

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## CHAPTER I

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# Genome-wide identification, expression profile and evolutionary relationships of TPS genes in the neotropical fruit tree species *Psidium cattleyanum*

Drielli Canal [1], Frank Lino Guzman Escudero [2], Luiza Alves Mendes [3], Marcia Flores da Silva Ferreira [4] & Andreia Carina Turchetto-Zolet [1]✉

**Terpenoids are essential for plant growth, development, defense, and adaptation mechanisms. *Psidium cattleyanum* (Myrtaceae) is a fleshy fruit tree species endemic from Atlantic Forest, known for its pleasant fragrance and sweet taste, attributed to terpenoids in its leaves and fruits. In this study, we conducted genome-wide identification, evolutionary and expression analyses of the terpene synthase gene (TPS) family in *P. cattleyanum* red guava (var. *cattleyanum*), and yellow guava (var. *lucidum Hort.*) morphotypes. We identified 32 full-length TPS in red guava (RedTPS) and 30 in yellow guava (YlwTPS). We showed different expression patterns of TPS paralogous in the two morphotypes, suggesting the existence of distinct gene regulation mechanisms and their influence on the final essential oil content in both morphotypes. Moreover, the oil profile of red guava was dominated by 1,8-cineole and linalool and yellow guava was enriched in α-pinene, coincident in proportion to TPSb1 genes, which encode enzymes that produce cyclic monoterpenes, suggesting a lineage-specific subfamily expansion of this family. Finally, we identified amino acid residues near the catalytic center and functional areas under positive selection. Our findings provide valuable insights into the terpene biosynthesis in a Neotropical Myrtaceae species and their potential involvement in adaptation mechanisms.**

*Psidium cattleyanum* Sabine (Myrtaceae), commonly known as araçá, cattley guava, strawberry guava, and cherry guava, is a fleshy fruit belonging to the Neotropical Myrteae tribe (Myrtaceae). The species is native to the Atlantic Forest, where it has readily adapted to a variety of climates, is associated with wet forests across the t ropics[1], occurs in areas under stress conditions[2,3], and is considered among the worst invasive species[4,5].

The genus *Psidium* is rich in essential oils[6,7], stored in the leaf secretory cavities[8–10], and traditionally used for extraction, with inexpensive resources and potential uses in the pharmaceutical and medicine industries[2,11]. These essential oils regulate environmental processes and ecological interactions between organisms, such as defense against herbivores and pathogens[11,12], protection against abiotic environments[13,14] and attraction of pollinators, especially in neotropical species with fleshy berries that serve as a food source[15,16].

*Psidium cattleyanum* species is divided into two morphotypes. The ripe fruits of red and yellow guava present red and yellow epicarps, respectively[17]. They also exhibit differences in antioxidant activity and phenolic

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content[18], leaf morphology, and phytochemistry, size, and and habit[1]. Previous studies have also detected considerably different oil profiles of yellow and red guava, which was attributed to differences in isolation techniques or the area of collection[22–25]. However, the genetic and evolutionary factors that can induce modifications in the secondary metabolism of the plant in the two morphotypes of *P. cattleyanum* remain largely unknown. Therefore, the study of genes of the biosynthetic pathway of these compounds in this species is highly relevant.

Because Myrtaceae species exhibit the highest concentrations and functional versatility of foliar terpenes among plants, significant efforts have been made to investigate the molecular mechanisms determining the structural diversity of terpene synthase (TPS) genes in this family. However, to the best of our knowledge, there are still no studies of these genes in Neotropical Myrtaceae species. The TPS family catalyzes the cyclization and rearrangement of geranyl diphosphate (GPP) or its cis-isomer neryl diphosphate (NPP) into monoterpenes (C10) and trans-geranyl diphosphate (GGPP) into diterpenes (C20) in the plastidic 2C-metil-D-eritritol-4-fosfato (MEP) pathway. In addition, farnesyl diphosphate (FPP) is converted into sesquiterpenes (C15) and triterpenes (C30) via the mevalonate (MVA) pathway in the cytosol, endoplasmic reticulum, and peroxisomes[26–29]. TPS controls not only the terpene chemodiversity present in plants but is also responsible for the unique composition of each taxon[30].

Recent studies have revealed that, among those with dried capsular fruits, the species of the Eucalypteae tribe, including *Eucalyptus grandis*, *E. globulus,* and *Corymbia citriodora*, contain the largest number of complete TPS genes reported in eudicotyledons (70, 69, and 89 complete genes, respectively). This is due to the key role of terpenes in defense over their long lifespans[26,29,31]. Terpene synthase genes have also been identified in *Melaleuca alternifolia* and *Leptospermum scoparium,* with 37 and 49 putative TPS genes, respectively[32,33]. The oil profile patterns in foliar terpenes across this species, which are common in forest woodlands, are the monoterpenes α-pinene and 1,8-cineole. Instead, fleshy-fruited species from Myrtaceae family have low foliar 1,8-cineole concentrations, with a greater diversity of abundant foliar sesquiterpenes[34].

In the present study, we aimed to conduct a comprehensive genome-wide analysis of TPS genes in *P. cattleyanum* to gain insights into the underlying mechanisms responsible for the differences in terpenoid biosynthesis and in the essential oil profiles in two morphotypes. Based on genomic and transcriptomic data, we identified the TPS gene repertoire and revealed its expression pattern in two *P. cathleyanum* morphotypes. We also examined the expansion and diversification of the TPS gene family among the Myrtaceae species. Finally, we investigated key amino acids using positive selection analysis to understand their effects on product specificity and consequently explain the chemical variability of the essential oil compounds. Our findings provide a foundation for deciphering TPS biosynthesis in *P. cattleyanum* and diversification of the two morphotypes. This knowledge will contribute to further studies on natural populations and the evolution of the Myrteae tribe, providing evidence of the successful distribution and adaptation of these species.

## Results

### Genome-wide identification of putative terpene synthases.

We performed a genome-wide sequence homology search to identify the complete repertoire of TPS genes across the *Psidium cattleyanum* morphotype genomes. The genomes were assembled separately for comparison. Based on the conservation of hidden Markov model (HMM) profiles and BLAST searches, we identified 110 loci in the red genome (RedTPS) (Supplementary Table S1) and 106 loci in the yellow genome (YlwTPS) (Supplementary Table S2). Three RedTPS and seven YlwTPS sequences were excluded from further analysis due to the presence of premature stop codons, lack of both C and N terminal domains, presence of less than three exons, and one gene that presented 38 exons likely to be pseudogenes, partial genes, or assembly errors (Supplementary Table S3). In YlwTPS, 28 lost the C-terminal domain (PF03936), 45 lost the N-terminal domain (PF01397), and 33 of them contained two domains. In RedTPS, 27 lost the PF03936 domain, 49 lost the PF01397 domain, and 34 of them contained two domains (Supplementary Tables S1, S2). Of the remaining TPS gene models, only 32 RedTPS and 30 YlwTPS were classified as full-length putative loci coding genes (Supplementary Tables S1, S2). The number of TPS may be underrepresented due to incomplete sequences or atypical gene structures obtained and in part due to draft genome assembly.

To identify putative orthologs between the two morphotypes, we created a sequence percentage identity matrix (Supplementary Table S4), and genes containing the top hits are shown (Supplementary Table S5). Only four TPS partial genes had identical sequences in the two genomes (Pca_red_g91813 and Pca_ylw_g91315; Pca_ red_g71488 and Pca_ylw_g60043; Pca_red_g21900 and Pca_ylw_g23854; Pca_red_g46186 and Pca_ylw_g20612). In addition, sequence identity among TPS genes between the two morphotypes was considerably lower, with only nine full length genes having greater than 90% amino acid identity (Supplementary Table S5). However, comparing partial and full genes, only 23 showed > 90% identity. The number increased when comparing only partial genes, where 29 genes showed > 90% identity (Supplementary Table S5).

Most TPS genes of subfamilies TPS-a, TPS-b, and TPS-g contained six to nine exons (Fig. 1a), with exceptions (Supplementary Tables S1, S2). Genes from the remaining subfamilies, TPS-c, TPS-e, and TPS-f, contained 7–14 exons (Fig. 2A). Moreover, only one full YlwTPS (Pca_ylw_g56204) and four RedTTPS (Pca_red_g44464, Pca_red_g43593, Pca_red_g28651, and Pca_red_g25997) lacked the highly conserved aspartate-rich motif "DDXXD" (Supplementary Tables S1, S2). The TPS-c subfamily is present in land plants and is characterized by the "DXDD" motif but not the "DDXXD" motif in their proteins, which was detected in only one RedTPS and two full Y lwTPS[26]. The second motif in the C-terminal domain, "NSE/DTE", is less conserved in TPS and presents the variation "(L,I) × (D,N,G)D(F,I,L) × (S,T,G,A)xxxE".

In the clade corresponding to the TPS-b subfamily monoterpene synthases, using different algorithm predictors, we found that only five full RedTPS and three full YlwTPS have an N-terminal transit peptide required for plastidial targeting (Supplementary Table S6).

We identified seven RedTPS and five YlwTPS with the N-terminal domain containing an "RRX8W" motif. In addition to these motifs, there is a highly conserved arginine-rich "RXR" motif. The TPS-g (Pca_ylw_g32591; Pca_red_g25997) subfamily is closely related to TPS-b; however, it lacks the conserved "R(R)X8W" motif in its encoded proteins, and its members may function in producing acyclic mono-, sesqui-, and diterpene p roducts[26].

