UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE GENÉTICA

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

Identificação e caracterização de genes envolvidos na biossíntese de lipídios em *Eugenia uniflora* L. (Myrtaceae) e seu potencial envolvimento com mecanismos adaptativos na espécie

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Orientadora: Prof.ª Dr.ª Andreia Carina Turchetto Zolet

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Dissertação submetida ao Programa de Pós-graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do grau de Mestre em Genética e Biologia Molecular.

Orientadora: Prof.ª Dr.ª Andreia Carina Turchetto Zolet

Porto Alegre, abril de 2019

“Existem muitas hipóteses em ciência que estão erradas.
Isso é perfeitamente aceitável,
elas são a abertura para achar as que estão certas”

Carl Sagan

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dar e é tanto assim que eu me considero parte de suas famílias, sei que posso contar com elas e elas comigo, seu apoio não tem limites e acredito que o céu é pouco para vocês, muito obrigado.

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RESUMO

Eugenia uniflora L., conhecida popularmente como pitanga, pitangueira ou cereja do Brasil, pertence à família Myrtaceae e sua distribuição ocorre ao longo dos domínios da Floresta Atlântica. *E. uniflora* é uma espécie versátil que ocorre em diferentes ambientes e apresenta variação no fenótipo, provavelmente como uma resposta ao ambiente. Essas características, e outras mais, a postulam ela como um bom modelo para estudos de variação adaptativa de populações naturais. Nesse sentido os lipídios podem ter um papel nos processos de adaptação das espécies por estarem envolvidos em uma extensa gama de reações metabólicas, exercendo papéis essenciais no desenvolvimento das plantas, como componentes das membranas celulares, armazenamento, moléculas sinalizadoras, entre outros. Os genes da rota da biossíntese de lipídios em plantas já foram reportados por participar em respostas a estresses ambientais. Entendendo que a adaptação depende do potencial das espécies de se adaptar e como a plasticidade fenotípica age a sinais ambientais conseguindo benefícios de ajuste para as espécies. Dessa forma, a presente dissertação teve como objetivo identificar e caracterizar genes envolvidos na rota de síntese de lipídios em *Eugenia uniflora* e verificar o seu potencial envolvimento em mecanismos adaptativos na espécie. Para isso, foram identificados e caracterizados os homólogos de *GPAT* em *E. uniflora*, utilizando os dados do transcriptoma através de diversas ferramentas de bioinformáticas como Blast2GO, Blastx ou ORFfinder e a base de dados Phytozome. Com as sequências obtidas foram realizadas análises filogenéticas, junto com os genes GPATs de *Arabidopsis thaliana* e *Eucalyptus grandis* já caracterizados, para a identificação dos ortólogos de GPATs e identificação dos domínios de proteínas conservadas. Além disso, também foram realizadas análises filogenéticas com as espécies do clado Rosidae que possuem genoma sequenciado para reconstruir as relações evolutivas dessa família gênica incluindo os genes de *E. uniflora*. Análises histoquímicas e morfológicas das folhas para a identificação de lipídios foram realizadas em indivíduos de duas populações. Para verificar o padrão de expressão dos genes *GPAT* nas populações de *E. uniflora* provenientes de distintos ambientes, foram realizadas análises de RT-qPCR. Sete genes GPATs putativos foram identificados em *E. uniflora.* As análises filogenéticas revelaram um ortólogo de GPAT1 (Euni_k21137491), dois ortólogos de GPAT2/3 (Euni_k21131380, Euni_k25280104), um ortólogo de *GPAT* 4/8 (Euni_k21284220), um ortólogo de *GPAT* 6 (Euni_k21135418) e dois ortólogos GPAT9 (Euni_k25121268, Euni_k25344309). Essas sequências mostraram relações evolutivas que resultaram de processos de duplicação e diversificação quando analisadas com

genes ortólogos com espécies do clado Rosidae. Os primers desenhados para RT-qPCR foram testados em ambas as populações com fenótipos característicos dos ambientes provenientes, porém, os resultados da expressão ainda estão em andamento. As análises histoquímicas e morfológicas mostraram que a anatomia das folhas é semelhante entre as duas populações analisadas. Foi evidenciado a presença de lipídios (cutina) na cutícula da espécie sendo mais espessa nos indivíduos da população de Mata ciliar do que naqueles de restinga. Estes resultados possibilitaram explorar um caminho a mais para fazer inferências sobre a evolução adaptativa de populações naturais.

Palavras-chave: GPAT (*sn-Glycerol-3-phosphate 1-O-acyltransferase*), *Eugenia uniflora*, Biossíntese de lipídios, Adaptação, Evolução, Myrtaceae

ABSTRACT

Eugenia uniflora L., popularly known as pitanga, pitangueira or cherry tree of Brazil, belongs to the family Myrtaceae and its distribution occurs along the Atlantic Forest domains. *E. uniflora* is a versatile species, that occurs in different environments and presents variation in the phenotype, probably as a response to the environment. These characteristics and others postulate as a model for studies of adaptive variation of natural populations. In this sense, lipids can be implicated in the adaptation processes of the species, since they are involved in a wide range of metabolic reactions, playing essential roles in the development of plants, such as: cell membrane components, storage, signaling molecules, among others. The genes of the lipid biosynthesis pathway in plants have been reported to participate in responses to environmental stresses. Understand that adaptation depends on the adaptive potential of the species and how phenotypic plasticity acts on the environmental signals, obtaining benefits for the species. Thus, the present dissertation aimed to identify and characterize genes involved in the route of lipid synthesis in *Eugenia uniflora* and to verify its potential involvement in adaptive mechanisms in the species. To that end, homologues of *GPAT* in *E. uniflora* were identified and characterized using transcriptome data through various bioinformatics tools such as Blast2GO, Blastx or ORFfinder and Phytozome database. With the sequences obtained, phylogenetic analysis were carried out, together with *GPAT* genes of *Arabidopsis thaliana* and *Eucalyptus grandis* already characterized, for the identification of orthologs of *GPATs* and identification of conserved protein domains. In addition, phylogenetic analyzes were also carried out with Rosidae clade species that have a sequenced genome to reconstruct the evolutionary relationships of this gene family including those of *E. uniflora* within the clade. Histochemical and morphological analysis of the leaves for the identification of lipids were performed in individuals from the same populations. To verify the expression pattern of *GPAT* genes in populations of *E. uniflora* from different environments, RT-qPCR analysis were performed. Seven putative *GPAT* genes were identified in *E. uniflora*. Phylogenetic analyzes revealed a *GPAT* ortholog of *GPAT1* (Euni_k21137491), two *GPAT2* / 3 orthologs (Euni_k21131380, Euni_k25280104), *GPAT* 4/8 ortholog (Euni_k21284220), *GPAT6* ortholog (Euni_k21135418) and, two *GPAT9* orthologs (Euni_k25121268, Euni_k25344309). These sequences showed evolutionary relationships that are of resulted in duplication and diversification processes when analyzed with orthologous genes of Rosidae species. The primers designed for RT-qPCR were tested in both populations with phenotypes characteristic of the coming environments, however,

the expression results are still in progress. Histochemical and morphological analysis showed that the anatomy of the leaves is similar between the two populations analyzed. It was evidenced the presence of lipids (cutin) in the cuticle of the species being more present in the individuals of the Riparian forest population than in those of Restinga. These results enabled us to explore an additional way to infer about the adaptive evolution of natural populations.