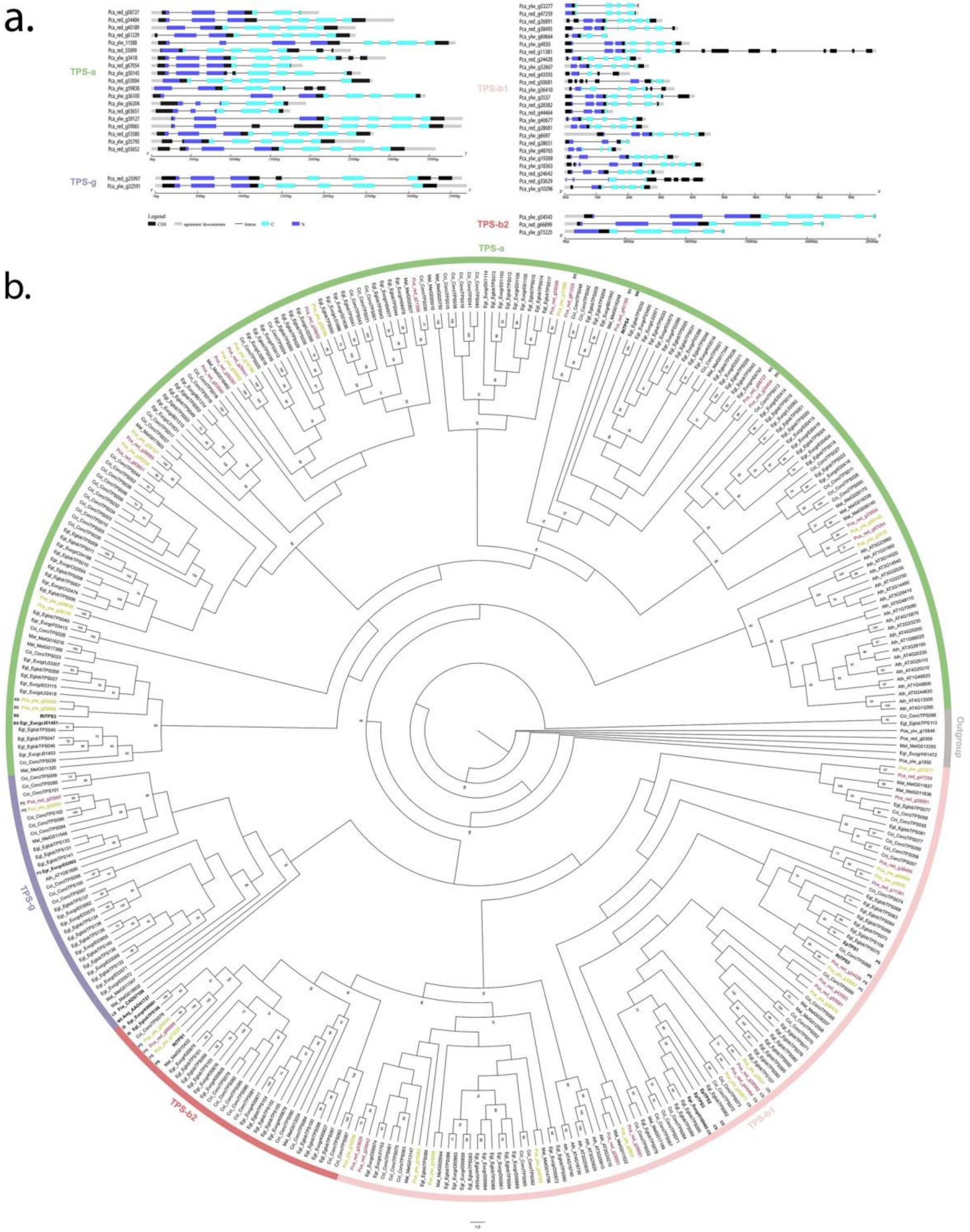

**Figure 1.** Phylogeny and gene structure of TPS from secondary metabolism. (**a**) Conserved domains in TPS genes and their consensus sequences from *P. cattleyanum.* (**b**) Phylogenetic tree of the Tps-a, Tps-b and Tps-g subfamilies from *P. cattleyanum* genome and characterized representative TPS from other Myrtaceae species. This tree was constructed through maximum likelihood analysis comparing the red and yellow morphotypes

(Pca_red and Pca_ylw), *C. citriodora* subsp. variegata (Cci), *E. grandis* (Egr), *E. globulus* (Egl), *M. alternifolia* (Mal) and *A. thaliana* (Ath). Functional characterized terpene synthases are written in bold. Bootstrap values supported by < 60% are noted by number. A few species from TPS-c clade were used as the outgroup.

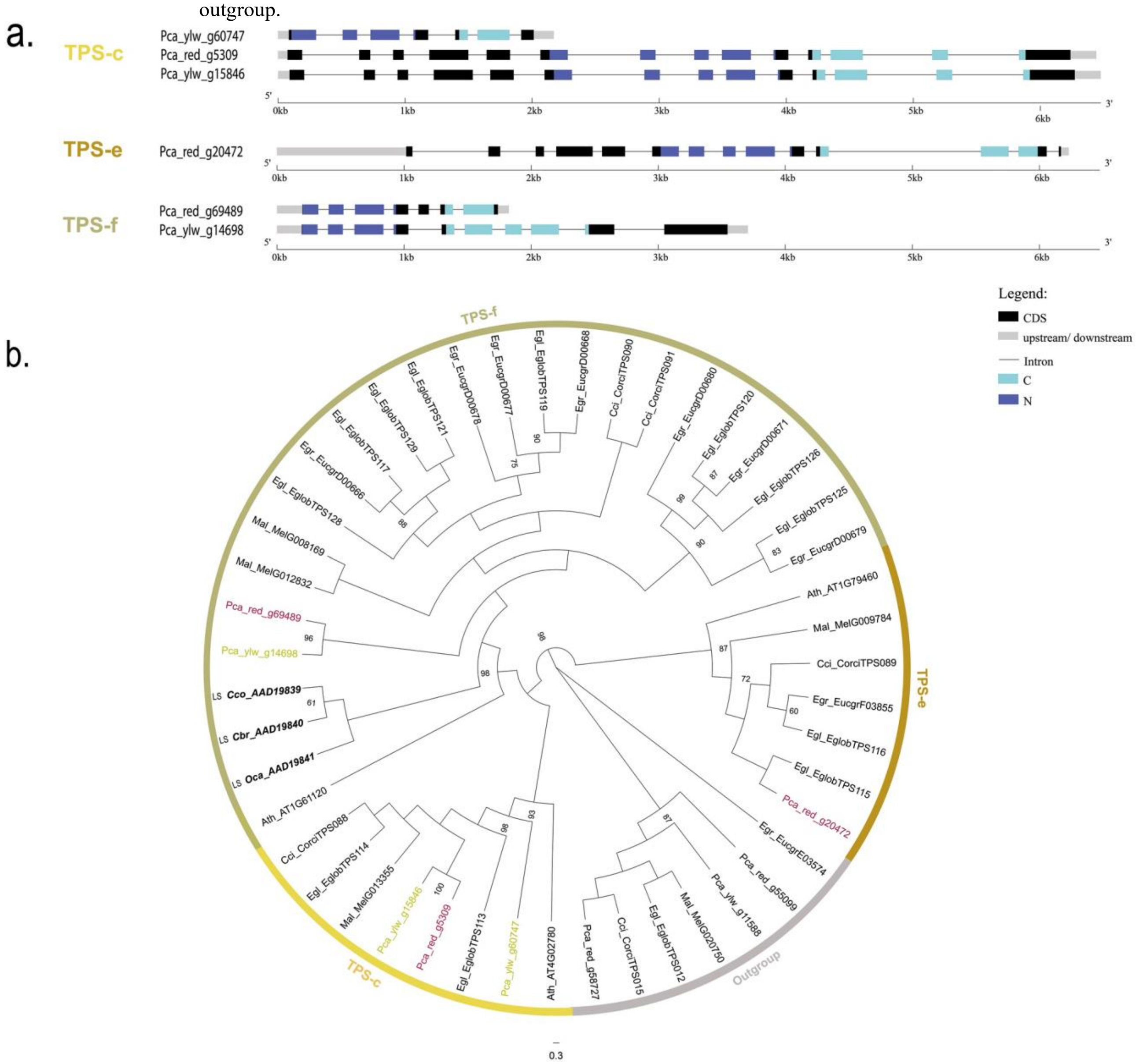

**Figure 2.** Phylogeny and gene structure of the TPS from primary metabolism. (**a**) Conserved domains in TPS genes and their consensus sequences from *P. cattleyanum.* (**b**) Phylogenetic tree of the Tps-c, Tps-e and Tps-f subfamilies from *P. cattleyanum* genome and representative TPS from other Myrtaceae species. This tree was constructed through maximum likelihood methods comparing the red and yellow morphotypes (Pca_red and Pca_ylw), *C. citriodora* subsp. variegata (Cci), *E. grandis* (Egr), *E. globulus* (Egl), *M. alternifolia* (Mal) and *A. thaliana* (Ath). Functional characterized terpene synthases are written in bold. Bootstrap values supported by > 60% are noted by number. A few species from TPS-a clade were used as the outgroup.

**Molecular evolutionary analysis.** To accurately classify the members of the *P. cattleyanum* TPS gene family based on sequence relatedness as well as functional assessments, we first collected 164 sequences of full-length TPS genes (containing the two TPS domains and having sequence lengths greater than 200 amino acids) from previous studies of species functionally characterized *A. thaliana* and *E. grandis* (Myrtaceae family) (Supplementary Fig. S2).

The topology of the phylogenetic tree allowed us to divide TPSs into subfamilies belonging to secondary metabolism, clustered with subfamily TPS-a, which produces sesquiterpenes (C15) with 14 RedTPS and 12 YlwTPS (Table 1, Fig. 3), and TPS-b, which encodes enzymes that produce monoterpenes with 14 RedTPS and 14 YlwTPS. Only one TPS-g gene was found in each morphotype, which predominantly produced acyclic mono-, sesqui-, and diterpenes (Table 2, Fig. 1b). In the cluster representing primary metabolism, a single gene, TPS-c, which produces diterpenes (C20), was found in *P. cattleyanum* red morphotype, while two were found in the yellow morphotype (Table 2; Fig. 2B). In the TPS-e/f subfamily, which produces mono-, sesqui-, and diterpenes, a single gene was found in the yellow morphotype, whereas two were found in the red morphotype. Our analysis including other Myrtaceae TPS genes showed that all TPS proteins identified in this study clustered into monophyletic-specific clades related to the subfamilies. The TPS-a and TPS-b subfamilies were the most expanded, accounting for approximately 80% of the total TPS full length genes identified (Fig. 3).

| N | Compound[a] | RI[b] | Content (%)[c] | Classification[d] | |
|---|---|---|---|---|---|
| Red | Yellow | | | | |
| 1 | α-Pinene | 930 | 10.0 | 35.4 | MH |
| 2 | β-Pinene | 972 | – | 3.2 | MH |
| 3 | β–Myrcene | 991 | – | 9.5 | MH |
| 4 | 1,8-Cineole | 1028 | 59.5 | 22.4 | MO |
| 5 | β-Ocimene | 1039 | 2.9 | – | MH |
| 6 | γ-Terpinene | 1058 | 4.1 | – | MH |
| 7 | Linalool | 1100 | 9.6 | 3.7 | MO |
| 8 | α-Terpineol | 1189 | 5.6 | 2.7 | MO |
| 9 | β-Caryophyllene | 1414 | 2.5 | 2.7 | SH |
| 10 | Nerolidol | 1563 | 2.4 | – | SO |
| 11 | Caryophyllene oxide | 1579 | – | 6.6 | SO |
| 12 | Viridiflorol | 1598 | – | 2.2 | SO |
| 13 | Aromadendrene epoxide | 1633 | – | 2.5 | SO |
| Total | | | 96.6 | 90.9 | |

**Table 1.** Chemical constituents of leaf oil from red and yellow morphotypes of *Psidium cattleyanum*. [a] Major compounds listed in the elution order using Rtx®-5MS column. [b] RI: Retention index determined by the normalization of retention times with respect to an n-alkane mixture (C7–C40)[87–89]. [c] Compounds with a relative area of > 2% were identified. [d] Terpenic classification: oxygenated monoterpene (MO), hydrogenated sesquiterpene (SH), oxygenated sesquiterpene (SO).

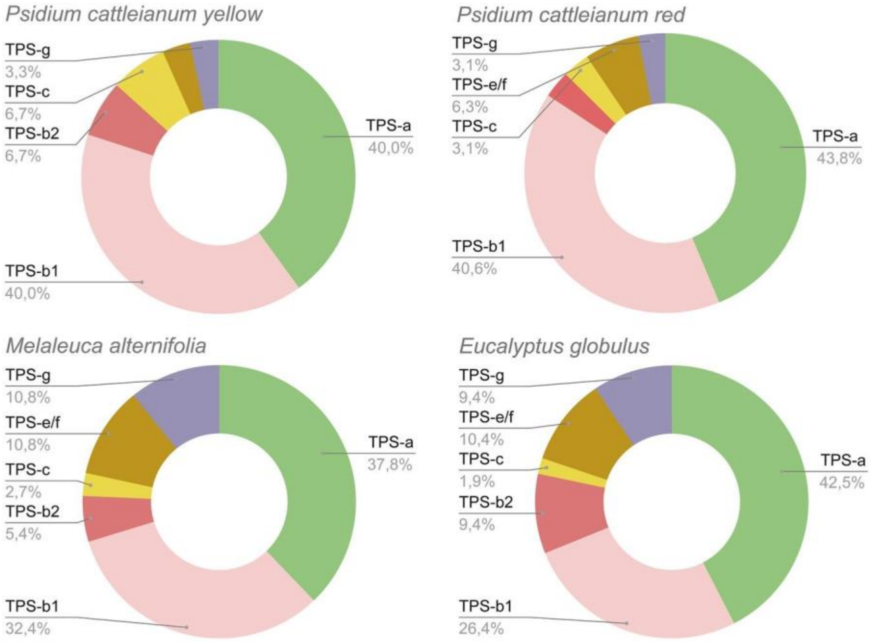

**Figure 3.** Proportion of TPS gene subfamilies found in Myrtaceae species. The number of genes in each subfamily relative to the total number of genes indicates the proportion of TPS genes. *Psidium cattleyanum* had the highest proportion of TPS-b1 genes (~ 40%).

| | | | Full length | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species | Total TPS gene models | Putative full length | a | b | c | d | e/f | g | h |
| *Psidium cattleyanum red* | 110 | 32 | 14 | 14 | 1 | 0 | 2 | 1 | 0 |
| *Psidium cattleyanum yellow* | 106 | 30 | 12 | 14 | 2 | 0 | 1 | 1 | 0 |
| *Corymbia citriodora* | 127 | 84 | 38 | 33 | 1 | 0 | 3 | 9 | 0 |
| *Melaleuca alternifolia* | 37 | 37 | 14 | 14 | 1 | 0 | 4 | 4 | 0 |
| *Eucalyptus globulus* | 143 | 103 | 44 | 37 | 2 | 0 | 10 | 10 | 0 |
| *Eucalyptus grandis* | 172 | 70 | 38 | 15 | 1 | 0 | 8 | 8 | 0 |
| *Leptospermum scoparium* | 49 | 23 | 9 | 7 | 1 | 0 | 4 | 2 | 0 |

**Table 2.** Numbers of TPS genes in Myrtaceae species.

Site model selection analyses indicate sites that evolve under positive selection fit the data significantly better than the respective null models (M8 vs. M7: LRT = 14.46, df = 2, p = 0.001), however, the posterior probability was low (p < 0.55) (Supplementary Table S9). Therefore, positive selection may only occur during specific stages of evolution or in particular branches, we tested a branch-specific model to detect positive selection in the three clades formed in the TPS-b subfamily, which were fixed as foreground branches. Clade 1 contained only TPS-b1 genes from *Eucalyptus* and *Psidium* species. Clade 2 contained only TPSb-1 genes from *Psidium* species. A third clade contained some genes from *Populus*, *Vitis*, and *Eucalyptus* which were grouped with three pinene synthase genes from *Psidium* and classified as the only TPS-b2 genes. The one-ratio branch model indicated an overall purifying selection for TPS evolution (ω mean values smaller than 1.0). We also investigated selective pressure using the branch site model according to the likelihood ratio tests (LRT) and comparisons of clade 1 (p = 0.26), clade 2 (p < 0.05), and clade 3 (p < 0.05), indicating that some sites were statistically significant (Supplementary Table S12). However, only five residues were strongly identified to be under positive selection in clade 2, located in the N-terminal portion of TPSb-1 genes of *Psidium*, including residue 121, with an aspartate (D) and the alteration to a leucine (L) in the foreground branches, and residue 124 with the most commonly found lysine (K), arginine (R), or tryptofan (W) and its alteration to alanine (A) in foreground branches. We also detected residues 222 with a cysteine (C) and alteration to valine (V) or leucine (L) in the foreground branches, and site 279 with a threonine (T) or isoleucine (I) that presented an alteration to cysteine (C) or tyrosine (Y) in the foreground branches, around "RDR" and "DDXXD" motifs in the C-terminal portion (Fig. 4). Clades 1 and 3 show the residuals with weak signs of positive selection.

**Global and differential expression analysis associated with the terpene biosynthesis.** To gain more insight into the TPS biosynthetic pathway, global and differential expression profiles were evaluated on TPS genes from RNA samples extracted from its leaves and compared in two morphotypes. After library construction, Illumina sequencing, and assembly, approximately 84 and 86 million paired end reads already cleaned were generated for yellow and red morphotypes, respectively.

Looking at total gene expression across the two morphotypes, approximately 30% of TPS genes were expressed in leaves (transcripts anchored in 35 genes in the red genome and transcripts anchored in 30 genes in the yellow genome). Genes that showed some expression patterns fell into five clades (Supplementary Tables S7, S8). We found 17 full-length and 18 partial TPS genes with evidence of expression in the red genome and 13 full-length and 18 partial TPS genes in the yellow genome.