Keywords: GPAT (*sn-Glycerol-3-phosphate 1-O-acyltransferase*), *Eugenia uniflora*, Lipid biosynthesis, Adaptation, Evolution, Myrtaceae

1. INTRODUÇÃO

1.1 *Eugenia uniflora* L. um bom modelo para estudos de evolução e adaptação

Além de serem organismos fascinantes, as plantas proporcionam importantes oportunidades para o estudo dos mecanismos e processos evolutivos de adaptação a diversas condições ambientais. Existem muitos exemplos de espécies de plantas que apresentam uma distribuição em ambientes heterogêneos e, consequentemente, essas espécies devem estar aptas a desenvolver e aprimorar mecanismos para suportar os fatores bióticos e abióticos dominantes em cada tipo de ambiente. *Eugenia uniflora* L (**Figura 1**) é um exemplo de espécie que ocorre em ambientes heterogêneos ao longo dos Domínios da Floresta Atlântica (DFA). Ocorre no Brasil desde o Rio Grande do Sul até Pernambuco, bem como no nordeste da Argentina, Uruguai e Paraguai (Sobral *et al.*, 2013; Rotman, 1995). A capacidade de *E. uniflora* de desenvolver-se em diferentes habitats, é uma característica relevante que torna essa espécie um interessante modelo para estudos de variação adaptativa em plantas (Turchetto-Zolet *et al.*, 2016). Além disso, a espécie possui importância econômica, por apresentar produção de compostos secundários usados na indústria farmacêutica e cosmética, e desempenhar um papel ecológico relevante quanto aos seus dispersores e polinizadores

Eugenia uniflora L. conhecida popularmente como pitanga, pitangueira ou cereja do Brasil é uma espécie pertencente à família Myrtaceae, uma das famílias botânicas mais ricas em espécies no mundo, com aproximadamente 5800 espécies, 144 gêneros e classificadas em 17 tribos (Wilson *et al*., 2005; Wilson, 2011; Vasconcelos, *et al*., 2017; WCSP, 2018). Essa família apresenta uma ampla distribuição no hemisfério sul e é um dos componentes florísticos relevantes nas áreas de maior diversidade de espécies no Brasil (Oliveira-Filho & Fontes, 2000; Thornhill *et al*., 2015; Flora do Brasil, 2018). Em ambientes neotropicais, a maior diversidade de Myrtaceae é representada pela tribo Myrteae com 2500 espécies e 51 gêneros (Wilson *et al*., 2005, Lucas *et al*., 2007; WCSP, 2018; Vasconcelos, *et al*., 2017). Dentro de Myrteae, o gênero *Eugenia,* que inclui espécies de árvores ou arbustos com uma distribuição pantropical, é um dos mais diversos com cerca de 1200 espécies (WCSP, 2019) que diversificaram na América do Sul há 20 milhões de anos, sendo por exemplo: *E. involucrata*, *E. brasiliensis*, *E. pluriflora*, entre outras endêmicas da floresta Atlântica (Sobral *et al.*, 2015; Mazine *et al*., 2014; Stehmann, J. R. 2009; Mazine 2006). Espécies do gênero *Eugenia* têm sido descobertas e estudadas em

diversas áreas de interesse como a Restinga e o Cerrado. *E. uniflora* foi descrita pela primeira vez pelo Carolus Linnaeus no *Especies Plantarum* em 1753.

A espécie ocorre em ambientes tropicais e subtropicais da América do Sul, sendo nativa da Mata Atlântica, uma das áreas consideradas como prioritária para a conservação e para hotspots da biodiversidade (Myers *et al*., 2000; da Fonseca, 1985). Estima-se que é a segunda maior floresta tropical na América do Sul, cobrindo uma área de mais de um milhão de quilômetros quadrados ao longo da costa brasileira e se estendendo até o leste do Paraguai e nordeste da Argentina (Ribeiro *et al.,* 2009; Oliveira-Filho & Fontes, 2000; Joly *et al*., 1999)

Eugenia uniflora distribui-se naturalmente em diferentes regiões fitogeográficas tropicais e subtropicais dos DFA (**Figura 2**), onde existe uma alta taxa de endemismos locais (Ribeiro *et al.,* 2009).

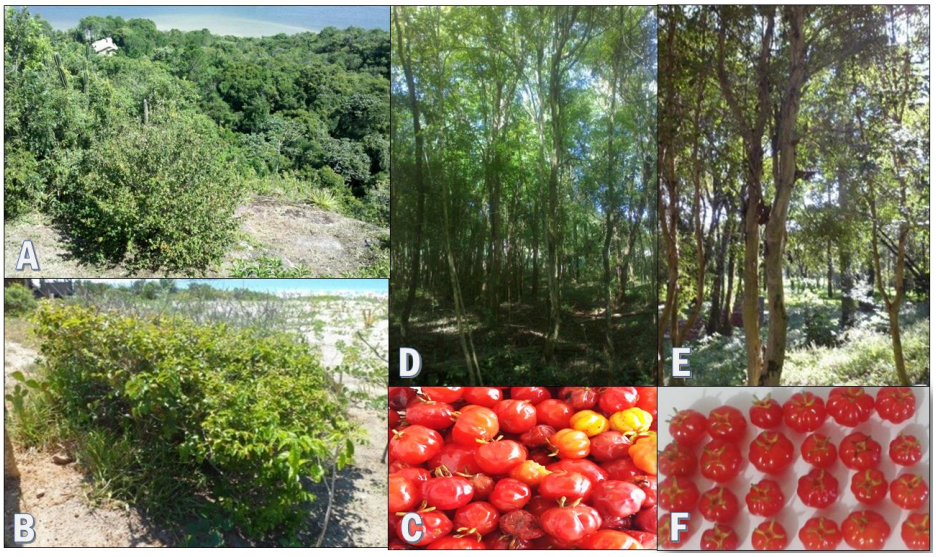

Figura 1. Habitats, hábitos e frutos de *Eugenia uniflora* L. (**A-B**) Subarbustos de *E. uniflora* em ambientes de Restinga na praia de Grumari-Rio de Janeiro. (**C)** Frutos maduros de *E. uniflora* da Restinga. (**D-E**) Arvoretas de *E. uniflora* em ambientes de Mata Ciliar no município de Frederico Westphalen-Rio Grande do Sul. (**F**) Frutos maduros de *E. uniflora* da Mata Ciliar. Fonte: Arquivo pessoal

Estes ambientes são edafoclimáticamente heterogêneos, devido a suas formações geomorfológicas, composição de solo, disponibilidade de água e nutrientes (**Figura 3**), abrangendo as formações vegetais: Mata Atlântica (floresta tropical), floresta semidecídua (Oliveira-Filho & Fontes, 2000), estepe campestre (Roesch *et al*., 2009) e o ecossistema de restinga adjacente (Scarano, 2002) das regiões norte e nordeste do Brasil, norte da Argentina e Uruguai. A espécie apresenta morfologias variáveis que vão desde arbusto ou pequena árvore na vegetação da planície costeira arenosa (Restinga) no sudeste e sul do Brasil (**Figura 1 A**, **B**),

até árvores ou arvoretas de 15 metros, na vegetação ribeirinha (Mata Ciliar) da região Sul (sul do Brasil, Paraguai, Uruguai e Argentina) (**Figura 1 D**, **E**) (Rattmann *et al*., 2012)