A heat map showing differential gene expression using DESeq2 based on |log2Fold Change |≥ 1 and FDR < 0.05 in the red and yellow morphotypes, with two biological replicates in leaves, is shown in Fig. 4. As the two genomes were assembled separately and belonged to the same species, two heatmaps were generated, anchoring all transcripts in the red genome (Fig. 5A) and all transcripts in the yellow genome (Fig. 5B). Therefore, statistical analysis can be performed and then compared. Among these, 19 gene sequences were upregulated in the red morphotype, with only 10 full TPS genes (Fig. 5C; Supplementary Tables S7, S8). In the yellow morphotype, 32 TPS genes were upregulated, but only 14 were full TPS. A total of 12 TPS genes showed the same expression pattern between the two transcriptome comparisons and > 90% of identity, indicating that the same gene was found in the different genome assemblies.

**Terpenoid profling in *Psidium cattleyanum* leaves.** The leaves of *Psidium cattleyanum* were examined for chemical compositions of the volatile terpene compounds, to investigate the genetic influence on the chemical variations of the oil content between the two morphotypes. The content of each terpenoid was calculated as a percentage of the total essential oil using gas chromatography with a flame ionization detector (GC-FID) and gas chromatography coupled to mass spectrometry (GC–MS) approaches. Thirteen compounds were identified, and the most abundant monoterpenes in both morphotypes were 1,8-cineole, α-pinene, linalool, and α-terpineol (Table 1; Supplementary Fig. S1A). Although these compounds were commonly found, they showed significant quantitative variation. For example, the α-pinene showed a large difference of 35.4% in yellow and only 10.0% in red morphotype; 1,8-cineole showed a difference of 59.5% in the red and 22.4% in yellow morphotype, whereas linalool showed a difference of 9.6% and 3.7% in the red and yellow morphotypes, respectively.

In addition to quantitative variations, the plants used also showed qualitative variations in the chemical composition of their essential oils. The hydrogenated monoterpenes β-ocimene (2.9%) and γ-terpinene (4.1%) were observed only in red morphotype essential oil, and the oxygenated sesquiterpene nerolidol (2.4%) (Supplementary Fig. S1B). The hydrogenated monoterpenes β-pinene (3.2%) and β-myrcene (9.5%) were observed only in yellow morphotype, and the oxygenated sesquiterpenes caryophyllene oxide (6.6%), aromadendrene epoxide (2.5%), and viridiflorol (2.2%) (Supplementary Fig. S1C).

## Discussion

The Myrtaceae family is recognized for its great potential to produce volatile oils of economic interest[35]. The identification of photochemical profiles of some species combined with genomic studies, revealing a high diversity of TPS genes that control the synthesis pathways of these compounds and are responsible for the various biological activities of essential o ils[28,29,31,36].

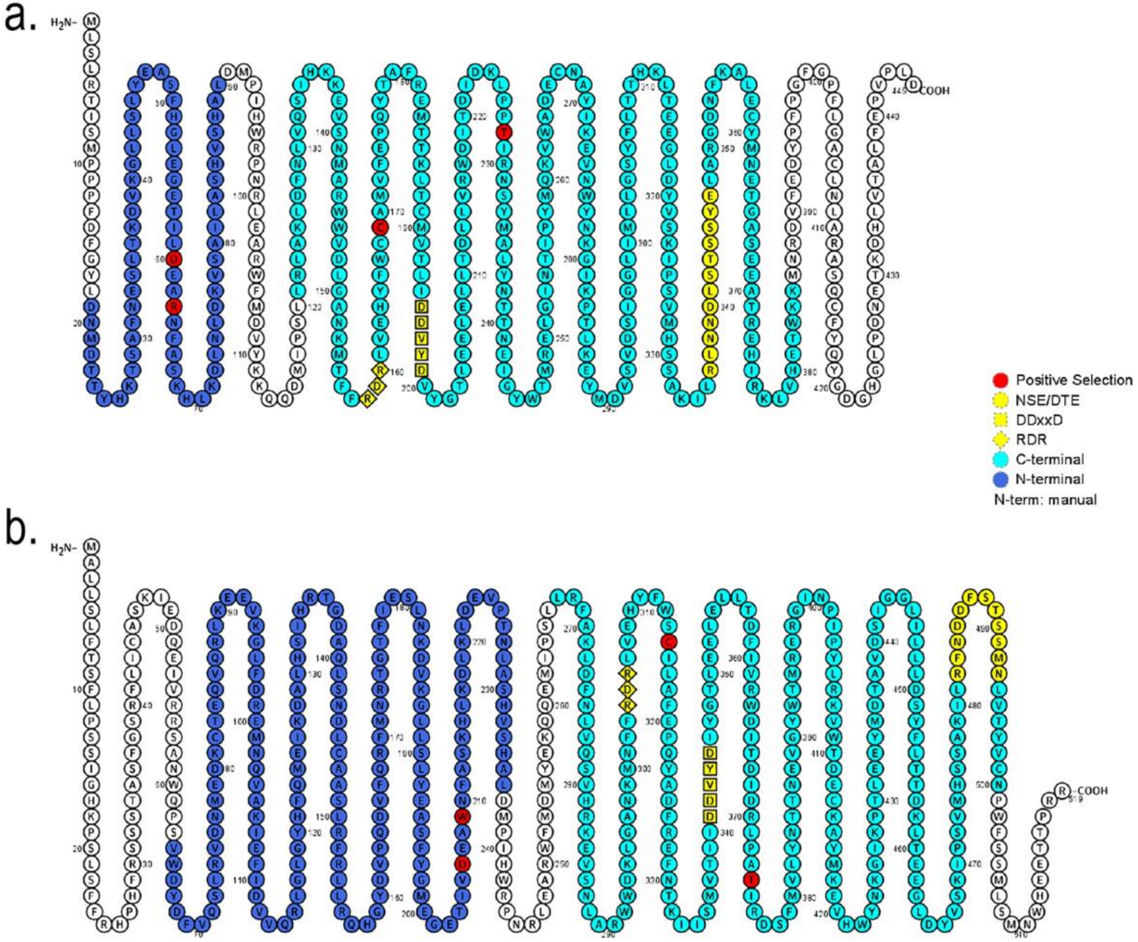

**Figure 4.** Positive selected sites in TPS-b1 branch including only *Psidium* genes. (**a**) The pinene synthase sequence Pca_red_g24428 representing clade 2 as foreground clade. (**b**) The linalool synthase sequence Pca_red_g28382 represents clade 2 as foreground clade. Amino acids that were identified on positive selection (red circles) are demonstrated on the protein sequence of these representative species corresponding to the sites in each alignment presented on Supplementary Table S12. Also, the representation of mainly motifs of the entrance of the active site (yellow square, circles, and triangle) represented for the "DDXXD", "NSE/DTE" and "RXR" domains.

In this study, the TPS family has been characterized in *Psidium cattleyanum*, a fleshy-fruited species from the Myrtaceae family, for the first time at the genomic and transcriptomic levels. It reveals a low number of putative functional full-length TPS genes (32 RedTPS and 30 YlwTPS) required for this species associated with wet forests across the neotropics, when compared with the woody-fruited species (Table 2) from open forest and woodland, such as Eucalypteae tribe, including *Eucalyptus grandis* (70 full length TPS), *Eucalyptus globulus* (103 full length TPS), and *Corymbia citriodora* (84 full length TPS), all species with the diversity center in the Asia and Oceania[37]. These species are predicted to defend their leaves much more strongly. Moreover, the relatively long lifespan of eucalyptus (well over 200 years)[33] compared to *Psidium* (approximately 40 years)[38], may drive further gene diversification as the need to adapt to long-term environmental changes. These results imply that evolutionary forces have acted differently upon lineages since they diverged from their most recent common ancestor more than 70 million years a go[1,39].

Partial genes might be considered non-functional, even though some of their incomplete sequences could have resulted from poor sequencing techniques. Still, the redundancy of TPS genes has been observed in many other plants, e.g., in grape (*Vitis vinifera*) there are 152 TPS-like genes, but only 62 full length TPS, with two domain structures[40] where tandem duplication rates for both domains (~ 90%) are the main mechanisms for family expansion. In *E. grandis* there were 70 full-length TPS, but seven had only the PF01397 domain where gene losses were mostly related to tandem duplications (71.4%) and less related to segmental duplication (3.9%) events, and 22 TPS with only the PF03936 domain more related to tandem duplication (71.7%) and fewer segmental duplication events (4.3%)[41]. We observed the same pattern in *Psidium cattleyanum*, where 28 RedTPS and 27 YlwTPS had only the PF01397 domain and 48 RedTPS and 45 YlwTPS had only the PF03936 domain (Supplementary Table S9). These data suggest that domain loss has been a common event in plants during the evolution of the TPS gene family, with the loss of the PF01397 domain being more frequent in the Myrtaceae family and plants in general than the loss of the PF03936 domain[41]. The functionality of these single domain containing TPS is not yet known, but more investigation on regulatory mechanisms, expansion history, and evolutionary advantage of the domains separately should provide a comprehensive view of the impact of partial genes in the diversification of TPS in plants[26].