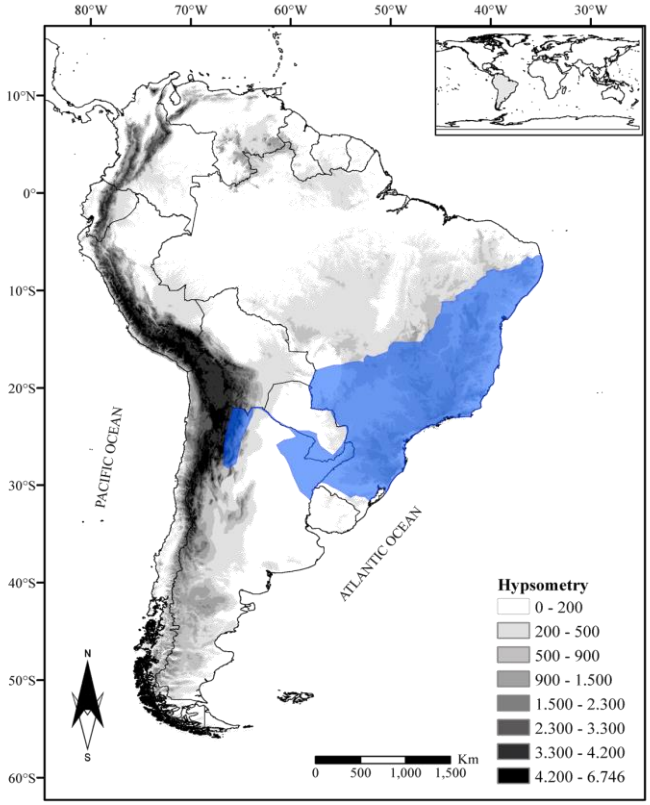

Figura 2. Mapa da distribuição da *Eugenia uniflora* na América do Sul. Extraído e adaptado de Turchetto-Zolet *et al.*, 2016

Eugenia uniflora é uma espécie com diversos usos e propriedades biológicas como relatado em diversos estudos, seja como planta ornamental, medicinal (por conter propriedades anti-fúngicas, anti-hipertensivas e anti-inflamatórias), de importância na indústria da beleza (metabólitos secundários como óleos essenciais) ou alimentícia pelos seus frutos comestíveis (**Figura 1 C**, **F**) para diversos animais (Rodrigues *et al*., 2013; Rattman *et al.*, 2012; Santos *et al*., 2012; Costa *et al.*, 2009; Oliveira *et al*., 2008; Fiuza *et al.*, 2008; Schapoval *et al*., 1994). *E. uniflora* também desempenha um papel importante para a recuperação de áreas degradadas, o que torna a espécie atrativa em âmbitos comerciais e ecológicos. Estudos filogenéticos recentes sugerem que *E. uniflora* está proximamente relacionada com *E. brasiliensis* Lam, *E. neomyrtifolia* Sobral, *E. sulcata* Spring ex Mart. e *E. pitanga* Kiaersk. (Vasconcelos *et al.*, 2017; Mazine *et al*., 2014; Wilson, 2011).

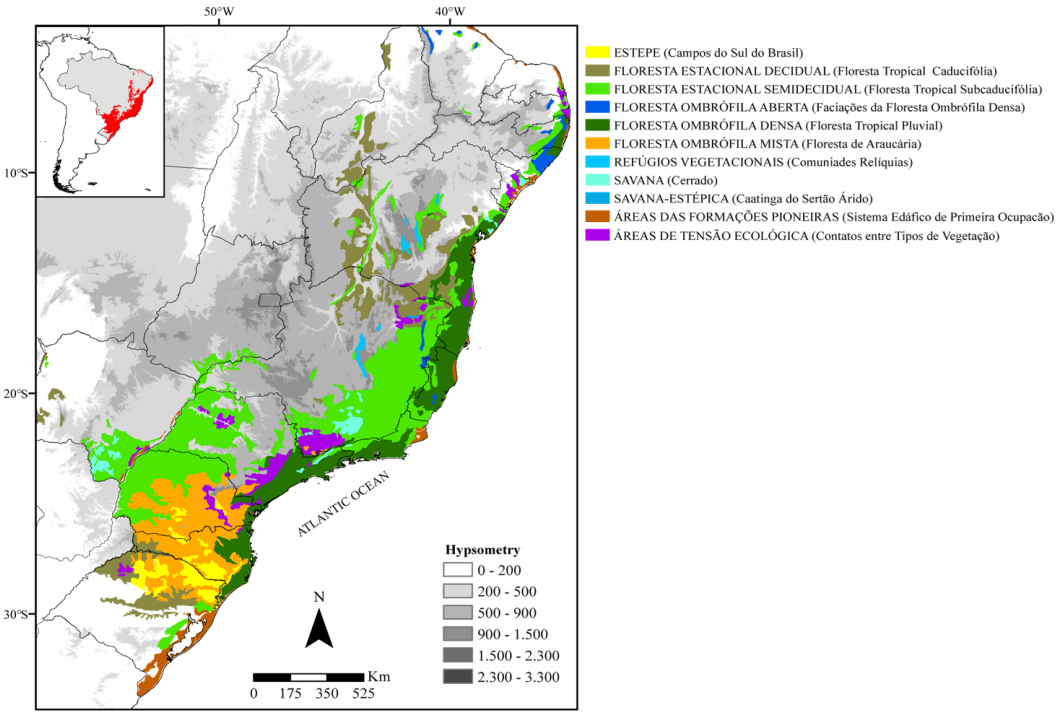

Figura 3. Cobertura original da Floresta Atlântica e seus diferentes domínios. Adaptado da Fundação SOS Mata Atlântica

Nos últimos anos foram realizados estudos de variabilidade e datação molecular para revelar a diversidade e diferenciação genética de *E. uniflora*, (Turchetto-Zolet *et al*., 2016; Ferreira-Ramos *et al*., 2008, 2014; Salgueiro *et al*., 2004; Margis *et al*., 2002), além de estudos sobre a regulação da expressão gênica e vias metabólicas (Vetö *et al.*, não publicado; Anton *et al.*, não publicado; Guzmán *et al*., 2014) dessa espécie.

Portanto, o entendimento sobre a história de vida das espécies, a preservação dos seus diferentes linhagens, a história demográfica das populações, identificação de centros de diversidade genética, os efeitos das oscilações das dinâmicas ecológicas e climáticas (Martins 2011; Turchetto-Zolet *et al*., 2013) em biomas como a Floresta Atlântica, que atualmente está totalmente fragmentado, permite identificar e entender mecanismos evolutivos que irão nos ajudar a preservar as espécies com as mudanças ambientais e quais foram os eventos capazes de gerar e manter a variabilidade das espécies de plantas, que foram adequados para conferir a adaptação da família Myrtaceae e mais especificamente de *E. uniflora*.

1.2 Uma abordagem sobre adaptação

As plantas oferecem múltiplos caminhos para estudar os processos evolutivos e de adaptação que tem acontecido em cada um dos taxa, abrangendo diversos fatores (condições ambientais, dinâmica ecológica, variação estrutural ou populacional, etc.) que possibilitam compreender o contexto atual e, portanto, testar hipóteses que refletem sua história ecológica e evolutiva em um determinado período.