Transcriptome examination revealed that out of 32 full-length RedTPS, 10 genes were upregulated in the red morphotype. Among the 30 full-length YlwTPS, only 14 genes were upregulated in the yellow morphotype. This demonstrates that the differential expression patterns in the two morphotypes can also contribute to the final terpene content in the leaves (Fig. 5). The high abundance of transcripts in this study (FPKM, Fragments Per Kilobase of exon per Million reads) from the TPS-a and TPS-b1 subfamilies in the transcriptome indicated their involvement in the formation of mono and sesquiterpenoid volatiles in leaves.

A comparison of the essential oil composition revealed the presence of oxygenated monoterpenes on leaves of *P. cattleyanum*, where the major compound was α-pinene (35.4%) in yellow morphotype and the 1,8-cineole (59,5%) and linalool (9.6%) in the red morphotype. This variation in the essential oil of *P. cattleyanum* morphotypes have also been previously described in native species in southern Brazil[24,25]. In cultivated plants of *P. cattleyanum* in different parts of the world, previous studies have identified the chemical composition with β-caryophyllene, a hydrocarbon sesquiterpene, as the main component[7,23,25,42–45], which was also found in smaller amounts in both morphotypes in this work.

The variations found between the two morphotypes in this study reflect a genetic and evolutionary origin. The identification of chemotype phenotypes (qualitative variability in foliar essential oil composition) within a single species has already been reported among different varieties or ecotypes of other species[46–49], mainly when a significant shift in the relative concentrations involved more similar compounds, such as cineole and pinene[50]. The yellow morphotype tends to be found at slightly lower elevations than the red m orphotype[17,21], this could reflect environmental a daptation[48] and in the terpenes plasticity[51].

In the TPS-a subfamily that encodes only sesqui-TPSs found in both eudicot and monocot plants[40], phylogenetic analysis revealed two YlwTPS (Pca_ylw_g29958 and Pca_ylw_g20359) closely related to RtTPS3 (AXY92168)[52] and in the same branch of the gene EgranTPS038 (Euc_Eucgr_J01451) of *E. grandis*. In addition, four RedTPS (Pca_red_g40189, Pca_red_g58727, Pca_red_g34404, and Pca_red_g61229) were found in the same branch as RtTPS4 (AXY92169)[52]; both belong to a branch of the betacaryophylene synthase (BS) (Fig. 6).

The monophyletic TPS-b subfamily is divided into two groups. The TPS-b1 clade contains putative cyclic monoterpene synthases, with transit peptides positioned upstream of the "RRX8W" motif and therefore has a high probability of localizing in the plastids[29]. The subfamily had the highest number of full-length genes (40%) as a proportion of the total number of TPS genes compared to *Melaleuca alternifolia* (32.4%) and *Populus trichocarpa* (31.2%) (Fig. 3). The high proportions of the TPS-b1 subfamily could be indicative of rapid ongoing evolution and lineage-specific gene family expansion of this subfamily in warm subtropical habitats, particularly for protection from damage caused by rapid temperature fluctuations[53,54]. Some terpenes can act by selecting the defense of antimicrobial secondary metabolites such as cyclic monoterpenes[32]. This suggests that subfamilies of TPS-b1 expansion might be related to species or ecotype diversification, enabling quick adaptation in response to environmental changes.

The other TPS-b subfamily contains putative isoprene/ocimene (C5, C10) synthases, described as TPS-b2[32] and has few genes in P*sidium cattleyanum* (6.6% in yellow and 3.1% in red morphotype) as a proportion of the total number of TPS genes compared with *Eucalyptus globulus* (9.4%) and *Melaleuca alternifolia* (5.4%) (Fig. 3)[29,32,55]. However, when including genes functionally characterized in the TPS-b2 clade, the relationships among *Psidium* genes were not entirely congruent because we detected these three genes (Pca_ylw_g54543, Pca_ red_g66899, and Pca_ylw_g73225) positioned in the same clade as *Rhodomyrtus tomentosa* RtTPS1 (AXY92166), characterized as pinene synthase (PS), a cyclic terpene[52], despite the high support (bootstrap value of 97) in the same branch of acyclic EglobTPS106, functionally characterized as isoprene synthase[29] (Fig. 1b). Including more TPS from Myrtaceae species from neotropics and functionally characterizing the *Psidium* genes should clarify their role and division within the clade TPS-b.

Two other genes (Pca_ylw_g32667 and Pca_red_g24428) clustered together with RtTPS2 (AXY92167) and EpTPS1 (MK873024) in the TPS-b1 branch and were related to pinene synthase (Fig. 6). Moreover, four genes (Pca_red_g44464, Pca_red_g28382; Pca_ylw_g3537, Pca_ylw_g40677) were in the same branch as EpTPS2 and EpTPS3 from *Eucalyptus polybractea* belonging to the CS TPS-b1 clade[56], and one gene from *E. grandis* monoterpene synthase (XP_010046521), which has similarity (95% amino acid identity) to CS that produces 1,8-cineole an in vitro assay using GPP as substrate[57]. Analysis of transcript abundance showed that the gene Pca_red_g28382 was highly expressed in leaves. The dominant product of many characterized CS enzymes is 1,8-cineole; however, they also produce small amounts of limonene, β-myrcene, sabinene, β-pinene, α-pinene, and α-terpineol. This group of compounds synthesized by CS is known as the 'cineole cassette', which has been reported in many plants[58,59]. Therefore, as multiple TPS genes are often expressed in the same tissue and many of these TPS' have overlapping ranges of products, it is not easy to identify the action of individual TPS enzymes on the profile of the terpene observed in that tissue[60].

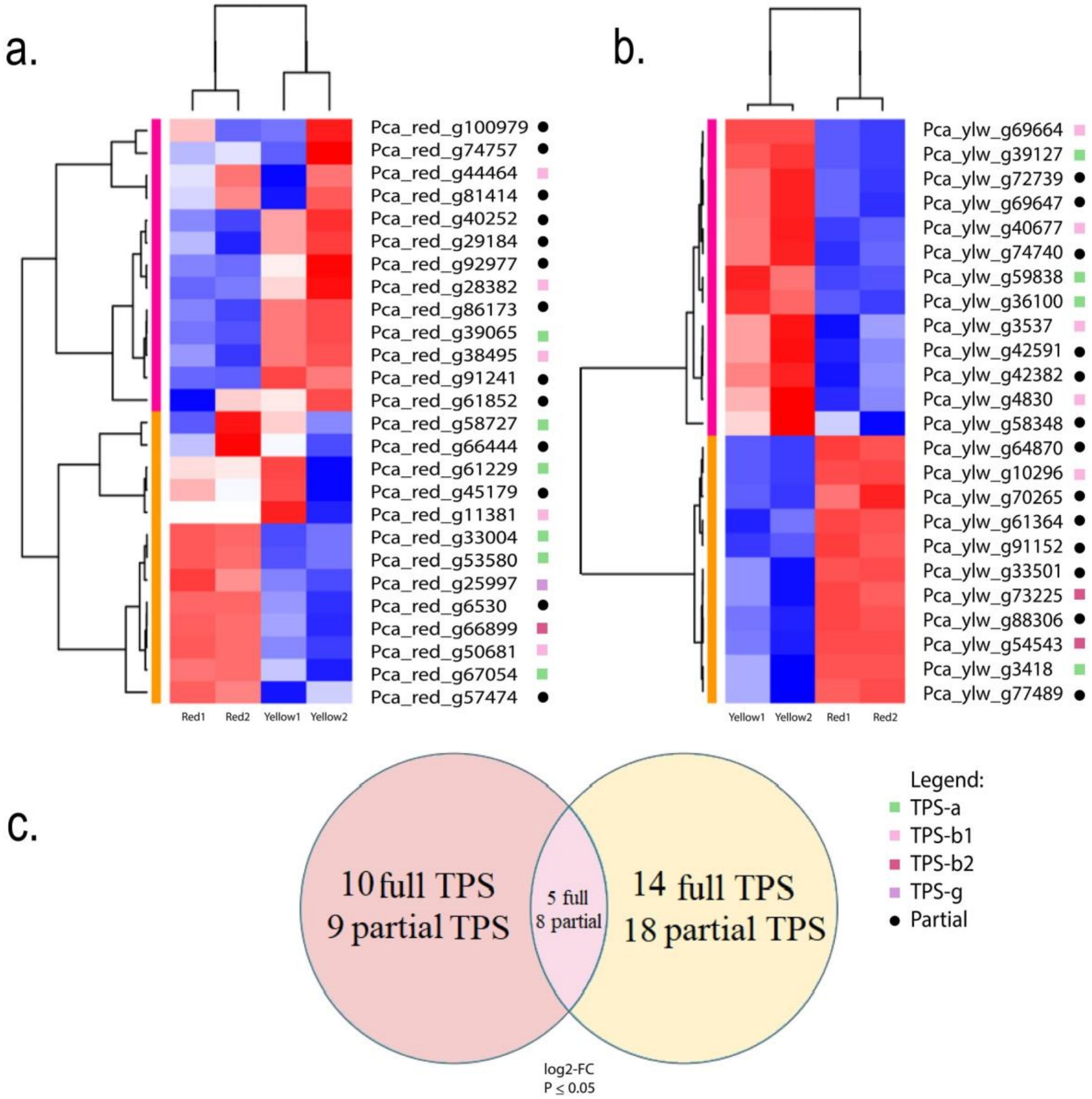

**Figure 5.** Differential expression of terpene synthase genes of *P. cattleyanum* leaves. (**a**) Red morphotype genome. (**b**) Yellow morphotype genome. The blue colored cells indicate the Log2 transformed FPKM (unit of fragments per kb of exon per million mapped reads) with no expression and value zero in this tissue (downregulated), and red color indicates a higher percentage of total expression for a given gene (up-regulated). Squares represent full-length genes and black dots represent partial genes. (**c**) Veen Diagram representing the up-regulated unique transcripts common between the morphotypes.

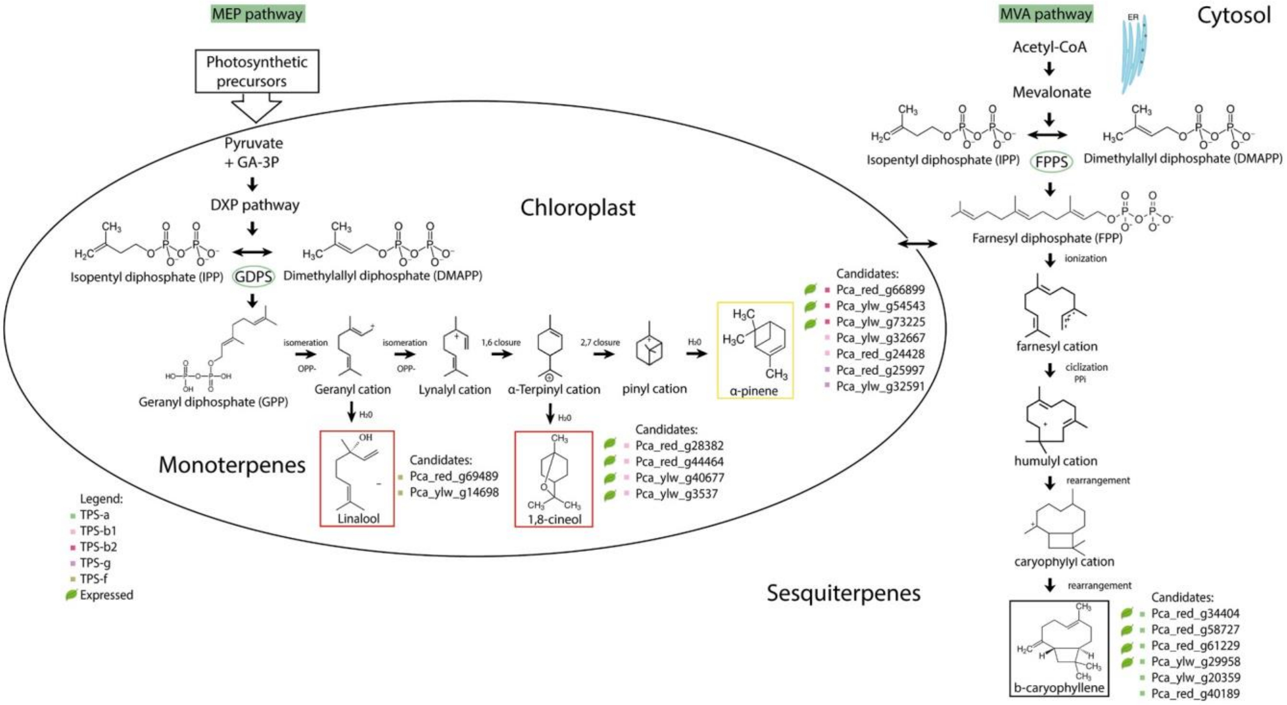

**Figure 6.** A schematic view of putative terpene synthase genes involved in α-pinene, 1,8-cineol, linalool and β-caryophyllene biosynthesis in *P. cattleyanum* red and yellow morphotypes. *DXP* 1-deoxylulose 5-phosphate, *DMAPP* dimethylallyl diphosphate, *IPP* isopentenil diphosphate, *GPPS* geranyl diphosphate synthase, *GPP* geranyl diphosphate, *FPPS* farnesyl diphosphate synthase, *FPP* farnesyl diphosphate, *ER* endoplasmic reticulum, *MEP* methyl erythritol 4-phosphate pathway, *MVA* mevalonate pathway.

Other expressed TPS-b genes Pca_ylw_g54543, Pca_red_g66899, and Pca_ylw_g73225 were positioned in the same clade as RtTPS1 of *Rhodomyrtus tomentosa*, and Pca_red_g24428 grouped with RtTPS2. Previous studies have shown the in vitro activity of RtTPS1 and RtTPS2, which mainly produce (+)-α-pinene and (+)-β-pinene with GPP, whereas RtTPS1 is also active with FPP, producing β-caryophyllene, along with a smaller amount of α-humulene[53]. This suggests that, depending on their expression profile or subcellular location, the enzymatic products of these TPS present in leaves can contribute to the different terpene mixtures found in the essential oil. We also detected the expression of four genes (Pca_red_g44464, Pca_red_g28382; Pca_ylw_g3537, Pca_ylw_ g40677) in the same branch of EpTPS2 and EpTPS3, belonging to the PS TPS-b clade.

There is a large diversity of Myrtaceae species, with α-pinene and 1,8-cineole being the dominant compounds in the leaves. The reaction cascade that leads to these two compounds includes the same carbocation intermediate, γ-terpinyl c ation[49]. There is evidence to show that the amino acid changes induced through site-directed mutagenesis can result in a different ratio of particular terpenes produced[47,61,62] and in natural systems, this might lead to different dominant compounds, such as the a-terpineol synthases of many species, which are the only characterized terpene synthases that are not 1,8-cineole synthases, but produce significant amounts of 1,8-cineole[59].

The TPS-g subfamily has two subclades encoding TPS' without the "R(R)X8W" motif, which facilitates isomerization of the geranyl cation in the linalyl cation. This subfamily is closely related to the TPS-b subfamily, and its members may function with the prevalence of acyclic monoterpene products. We also identified two genes from *Psidium* (Pca_red_g25997 and Pca_ylw_g32591) in the same branch as the functionally characterized PS of EgranTPS101[29] (Egr_EucgrE03562; Fig. 6).

We screened TPS genes to identify the LS based on functionally characterized enzymes from other plant species. Phylogenetic analysis demonstrated that only two genes (Pca_ylw_g14698 and Pca_red_g69489) are closely related to LS from the rosids *Clarkia breweri* (Cbr_AAD1984)*, Oenothera arizonica* (Oca_AAD1984)*,* and *Clarkia concinna* (Cco_AAD1983). They fall into the TPS-f synthase classification, proposed to be the most ancient, and could have been due to a relatively recent common ancestor, copalyl diphosphate synthase (CPS)[63], as evidenced by the sequence conservation of this region in the N-terminus of the protein (Fig. 6). In this study, LS gene expression was not observed in leaves. Monoterpene synthases of this subfamily are responsible for the conversion of GDP into the bulk of monoterpenes found in vegetative organs, whereas the subfamilies TPS-f and TPS-g are thought to be exclusively active in flowers, likely having a primary function in attracting insect pollinators[36,64]. In addition, other genes could be expressed when directly involved in plant defense against herbivores by attracting predators[65] or by directly driving herbivores away[66].

Depending on the extent to which gene function is affected, single-base substitutions may result in changes in terpene composition and profile, and if upstream pathway elements are involved, even in terpene concentrations [46,67]. To infer whether selection acted on the TPS-b subfamily, we used several statistical tests to compare clades on the phylogenetic tree. Codon substitution patterns with a maximum likelihood approach implementing a branch-site model indicated positive selection acting on a specific TPS-b1 branch, including some pinene and cineole synthase genes and other non-functionally assigned genes.

In particular, some positively selected sites are located in the N-terminal region, which controls substrate specificity. It is interesting to note that residue 224 contains an arginine (R) in the PS genes (Fig. 4A), whereas we observed an alteration to a tryptophan (W) residue in the CS genes (Fig. 4B). Conserved arginines close to the diphosphate moiety stabilize the evolving negative c harges[68]. The tryptophan residue contributes to stabilization of the cation and deprotonation of the s ubstrate[69]. In addition, the positively selected residues 222 and 279 were located around the aspartate-rich motif ("DDXXD") in the C-terminal half, which is important for the coordination of divalent ion(s), water molecules, and stabilization of the active site[70–72].

These results illustrate the importance of these residues to product spectrum of TPS genes, mainly in this case of PS and LS, that have the same carbocation intermediate, thereby differing in their profiles[46]. Future studies should investigate in detail how the active site promotes discrimination from other potential substrates. Analysis of this type of data could be used to better understand the diversity of terpene synthases and the role of different terpenes in mediating ecological interactions[34].

Several biological and pharmacological activities have been reported for pinene, cineol, and linalool, including anti-inflammatory and antinociceptive properties[11,73–75], anticancer[61,76,77], antifungal[78,79], antidiabetic[80], antioxidant, antimicrobial[77,81,82], antidepressive and neuroprotective[77], allelopathic[83], antibacterial, and insecticidal activity[84,85]. The high content of these compounds in the volatile oils of these species suggests that they could constitute an alternative commercial source of this compound[86].