Note-se que a variabilidade morfológica de *E. uniflora* e sua capacidade de desenvolver-se em diferentes habitats, são características relevantes que sugerem mecanismos evolutivos envolvidos na adaptação em resposta a variações ambientais. Um trabalho recente do grupo (Turchetto-Zolet *et al*., 2016) revelou padrões de estruturação populacional, diversidade genética e demografia histórica das populações de *E. uniflora* distintos nos diferentes ambientes da sua distribuição. Nesse contexto, Veto *et al.*, dados não publicados, evidenciaram 70 polimorfismos de nucleotídeos únicos (SNPs) associados a variações climáticas e estrutura populacional em *E. uniflora*. Certamente, um dos eventos complexos é a evolução da diversidade neotropical, onde temos divergência morfológica máxima ocorrendo, com "baixos" níveis de diversidade genética observável (Frankham, 1997), mas com evidências de que as alterações genéticas responsáveis pelas adaptações morfológicas envolvem alterações nos genes regulatórios, em vez dos genes estruturais (Takayama *et al.*, 2015; Perugganan *et al*.,2003)

A importância de compreender quais são os mecanismos evolutivos que conduzem à adaptação de espécies em resposta às variações ambientais por exemplo, contribuem para candidatar a *Eugenia uniflora* como modelo excepcional para este tipo de estudo, uma vez que possui elementos chaves que podem fornecer e ajudar a reconstruir os eventos que levaram a estes processos, devido à sua distribuição em locais de características ambientais e climáticas distintas, além de sua adaptação (Turchetto-Zolet *et al.*, 2016). Garantindo que as investigações de alguns organismos, utilizados como modelos, no caso desta espécie, consigam elucidar a amplitude de processos evolutivos que mantêm a variação fenotípica e genética nas populações naturais (Mitchell-Olds *et al*., 2007) e permitam conhecer mais a respeito dos mecanismos adaptativos que foram utilizados para manter suas populações nos diferentes ambientes onde estão presentes.

Nesse sentido, a evolução e a continua variação genética é o modo mais rápido de adaptação, acontecendo pela compatibilidade com o genoma. (Elmer & Meyer, 2011; Barrett & Schluter, 2008). Na biologia evolutiva, uma das explicações mais aceitáveis para as modificações nas taxas de diversificação tem como base à aquisição de novas características biológicas na linhagem (Donoghue, 2005). No caso da família Myrtaceae e mais certamente para a tribo Myrteae, as diferenças da morfologia do embrião em *Myrcia*, *Plinia* e *Eugenia* são catalogadas como vantagens adaptativas para esses gêneros pela rápida germinação das sementes e sua capacidade de armazenar amidos (Vasconcelos *et al.*, 2017; Landrum, 1986; Landrum & Stevenson, 1986)

Além disso, existem outros fatores que alteram o potencial das espécies de se adaptar aos diferentes ambientes, como as mudanças climáticas, que favorecem a expansão de habitats e estabelecimento de regimes, por exemplo, ao fogo (Antonelli & SanMartin, 2011), desencadeando a diversificação de plantas adaptadas nessas condições, como pode ter acontecido em diferentes períodos de esfriamento global nos continentes do sul (Simon *et al*., 2009; Crisp *et al.*, 2010; Bytebier *et al.*, 2011). Afiançando, que a adaptação local evolua assim, os organismos devem ser capazes de agir a sinais ambientais previsíveis, espécies com maior diversidade genética tenham uma série de benefícios de ajuste sobre os genótipos que não tem a possibilidade de alterar seus fenótipos (Anderson *et al*., 2010; Baythavong & Stanton, 2010)

Por outro lado, na literatura expõe-se que espécies com ampla distribuição geográfica apresentam diferenciação genotípica e fenotípica e, portanto, adaptação local (Neale & Kremer 2011; Alberto *et al*., 2013). Embora o tamanho geográfico das populações esteja correlacionado com a adaptação (Volis *et al*., 2015), a adaptação local pode ser observada em escalas espaciais menores (Eckert *et al*., 2015). A plasticidade fenotípica funciona simultaneamente com a seleção na maioria dos casos estudados (Kremer *et al*., 2014) e, portanto, pode desvirtuar as correlações entre adaptações locais e divergência fenotípica nas populações (Schmid & Guillaume, 2017).

Atualmente, o desenvolvimento de tecnologias de Sequenciamento de Nova Geração (*Next Generation Sequencing - NGS*) tem possibilitado a disponibilidade de bases de dados de sequências de diversas espécies (De Wit *et al*., 2015), facilitando o uso de análises multilocus para assim entender as diferentes estratégias de adaptação das plantas. No entanto, enfrentamos

uma tarefa sem precedentes, pois parte dessa diversidade alvo de estudos está em risco considerável de desaparecer devido à destruição do habitat e às mudanças climáticas (Antonelli & SanMartin, 2011; Hubbell *et al*., 2008; Svenning & Condit, 2008). Assim, pulando essas razões, é importante abordar, compreender e estudar quais são as características adaptativas básicas nos processos de adaptação nos leva a conhecer e a discernir sobre que elementos interagem com o genótipo e quais com o fenótipo.

Evidentemente não podemos ignorar o papel das barreiras geográficas, já que sem elas o intercâmbio biótico entre áreas ocorreria de contínuo, o que teria promovido a especiação por meio da adaptação gradual a altitudes levemente desiguais (Smith *et al.,* 1997) no caso da Amazônia e dos Andes. Se fora acontecido desse jeito, hoje nenhum padrão biogeográfico seria observado (Antonelli & SanMartin, 2011). Então, toda essa somatória de fatores vai contribuir com a variabilidade e essas características diversas evoluem como resultado de fatores ambientais impondo pressão seletiva sobre os indivíduos que habitam uma paisagem heterogênea (Schoville *et al*., 2012). Entender como aqueles processos agem no genoma de um organismo, e como ele evolui em resposta e os desafios ambientais aos quais foi exposto, é essencial para conhecer os processos que contribuíram para os padrões atuais de distribuição e diversidade das espécies resultado da adaptação a ambientes (Schoville *et al*., 2012; Feder & Mitchell-Olds 2003; Elmer & Meyer 2011; Rice *et al*., 2011)

Assim, nos últimos tempos o estudo de genes candidatos podem demonstrar e elucidar sobre o nível e a estrutura da variação genética adaptativa, permitindo a caracterização de genes potencialmente envolvidos em resposta na adaptação em diferentes eventos como, adaptação local, stress abiótico ou fenologia do brotamento (Lalagüe *et al*., 2014).

1.3 Lipídios uma alternativa para adaptação

Os lipídios são um grupo de biomoléculas estruturalmente diversas e de composição química variada, apresentando como característica comum serem insolúveis na água e solúveis em solventes orgânicos (Amaro *et al.,* 2015). Os lipídios incluem uma ampla variedade de compostos derivados de ácidos graxos, além de pigmentos e compostos secundários que não estão diretamente ligados ao metabolismo dos ácidos graxos (Li-Beisson *et al.,* 2013; Liang *et al.,* 2014). Cada célula vegetal contém uma gama diversificada de lipídios, localizados em estruturas e tecidos específicos, que interagem em diferentes e complexas vias ou rotas lipídicas. As plantas acumulam diversos ácidos graxos (Furt *et al.,* 2011) e, portanto, há muitas

hipóteses sobre a natureza das enzimas envolvidas na síntese desses compostos, além de quais são as vias e mecanismos que as regulam.

Tabela 1. Tipos de lipídios e sua função. Adaptado e traduzido de Buchanan *et al.*, 2015

Função	Tipo de lipídio envolvido
Componentes estruturais de membrana	Glicerolipídios
	Esfingolipídios
	Esteróis
Compostos de armazenamento	Triacilgliceróis
	Ceras
Compostos ativos em reações de transferência de elétrons	Clorofila e outros pigmentos
	Ubiquinona e plastoquinona
Fotoproteção	Carotenoides (Ciclo da xantofila)
Proteção de membranas contra danos causados por radicais livres	Tocoferóis
Impermeabilização e proteção de superfície	Ácidos graxos de cadeia muito longa, cadeia longa e seus derivados (cutina, suberina, ceras superficiais)
Modificação de proteína	
Adição de âncoras de membrana	
Acilação	Principalmente 14: 0 e 16: 0 ácidos graxos
Prenilação	Farnesil e geranilgeranil pirofosfato
Outros componentes de âncora de membrana	fosfatidilinositol, ceramida
Glicosilação	Dolichol
Sinalização	
Interno	Ácido abscísico, giberelina, brassinosteróides
	18:3 precursores de ácidos graxos do jasmonato
	Inositol fosfato
	Diacilglicerol
Externo	Jasmonato
Compostos de defesa e anti-alimentação	Atraentes de insetos voláteis
	Óleos essenciais
	Componentes de látex (borracha, etc.)
	Componentes de resina (Terpenos)

Os lipídios estão envolvidos em uma ampla gama de reações metabólicas, exercendo papéis fisiológicos importantes no desenvolvimento das plantas, como principais componentes das membranas celulares, armazenamento, camadas protetoras extracelulares e moléculas sinalizadoras (Waschburger *et al.*, 2018; Buchanan *et al.*, 2015; Chen *et al.*, 2011) (**Tabela 1**).