## Conclusion

In this study we identified putative TPS genes responsible for the formation of predominant essential oil compounds in *Psidium cattleyanum*. The chemotypic variability found in the red and yellow morphotypes confirm our hypothesis about the complex and polymorphic nature of the genes encoding the key enzymes regulating compound production and suggest adaptive genetic plasticity of the two morphotypes. The TPS-b clade has undergone substantial expansion compared to other subfamilies and includes some positively selected amino acid residues, evidence the monoterpene synthase genes are important for adaptation to *Psidium* at different niches. The present study provides the first insight into the genetic basis of TPS in *P. cattleyanum* morphotypes, gaining insights about the biodiversity in the Atlantic rainforest for further ecological genetic studies in the genus.

## Materials and methods

**Plant materials.** Young leaf samples of the yellow and red morphotypes were grown on the same open ground plot (in two 5-m long rows per cultivar) at the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). The plants were 20–25 years old during the sampling year (2020). The leaves were washed with distilled water, frozen, and stored at -18 °C until extraction of volatile compounds, immediately frozen in liquid nitrogen and stored at − 80 °C for further RNA extraction.

**Chromatographic profile of the essential oils.** We collected volatiles from the leaves of the two morphotypes under the same growth conditions and ambient temperature, in biological triplicates. Approximately 100 g of dry leaves from the two morphotypes, were extracted with 1000 mL of reverse osmosis water using a Clevenger apparatus [87], following four hours of extraction by hydro-distillation. Samples of the essential oils extracted from the leaves were analyzed using gas chromatography with a flame ionization detector (GC-FID) (Shimadzu GC-2010 Plus) and gas chromatography coupled to mass spectrometry (GC–MS) (Shimadzu GCMSQP2010 SE).

We conducted the analyses according to the following conditions: helium (He) as the carrier gas for both detectors, with the flow and linear speeds of 2.80 mL $min^{-1}$ and 50.8 cm $s^{-1}$ (GC-FID), and 1.98 mL $min^{-1}$ and 50.9 cm $s^{-1}$ (GC–MS), respectively; injection port temperature of 220 °C with a split ratio of 1:30; fused silica capillary column (30 m × 0.25 mm); stationary phase R tx®-5MS (0.25 µm film thickness); oven with an initial temperature of 40 °C, maintained for 3 min, then gradually increased by 3 °C $min^{-1}$ until 180 °C, where it remained for 10 min (total analysis time: 59.67 min); and FID and MS detector temperature of 240 °C and 200 °C, respectively[49]. The used samples were taken from the vials in 1 µL of a solution containing 3% essential oil dissolved in hexane with 0.1 mol $L^{-1}$ dimethylacetamide (DMA; external standard for reproducibility control).

The GC–MS analyses were performed using electron impact equipment with an impact energy of 70 eV, scanning speed of 1000, scanning interval of 0.50 fragments $s^{-1}$, and fragments detected from 29 to 400 (*m/z*). The GC-FID analyses were carried out in a flame formed by H2 and atmospheric air at a temperature of 300 °C. Flow rates of 40 mL $min^{-1}$ and 400 mL $min^{-1}$ were used for H2 and air, respectively. Identification of the compounds in the essential oils was accomplished by comparing the obtained mass spectra with those available in the spectral library database (Wiley 7, NIST 05, and NIST 05 s) and retention indices (RI). To calculate the RIs, we used a mixture of saturated alkanes C7–C40 (Supelco-USA) and adjusted retention time of each compound, obtained by GC-FID. The values calculated for each compound were compared with those reported in literature[88–90].

We calculated the relative percentage of each compound in the essential oil using the ratio between the integral area of the peaks and the total area of all sample constituents obtained via GC-FID analyses. The compounds with a relative area above 2% were identified and considered predominant if above 10%.

**Terpene synthase gene identification and annotation.** Initially, we used two terpene synthase-specific domains, PF01397 and PF03936, which represent respectively the N-terminal and C-terminal domains of TPS from the Pfam database (http://pfam.xfam.org/) [91], as queries to search for terpene synthase homolog genes in the *P. cattleyanum* yellow and red morphotypes predicted genes from their genomes (unpublished data). We analysed each morphotype separately using HMMER version 3.1[92]. We also performed a local BLASTP search for TPS genes in the *P. cattleyanum* reference genome based on functionally characterized genes[93,94]. We created a preliminary list of putative TPS genes based on hits with a high similarity (e-value < 1e − 05).

To better understand the structural sequence features of each gene, we used the open reading frame (ORF) Finder of NCBI (http://www.ncbi.nlm.nih.gov/orffinder/) to identify the ORFs for each sequence recovered. Gene structure was determined using the Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn) [95]. We confirmed the presence of functional domains based on the translation of gene sequences identified in Simple Modular Architecture Research Tool (SMART)[96]. Moreover, several algorithms were used to predict a putative transit peptide for chloroplast targeting in the N-terminal sequence upstream of the RRX8W motif (ChloroP 1.1[97], TargetP v.1.01[98], PCLR 0.9[99]). To determine the sequence diversity between the two morphotypes, a complete set of pairwise comparisons of protein sequences was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

**Phylogenetic reconstruction.** In this study, we first used terpene synthase protein sequences from fully sequenced genomes of *A. thaliana*[100] and *E. grandis*[29], to classify the putative genes found in *P. cattleyanum* according to the previous classification in the subfamilies TPS-a,-b,-c,-e/f, and -g by sequence similarity[26].

To examine the evolutionary history of TPS genes, a second analysis including more species (*E. grandis*, *E. globulus*, *A. thaliana*, *P. trichocarpa*, *V. vinifera*, *C. citriodora*, and *M. alternifolia*) was carried out. We generated a tree with TPS sequences related to primary metabolism (subfamilies -c, -e, and -f) with a total of 45 sequences and a second tree related to secondary metabolism (subfamilies a, b, g) including 360 sequences[29,32,55].

The functionally characterized pinene (RtTPS1 and RtTPS2 accession number AXY92166 and AXY92167, respectively) and caryophyllene synthases (RtTPS3 and RtTPS4 accession numbers AXY92168 and AXY92169) from *Rhodomyrtus tomentosa*[52], pinene synthase (EpTPS1 accession number MK873024) and 1,8-cineole synthases (EpTPS2 and EpTPS3 accession numbers MK873025 and QCQ05478) from *Eucalyptus polybractea*[56], beta cayophyllene synthase (Eucgr. J01451) from *E. grandis*[29], myrcene synthase from *Antirrhium majus* (AAO41727)[101], two isoprene synthase genes from *E. globulus* (EglobTPS106), *E. grandis* (Eucgr. K00881)[29] and five linalool synthases from *Oenothera californica* (AAD19841)[63], *Clarkia breweri* (AAD19840), *Clarkia concinna* (AAD19839), and *Fragaria x ananassa* (CAD57106)[102] were also included in the phylogenetic analysis to assess the homology of known TPS to *Psidium* genes.

For each dataset used to construct the trees, we first aligned the amino acid sequences of putative TPS genes using ClustalW implemented within MEGA v7.0 software package[103]. Due to high levels of variation and variable exon counts between taxa, we trimmed the alignment using Gblocks[104] with the following parameters: smaller final blocks, gap positions within the final blocks, and less strict flanking positions. We used the maximumlikelihood method implemented in PhyML v2.4.4[105] online web server[106] to perform the phylogenetic analysis. The JTT + G + F was the best-fit substitution model selected with ModelGenerator for protein analyses[107]. The confidence values in the tree topology were assessed by running 100 bootstrap replicates. Trees were visualized using Figtree v1.4.4[108].

**Molecular evolutionary analysis involving TPS-b.** To understand the molecular evolution at the amino acid level and the intensity of natural selection acting on metabolism in a specific clade, we used a tree based on codon alignment produced by the maximum-likelihood method using the software EasyCodeML[109]. We retrieved Coding Sequencing (CDS) sequences from TPS-b genes from *A. thaliana*, *E. grandis, P. cattleyanum, V. vinifera* and *P. trichocarpa* species in Phytozome v11 (http://phytozome.jgi.doe.gov/; last accessed November 2020), to use in positive selection analysis. The dataset included 76 sequences and 389 amino acids from five species. We performed statistical analysis using the CodeML program in PAML version 4.9 software using the site, branch, and branch-site models[110], implemented in EasyCodeML[109].

Parameter estimates (ω) and likelihoods cores[111] were calculated for the three pairs of models. These were M0 (one-ratio, assuming a constant ω ratio for all coding sites) vs. M3 (discrete, allowed for three discrete classes of ω within the gene), M1a (nearly neutral, allowed for two classes of ω sites: negative sites with ω0 < 1 estimated from our data and neutral sites with ω1 = 1) vs. M2a (positive selection, added a third class with ω2 possibly > 1 estimated from our data), and M7 (beta, a null model in which ω was assumed to be beta-distributed among sites) vs. M8 (beta and ω, an alternative selection model that allowed an extra category of positively selected sites)[112].

A series of branch models and branch site models were tested: the one-ratio model for all lineages and the two-ratio model, where the original enzyme functional evolution occurred. The branch-site model assumes that the branches in the phylogeny are divided into the foreground (the one of interest for which positive selection is expected) and background (those not expected to exhibit positive selection).

Likelihood ratio tests (LRT) were conducted to determine which model measured the statistical significance of the data. The twice the log likelihood difference between each pair of models (2ΔL) follows a chi-square distribution with the number of degrees of freedom equal to the difference in the number of free parameters, resulting in a p-value for this[113]. A significantly higher likelihood of the alternative model compared to the null model suggests positive selection. Positive sites with high posterior probabilities (> 0.95) were obtained using empirical Bayes analysis. If $\omega > 1$, then there is a positive selection on some branches or sites, but the positive selection sites may occur in very short episodes or on only a few sites during the evolution of duplicated genes; $\omega < 1$ suggests a purifying selection (selective constraints), and $\omega = 1$ indicates neutral evolution. Finally, naive empirical Bayes (NEB) approaches were used to calculate the posterior probabilities that a site comes from the site class with $\omega > 1$[112]. The selected sites and images of protein topology were predicted using Protter[114].

**Transcriptome analysis.** For expression analysis, we used the published RNA-Seq dataset from leaves for the yellow and red morphotypes of *P. cattleyanum*[115]. To verify the quality of reads and the presence of Illumina adaptors, we used the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Based on these data, we used the Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to eliminate read strings with a quality below 30 and adapter sequences.

Two replicates from the red morphotype and two from the yellow morphotype, corresponding to four RNAseq libraries, were aligned on the draft genome assembly of each morphotype (unpublished data) using TopHat2[116]. The read count tables mapped to each gene were generated using the featureCounts module of the Subread software [117], from the bam anchor files generated by TopHat2. The criteria used to create the counting tables were as follows: fragments (pairs of reads) were counted instead of individual reads, pairs of reads anchored on different chromosomes or anchoring on identical chromosomes but on different strands were not considered, and neither were the reads anchored in multiple places in the genome.

We used the DESeq2 package version 1.36[118] to perform statistical analysis and identify differential expression. We analyzed the counting tables using a false discovery rate (FDR) of 0.05, log2 fold change ≥ ± 1[119] and separated them into a group formed by the "up-regulated" genes and another formed by the "down-regulated" genes.

As the genome of each morphotype was assembled separately and corresponded to the same evaluated species in question, we performed two independent comparative transcriptomic analyses: a comparison of morphotype red leaf against yellow leaf anchoring in red morphotype genome (i) and in yellow genome (ii). We evaluated the differential expression considering each gene found in each morphotype and were able to detect more genes under differential gene expression (DGE), considering that some gene copies were detected only in one of the reference genomes.

**Ethical standards.** The yellow and red morphotypes of *Psidium cattleyanum* were sampled originally as part of the project "Genomics and Transcriptomics Analysis of *Psidium cattleyanum* Sabine (Myrtaceae)". The studied samples were collected in full compliance with specific federal permits issued by the approved by the Brazilian Ministry of Environment (MMA) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and approved by the Biodiversity Information and Authorization System (SISBIO 43338-2) and National System for Governance of Genetic Heritage and Associated Traditional Knowledge (SisGen A7B0331). The studied plants are kept in an ex-situ collection at the Federal University of Rio Grande do Sul (UFRGS). Exsiccates will be deposited in the ICN herbarium of UFRGS. As official authorities in Brazil reported, the species used in this study are not endangered or protected in the Rio Grande do Sul State, where the sampling occurred.

## Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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## Author contributions

A.C.T.Z. coordinated the project. D.C. designed and performed experiments, analyzed data, and wrote the manuscript. F.G. performed bioinformatic analysis of RNA-seq data. L.M. performed chromatographic experiments. A.C.T.Z. and M.F. provided critical suggestion and revised the manuscript. All authors read and approved the final manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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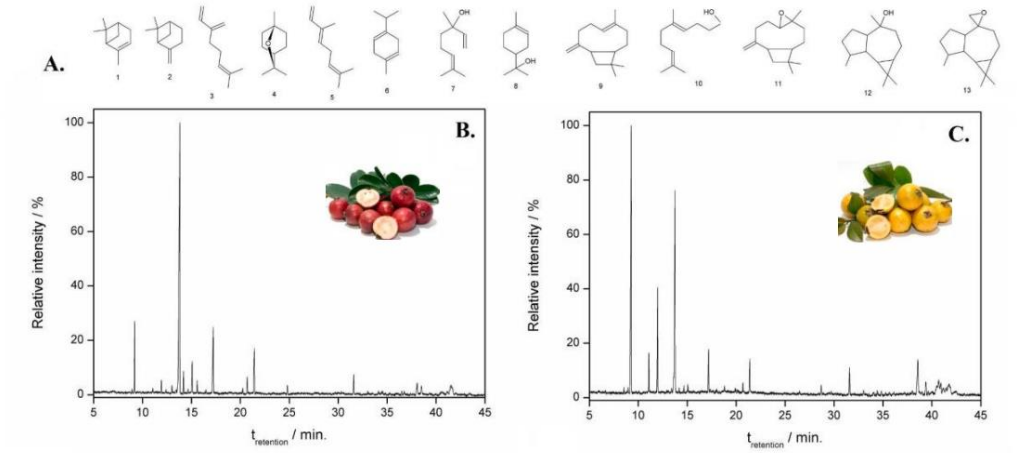
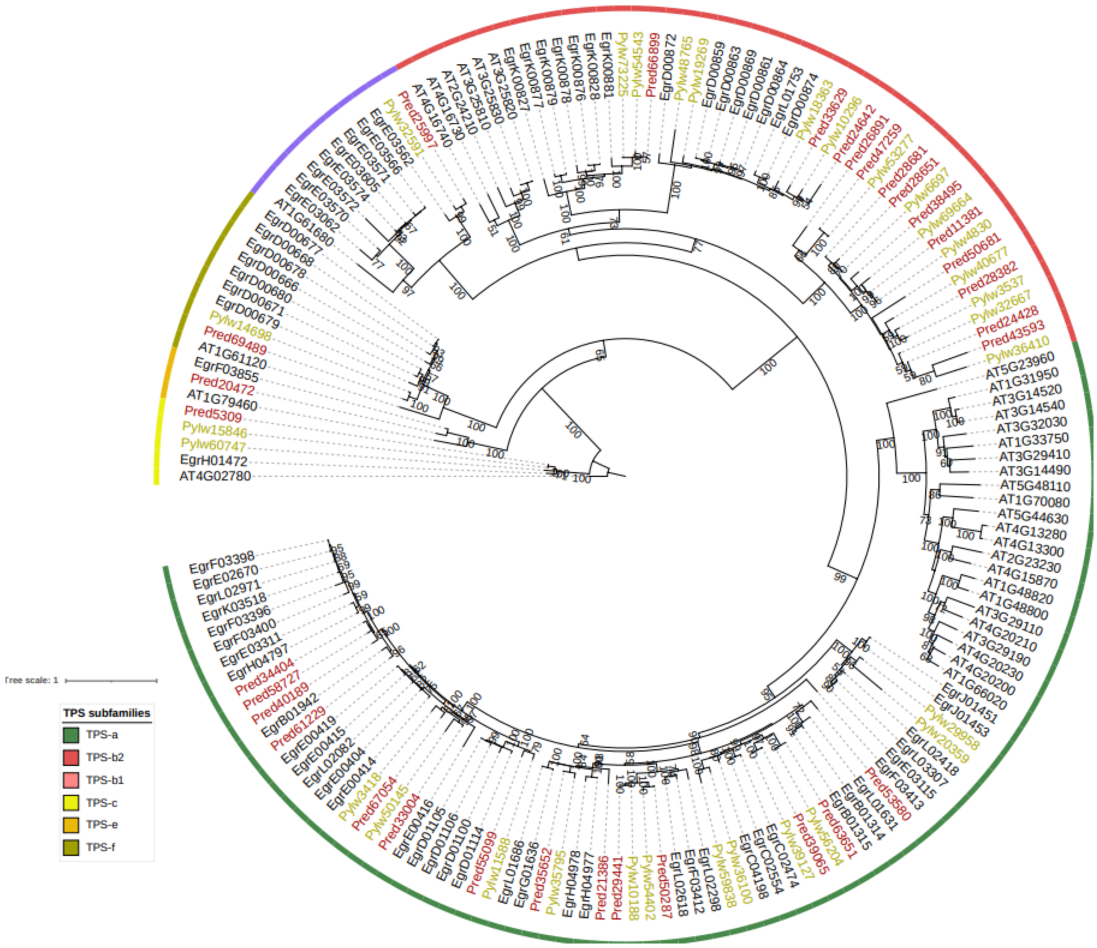
# SUPPLEMENTARY MATERIAL



Supplementar Figure S1. Chromatogram of *Psidium cattleyanum* essential oil. **A.** Red and **B.** Yellow morphotypes. **C.** Chemical structures of the compounds identified in the essential oil : 1) α-pinene; 2) β-pinene; 3) β-myrcene; 4) 1,8-cineole; 5) β-ocimene; 6) γ- terpinene; 7) linalool; 8) α-terpineol; 9) β-caryophyllene; 10) nerolidol; 11) caryophyllene oxide; 12) viridiflorol; 13) aromadendrene epoxide.



Supplementar Figure S2. Phylogenetic analysis of *Eucalyptus grandis*, *Arabdopisis thaliana* and *Psidium cattleianum* full length terpene synthase (TPS) enzymes using 164 sequences. The clusters correspond to TPS subfamilies: TPS-a, TPS-b, TPS-c, TPS-e/f and TPS-g. Protein alignments were conducted using the ClustalW algorithm and trees were inferred using maximum likelihood methods. The support values associated with the branches are bootstrapping, with values over 60% shown.