No caso das membranas, elas ajudam na compartimentalização celular, formadas por lipídios polares que impede a difusão livre de moléculas hidrofílicas entre as organelas celulares e impedem a difusão para dentro e para fora das células (Xu, 2013). Os lipídios também são fonte de reserva química de energia livre, já que os ácidos graxos sendo moléculas orgânicas mais reduzidas, tem um potencial maior para produzir energia quando comparadas com os carboidratos (Buchanan *et al.,* 2015). Os ácidos graxos são precursores de outros componentes relevantes do metabolismo vegetal, por exemplo, ceras que revestem e protegem as plantas do meio ambiente com derivados de ácidos graxos, ou a cutina das células epidérmicas que também é composta de ácidos graxos oxigenados esterificados (Chen *et al.,* 2014; Beisson *et al.*, 2012, Nawrath, 2002).

Nas plantas terrestres, a síntese de lipídios ocorre no retículo endoplasmático (ER), junto com outros processos relacionados a essas estruturas (Wang & Benning, 2012; Xu *et al.*, 2008). Os lipídios processados pelas plantas são os triacilgliceróis / triacilglicerídeos (TAG), são produzidos através da via de Kennedy ou rota do glicerol fosfato (Kennedy and Weiss, 1956**)**. Esse é o principal caminho para a biossíntese de triglicerídeos (Maraschin *et al.,* 2019) em vários organismos no ER (Durrett, 2013), que também servem como repositório para a biossíntese de ácidos graxos (AGs) e fosfolipídios (Gao *et al*., 2014). Esses TAG são compostos por três AGs esterificados ligados e uma molécula de glicerol nas posições *sn*-1, *sn*-2 e *sn*-3. Além de serem neutros e envolvidos em diferentes funções celulares, são a fonte de armazenamento primário de energia nas células eucarióticas (Maraschin, *et al.,* 2019; Sami *et al.,* 2014; Zweytick *et al.,* 2000).

Os TAG acumulam-se nas plantas em sementes, pericarpos, flores, pólen e folhas (Maraschin *et al.,* 2019; Reynolds *et al.,* 2015; Cagliari *et al.,* 2011; Knutzon *et al.,* 1995). TAGs quando armazenado nas sementes, contém ácido palmítico (16:0), ácido esteárico (18:0), ácido oleico (18:1Δ^{9}), ácido linoleico (18: 2$\Delta^{9,12}$) e ácido α- linolênico (ALA, 18:3 $\Delta^{9,12,15}$) (Iskandarov *et al.,* 2017; Chen *et al.,* 2016). Os TAGs contribuem para muitos estágios de desenvolvimento nas plantas. Eles são as principais reservas de AGs para a síntese de carboidratos e produção de energia durante a germinação de sementes, o estabelecimento inicial de plântulas e o desenvolvimento de órgãos pós-germinativos (Marchin *et al.,* 2017; Fan *et al.,* 2014; Mañas-Fernández *et al.,* 2013). A maior parte do TAG em sementes, armazenadas em gotículas lipídicas citosólicas (Turchetto-Zolet *et al.,* 2011; Stone *et al.,* 2004), é mobilizada

imediatamente após a germinação para sustentar o crescimento e desenvolvimento da plântula, antes do estabelecimento da fotossíntese.

Nesse contexto, genes que codificam enzimas que atuam na rota da biossíntese de lipídios em plantas já foram reportados por estarem envolvidos em respostas a estresses ambientais, já que muitos formas de lipídios fazem parte da constituição de membranas (Golldack *et al.*, 2011; Kader & Lindberg 2010) e também são formas de lipídios que exercem proteção às plantas (cutina e suberina) como relatado por Bourdenx *et al.*, 2011; Lee *et al.*, 2009; Pierrugues *et al.*, 2001. Já foi demonstrado que em condições de estresse salino as plantas reforçam as barreiras apoplásticas através da suberização (Kolattukudy, 2001). Os genes da rota de síntese de Lipídios já foram bem caracterizados em diversas espécies de plantas (Men *et al.*, 2017; Misra *et al.*, 2014; Arroyo-Caro *et al.*, 2013; Carma & Han, 2009), então a identificação e caracterização de genes envolvidos na biossíntese de lipídios em espécies promissoras como a *E. uniflora*, pode auxiliar no entendimento de processos adaptativos em populações naturais.

1.4 Família gênica *GPAT* (Glycerol 3 phosphate Acyltransferase) uma resposta para a adaptação?

sn-Glycerol-3-phosphate 1-O- acyltransferase (GPAT; Enzyme Commission [EC] 2.3.1.15) é a primeira enzima que participa da biossíntese de glicerolipídios e na síntese de novo da membrana (Yang *et al.*, 2012). Muitos estudos revelaram o papel fundamental das GPATs na biossíntese lipídica, pela associação ao crescimento, desenvolvimento e resistência das plantas ao estresse biótico e abiótico (Washburger *et al.*, 2018).

As GPATs catalisam a primeira etapa da biossíntese de TAG (**Figura 4**) pela acilação do glicerol 3-fosfato na hidroxila *sn*-1 ou *sn*-2 com um doador de acila, acil-CoA ou acil-ACP e gerando ácidos que podem atuar como moléculas de sinalização na regulação do crescimento celular (Men *et al.*, 2017; Sheng *et al*., 2015; Takeuchi & Reue, 2009). Esses ácidos são intermediários chave na biossíntese de todos os glicerolípidos, a partir dos ácidos lisofosfatídicos (LPAs). Numerosas isoformas destas enzimas são conhecidas e têm sido caracterizadas amplamente em bactérias, fungos, animais e plantas (Murata & Tasaka, 1997; Zheng & Zou, 2001; Gimeno & Cao, 2008; Zhang & Rock, 2008; Wendel *et al*., 2009; Chen *et al*., 2011), elas são expressas com distribuições específicas de tecidos e membranas e são regulados por diferentes vias, por exemplo metabólicas (Buchanan *et al.*, 2015).

De acordo com a literatura em *Arabidopsis thaliana* (L.) Heynh, planta modelo para diversos estudos, existem 10 genes anotados como *GPATs*: localizadas no plastídio, como a *GPAT* solúvel (Nishida *et al.*, 1993), e no retículo endoplasmático as nove *GPATs* restantes. Em plantas terrestres são encontradas todas as formas gênicas que codificam as enzimas *GPATs* (*GPAT1-9* e *GPAT* solúvel) sendo que as *GPAT* 1 a 8, são exclusivas das plantas terrestres; Dentre as *GPAT1-8* pelo menos cinco delas codificam aciltransferases *sn*-2 que não estão envolvidas na síntese de TAG (Maraschin *et al.*, 2019), mas participam da síntese de lipídios extracelulares (Li *et al.*, 2007; Beisson *et al.*, 2007; Beisson *et al.*, 2012).

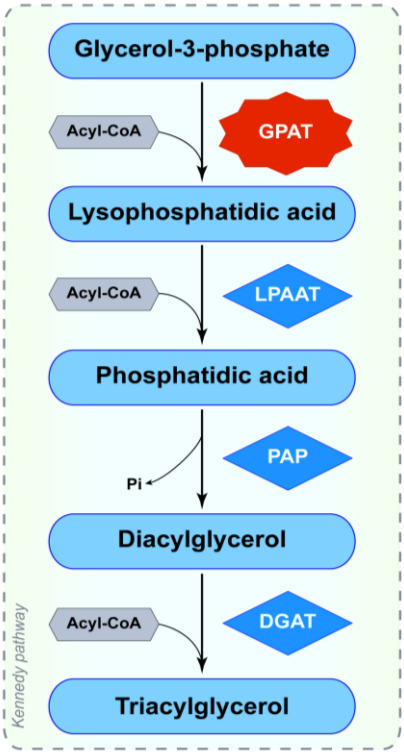

Figura 4. Esquema da biossíntese de triacilglicerídios (TAG) através da via glicerol fosfato, adaptado de Körbes *et al.*, (2016)

Estudos recentes, relatam um papel destacado da GPAT9 na biossíntese de TAG, sugerindo sua conservação ao longo da evolução das plantas e sua capacidade de interagir com outras enzimas envolvidas na biossíntese do glicerolipídios (Li-Beisson *et al.*, 2009; Zhang *et al.*, 2015). Washburger *et al.*, 2018 sugerem numa reconstrução filogenética (**Figura 5**) com 450 sequências que codificam os genes *GPATs* pertencentes a 39 espécies (algas e plantas) a estruturação de três clados: Clado I (GPAT9), Clado II (GPAT solúvel) e o clado III (GPAT 1-8).

As três isoformas que codificam as GPATs ligadas à membrana mitocondrial ou seja, GPATs 1 a 3, foram clonadas em *Arabidopsis* (Zheng *et al.*, 2003) e entre elas, o AtGPAT1 foi essencial para a diferenciação do tapete e a fertilidade masculina (Yang *et al.*, 2012; Chen *et al.*, 2014). Também se sabe que a GPAT4, 6 e 8 (provavelmente as mais ancestrais) estão envolvidas na síntese de poliésteres lipídicos extracelulares (tipo cutina e suberina) nas plantas terrestres (Li *et al.*, 2007; Li-Beisson *et al.*, 2009; Beisson *et al.*, 2012). Além disso, estudos relatam que a GPAT7 é induzida por ferimentos, produzindo um derivado de suberina (Yang, *et al.*, 2012) e o papel da GPAT5 no amoldamento de uma ampla gama de comprimento de

lipídios de cadeia ω-oxidada, envolvidos na síntese de suberina em raiz e semente (Beisson *et al*., 2007)

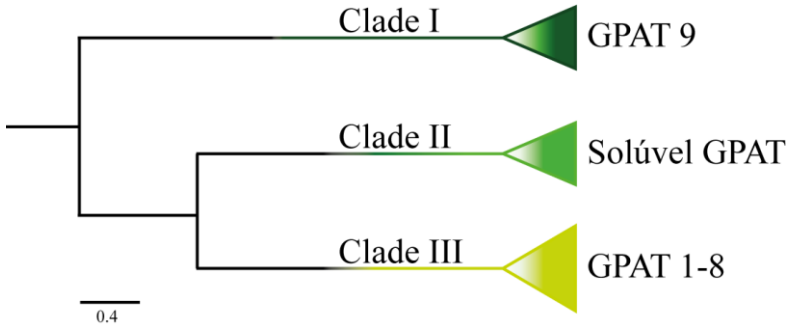

Figura 5. Relação filogenética entre sequências de proteínas GPAT de plantas e algas. Adaptado de Washburger *et al.*, 2018

A GPAT9 como exposto anteriormente, tem um papel destacado na biossíntese de TAG, já que está envolvido na biossíntese de lipídios na membrana e no armazenamento (Gidda *et al*., 2009), permitindo a síntese de glicerolipídios não plastidiais indicando a existência de complexos biossintéticos lipídicos situados no ER. (Yang *et al*., 2012; Zhang *et al.*, 2015). Finalmente, a GPAT solúvel tem preferência pelo substrato acil em plastos podendo controlar parcialmente a tolerância ao frio, mediando a composição do ácido graxo, afetando assim a fluidez da membrana do tecido do ar da planta (Nishida *et al*., 1987, 1993; Waschburger *et al*., 2018).

Então, existem evidências crescentes do papel relevante que tem a biossíntese de lipídios nas plantas e que as reações subsequentes podem ser chave nos processos adaptativos das plantas que ocorrem em ambientes heterogêneos pela diversificação de produtos gênicos envolvidos em diferentes tecidos, estruturas e entre espécies. Essas evidências levam a hipótese de que os genes que codificam enzimas envolvidas com a síntese de lipídios em plantas são genes potencialmente envolvidos em processos adaptativos em plantas como a *E. uniflora*. Dessa forma, a identificação e caracterização de genes da biossíntese de lipídios em *E. uniflora* consiste em um primeiro e importante passo para testar essa hipótese. Nesta dissertação foram estudados os genes que codificam enzimas GPAT, a qual é a primeira enzima da biossíntese de lipídios na rota de Kennedy e é codificada por uma família gênica que apresenta diversificação em plantas com vários parálogos que estão envolvidas exclusivamente com a síntese de cutina e suberina.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo geral deste trabalho foi identificar e caracterizar genes envolvidos na rota de síntese de lipídios em *Eugenia uniflora* e verificar o seu potencial envolvimento em mecanismos adaptativos na espécie.

2.2 Objetivos específicos

1. Identificar no transcritoma de *Eugenia uniflora* sequências homólogas a genes que codificam enzimas envolvidas na rota de biossíntese de lipídios em plantas;
2. Caracterização *in silico* dos potenciais genes identificados no transcritoma e anotação dos mesmos;
3. Realizar uma reconstrução filogenética com os genes identificados e caracterizados em *E. uniflora* e espécies de plantas proximamente relacionadas;
4. Analisar o perfil de expressão dos genes em plantas provenientes de dois ambientes contrastantes e cultivadas em casa de vegetação sob condições controladas.

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3. ESTRUTURA E ORGANIZAÇÃO DA DISSERTAÇÃO

Esta dissertação foi elaborada contendo um capítulo, assim intitulado:

Capítulo 1:

Identification, characterization and expression patterns of glycerol-3-phosphate acyltransferase (GPAT) genes of *Eugenia uniflora* L. reveal its potential involvement with adaptive mechanisms in the species

Ossman Barrientos-Diaz, Nicole Moreira Veto, Franciele Kulcheski, Alexandra Antunes Mastroberti, Andreia Carina Turchetto-Zolet.

O Capítulo 1 está apresentado em forma de um artigo científico em preparação e que será posteriormente submetido a uma revista da área, contemplando os objetivos da dissertação. Por isso a formatação do capítulo ainda não segue o modelo de nenhuma revista, as figuras e tabelas principais estão distribuídas ao longo do texto para facilitar a leitura e interpretação. As figuras e tabelas suplementares encontram-se no final do capítulo após as referências bibliográficas.

4. CAPÍTULO 1: Identification, characterization and expression patterns of glycerol-3-phosphate acyltransferase (GPAT) genes of *Eugenia uniflora* L. reveal its potential involvement with adaptive mechanisms in the species

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Artigo em preparação

Identification, characterization and expression patterns of glycerol-3-phosphate acyltransferase (GPAT) genes of *Eugenia uniflora* L. reveal its potential involvement with adaptive mechanisms in the species

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ABSTRACT

Eugenia uniflora L. is a Myrtaceae species that occurs in Atlantic Forest domain (AFD). Popularly known as pitanga or Brazilian cherry, *E. uniflora* can habit in heterogeneous environments being a good model to study adaptive evolution in AFD. Thus, identify and characterize genes involved in response to environmental factors in *E. uniflora* could help us to understand adaptive evolution in natural populations. Enzymes involved in lipid biosynthesis, such as GPAT (*sn* glycerol-3-phosphate 1-O-acyltransferase) are already known to be involved in abiotic stress response in plants. GPAT enzyme can act in different metabolic pathways and physiological functions. In this study, we aimed to identify, characterize and examine the expression profile of genes encoding GPATs in the pathway of lipid synthesis of *E. uniflora*. To identify and characterize the *GPAT* homologs in *E. uniflora*, we used the transcriptome data of this species and Blast2GO to make annotations using the Blastx, ORFfinder and Phytozome database, as well as detection of conserved protein domains. For analysis of gene expression, plant seeds from two populations representing two different environments were germinated in a greenhouse under controlled conditions. The RNA was isolated for RT-qPCR analysis using primers designed for the GPATs identified by *in silico* analysis. Finally, histochemical and morphological analyzes of the leaves of the species for lipid identification were carried out. The analyzes of Genetic Ontology and BLAST revealed contigs similar with the *GPAT* genes of *A. thaliana* and *E. grandis*. After alignments and phylogenetic analysis, we identified seven putative GPATs (Euni_k21131380, Euni_k25280104, Euni_k21137491, Euni_k21135418, Euni_k21284220, Euni_k25121268, Euni_k25344309) in *E. uniflora*, orthologs of *GPAT1*, *GPAT2/3*, *GPAT6*, *GPAT4/8* and *GPAT9*. Phylogenetic analyzes of the *GPAT* genes in Rosidae species, demonstrated evolutionary relationships with orthologous genes among the species studied and histochemical and morphological analysis showed the presence of lipids in the cuticle of both populations. Our results on the identification of the *GPAT* genes in *E. uniflora* together with their characterization, phylogenetic relationships and expression, histochemical and morphological analysis, allowed us routes on adaptive evolution in natural populations.

Keywords: *E. uniflora*, phylogenetic, adaptive, GPAT, lipid biosynthesis**.**

INTRODUCTION

Lipids are a structurally diverse group of molecules derived from fatty acids, pigments and secondary compounds that are vital for the normal functioning of a cell (Buchanan *et al.*, 2015). In plants there are several types of fatty acids and one of the challenges nowadays is to identify the genes that participate in these biochemical pathways and the mechanisms they regulate (Maraschin *et al.*, 2019; Vance & Vance, 2008). Recent studies have demonstrated the important role of lipids for plants in response to different physiological and environmental conditions (eg. protection and stress responses) (Benadjaoud *et al.*, 2013; Martin *et al.*, 2014; Fan *et al.*, 2014; Beisson *et al.*, 2007; Xu *et al.*, 2003). To give an example, the extracellular lipids suberin and cutin are deposited by some types of plant cells acting as barriers of protection. In the case of cellular membranes, alterations in lipids may affect various properties of the membrane, including its fluidity, thickness, permeability, and packing, and thus affect the clustering of important membrane proteins, which are sensitive to environment conditions (Escribá *et al*., 2008). Considering that each plant species naturally exhibits its own optimal temperature range for growth and reproduction, and thus, extreme variations can rapidly exert a thermodynamic influence on the intracellular macromolecules (nucleic acids and proteins) and substructures of plant cells (Ruelland & Zachowski, 2010). So, this lipid barrier is suggested to be involved in the adaptation of plants to a terrestrial environment (Rensing *et al*., 2008), additionally evolutionary adaptation is the most likely way for natural populations to act on rapid climate change (Hoffmann & Sgro, 2011). In this way, the genes involved in lipid biosynthesis can be potential candidates in mechanisms of plant adaptation in nature.

The *GPAT* gene family encodes the GPAT enzyme, an important enzyme in glycerolipid biosynthesis that is involved in different metabolic pathways and physiological functions (Yang *et al*., 2012). GPAT is also a key enzyme in the pathway for the *de novo* synthesis of membrane lipids and storage because it is the first enzyme involved in this process (Chen *et al*., 2014). The GPAT activity in plants has been observed in three different subcellular compartments: endoplasmic reticulum (ER), mitochondria and plastid (Gidda *et al*., 2009). The *GPAT* genes have already been cloned and isolated from some plant species (Men *et al.*, 2017; Chen *et al*., 2014; Yang *et al.*, 2012; Gidda *et al.*, 2009). The *GPAT* gene family is a diversified family in plants. At least 10 paralogous genes can be found in plants genomes studied until now. The model plants Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) present 10 (Murata & Tasaka, 1997) and 16 members (Men *et al.*, 2017), respectively. It is known from a recent

publication on evolution and diversification of *GPAT* gene family in the plant kingdom that putative homologs of *GPAT1-8*, *GPAT9* and plastidial soluble *GPAT* were identified in the 33 species of plants studied (Washburger *et al.,* 2018). The evolutionary relationships of GPATs revealed that they are grouped in three main clades in the phylogeny. One including GPAT9 and the other two including the plastidial soluble *GPAT* and *GPATs1-8*, respectively. GPAT9 are involved in plant membrane and storage lipid biosynthesis (Gidda *et al*., 2009; Chen *et al*., 2011; Shockey *et al*., 2015). The plastidial form of GPATs are essential to the *de novo* biosynthesis of glycerolipids within chloroplasts (Ohlrogge & Browse, 1995). GPAT1-8 genes are exclusively found in plants and can encode enzymes that are associated with the biosynthesis of other lipids, such as cutin and suberin (Yang *et al.,* 2012). The diversification of GPAT1-8 in plants and its involvement in the composition and quantity of cutin or suberin make them potential candidates in mechanisms of plant adaptation. It could be speculating that the activation of *GPAT* gene expression induced by stress linked to the regulation of plant metabolism and development, may be one of the adaptation mechanisms generated by plants to overcome adverse conditions for favorable growth (Cui *et al*., 2019; Niu & Xiang, 2018).

Certainly, adaptation is the property of a biological system that, through changes in population frequency and homeostasis, is functional on a wide range of stimulus and neutral levels in system parameter variations. (Briat *et al.,* 2016). Thence, the investigations with several organisms elucidate the breadth of evolutionary processes that maintain the phenotypic and genetic variation in the natural populations (Mitchell-Olds *et al*., 2007). There are several factors that alter the potential of species to adapt to different environments. In the case of climate changes, which favor habitat expansion and establishment of regimes (Antonelli & SanMartin, 2011), it can triggering the diversification of plants adapted to certain conditions, such as happened in the southern continents in different periods of global cooling (Simon *et al*., 2009, Crisp *et al*., 2010, Bytebier *et al*., 2011).

Thus, a sum of factors will contribute to variability and these diverse characteristics evolve as a result of environmental factors imposing selective pressure on the individuals that inhabit a heterogeneous landscape (Schoville *et al*., 2012)

In that sense *Eugenia uniflora* L., is a versatile species that occurs in heterogeneous environments within the Atlantic Forest domain (Turchetto-Zolet *et al*., 2016; Salgueiro *et al*., 2004; Scarano, 2002), considered one of UNESCO's five priority biodiversity hotspots (Myers *et al*., 2000; Oliveira-Filho & Fontes, 2000). Popularly known as pitanga or Brazilian cherry, it

belongs to the Myrtaceae family (Sobral *et al*., 2015; Legrand & Klein, 1969), one of the most important families of plants of this biome, native of South America and common in regions with tropical and subtropical climate (Sobral *et al*., 2013; Amorim & Alves 2012; Govaerts *et al*., 2010; Ferreira-Ramos *et al*., 2007; Silva *et al*., 2005; Wilson *et al*., 2005; Margis *et al*., 2002). Recent genomic studies of the diversification, population structure and for identification of genes involved in the response to abiotic factors in *E. uniflora* have postulated the species as a good model to study adaptive evolution (Veto *et al*., unpublished; Vasconcelos *et al*., 2017; Turchetto-Zolet *et al*., 2016). This species presents variable morphologies ranging from shrub to 15-meter trees in Restinga and Riparian forest, respectively, in the South region (southern Brazil, Paraguay, Uruguay and Argentina) (Rattmann *et al*., 2012). Recent studies have revealed several biological properties of this species (Rodrigues *et al*., 2013; Jucoski *et al*., 2013; Oliveira *et al*., 2008).

In this study, the *GPAT* genes were identified and characterized in the native species *Eugenia uniflora*. We further analyzed their phylogenetic relationships with GPATS of other plants, and their expression profiles in individuals of populations of *E. uniflora* from contrasting environments. We also performed anatomical and histochemical analysis of leaves of *E. uniflora* in order to compare the anatomical structure of individuals from such contrasting environments. This evidence leads to the hypothesis that genes encoding enzymes involved in plant lipid synthesis are genes potentially involved in adaptive processes in plants such as *E. uniflora*.

METHODS

Selection of candidate genes of GPAT

The transcriptome of *Eugenia uniflora* (Guzmán *et al*., 2014) was used to identify the candidate genes involved in the adaptation of the species. The BLAST searches were performed against the unigenes of the transcriptome of *E. uniflora* using the already identified genes of *GPAT* in *Arabidopsis thaliana* (AT1G06520.1 (ATGPAT1, GPAT1), AT1G02390.1 (ATGPAT2, GPAT2), AT4G01950.1 (ATGPAT3, GPAT3), AT1G01610.1 (ATGPAT4, GPAT4), AT3G11430.1 (ATGPAT5, GPAT5), AT2G38110.1 (ATGPAT6, GPAT6), AT5G06090.1 (ATGPAT7, GPAT7), AT4G00400.1 (AtGPAT8, GPAT8), AT5G60620.1 (GPAT9) and *Eucalyptus grandis* W. Hill ex Maiden (Eucgr.A01977.1, Eucgr.G02949.1, Eucgr.F04388.1, Eucgr.I01507.1, Eucgr.B03397.1, Eucgr.G01836.1, Eucgr.K03558.1,

Eucgr.G01837.1, Eucgr.F04389.1, Eucgr.F01545.1, Eucgr.A00515.1, Eucgr.A00496.1, Eucgr.E00121.1, Eucgr.E01329.1, Eucgr.J01235.1, and Eucgr.C02495.1) recovered from the Phytozome database (https://phytozome.jgi.doe.gov/) as queries. In this way, the identification of the gene ontology of the candidate genes was processed through BLAST2go. The open reading frame (ORF) and protein prediction for each sequence recovered were further analyzed using ORFfinder tool and Blastx (NCBI). Based on these analyses we performed the initial annotation of the candidate *GPAT* genes of *E. uniflora*. The identification of orthologous for each *GPAT* gene was further performed based on phylogenetic analysis.

Identification of *GPAT* genes from species of Rosidae clade

To identify the *GPAT* genes homologs of the clade Rosidae, we first retrieved the sequences of *GPAT* genes characterized in *A. thaliana* in the Phytozome database. Then, these sequences (GPAT1 [AT1G06520], GPAT2 [AT1G02390], GPAT3 [AT4G01950], GPAT4 [AT1G01610], GPAT5 [AT3G11430], GPAT6 [AT2G38110], GPAT7 [AT5G06090], GPAT8 [AT4G00400] and GPAT9 [AT5G60620], except soluble GPAT) were used as a reference to search for available GPATs sequences for Rosidae clade species using TBLASTx in the same database.

We searched BLAST against 10 genomes of plant species and proteomes (*Aquilegia coerulea* E. James (outgroup), *Arabidopsis thaliana* (L.) Heynh, *Brassica rapa* L., *Eucalyptus grandis* W. Hill ex Maiden, *Glycine max* (L.) Merr., *Manihot esculenta* Crantz, *Medicago truncatula* Gaerth., *Populus trichocarpa* Torr. & A. Gray ex Hook., *Phaseolus vulgaris* L, *Ricinus communis* L.) available in Phytozome. The CDS, the genomic DNA and the amino acid sequences corresponding to each GPAT were downloaded from the Phytozome database (Table S1). The taxa were indicated by acronyms consisting of three letters (eg. *Eucalyptus grandis*: Egr, where the first letter is the initial letter of the genus and the next two letters are the first two letters of the specific name of the species). The sequences were identified in all analyzes using the acronym followed by the access number to the protein (for example, Egr_Eucgr_E00121 corresponds to *Eucalyptus grandis*). The gene names of the *A. thaliana* GPATs reported above were added before the acronym, and the access number (for example, GPAT1-Ath_AT1G06520 corresponds to *A. thaliana* GPAT1). Supplementary Table S1 provides a detailed description of the sequences used in this study, including their corresponding name species and access numbers.

Sequence alignment and phylogenetic analyses

All the nucleotide and protein sequences were aligned using MUSCLE (Edgar, 2004) implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 (Kumar *et al*., 2016). The multiple sequence alignments were inspected and edited in the Gblocks server version 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) and positions aligned and selected by the software in the final analysis were used for phylogenetic analysis. The phylogenetic relationships were reconstructed according to protein sequence alignments using a Bayesian method performed in BEAST1.8.4 (Drummond *et al*., 2012). To select the best protein evolution model, ProtTest 3.4.2 was used (Darriba *et al*., 2011), which indicated JTT + I + G as the best model for the protein sequence data set. Birth-death processes were selected as a tree prior before the Bayesian analysis and executed for 50,000,000 generations with Monte Carlo algorithms of the Markov chain (MCMC) for the amino acid sequences. Tracer 1.6 (Rambaut *et al*., 2014; http://beast.bio.ed.ac.uk/Tracer) was used to verify the convergence of Markov chains and the appropriate effective sample sizes (> 200). Finally, consensus trees were generated using TreeAnnotator 1.8.4 (Drummond *et al*., 2012) with the maximum credibility of the clade, average nodes heights and discarded burnins. The trees were visualized and edited in FigTree 1.4.3 (Rambaut & Drummond 2009).

Detection of protein conserved domains

The potential transmembrane domains (TMD) in GPAT protein sequences identified in *E. uniflora* were predicted using the PROTTER (Omasits *et al*., 2014) and TMHMM-2.0 software package (Krogh *et al*., 2001) developed by the CBS prediction servers (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and where compared with the well characterized GPAT sequences from *A. thaliana*.

Plant material and experimental conditions

The seeds of *Eugenia uniflora* were collected in two different edaphoclimatically environments in natural populations: riparian forest in the state of Rio Grande do Sul, Brazil (27 ° 27'13.99"S; 53° 28'9.01"W) and in Restinga in the State of Rio de Janeiro, Brazil (23º02'55.53"S; 43º31'27.84"W) (**Fig. 1**). The seeds were grown under controlled environmental conditions in the greenhouse of the Biotechnology Center and Genetics Department of the Biosciences Institute of the Federal University of Rio Grande do Sul

(UFRGS) in Porto Alegre, Brazil. Four months after germination, five plants from each of the environments were selected to perform RNA extraction.

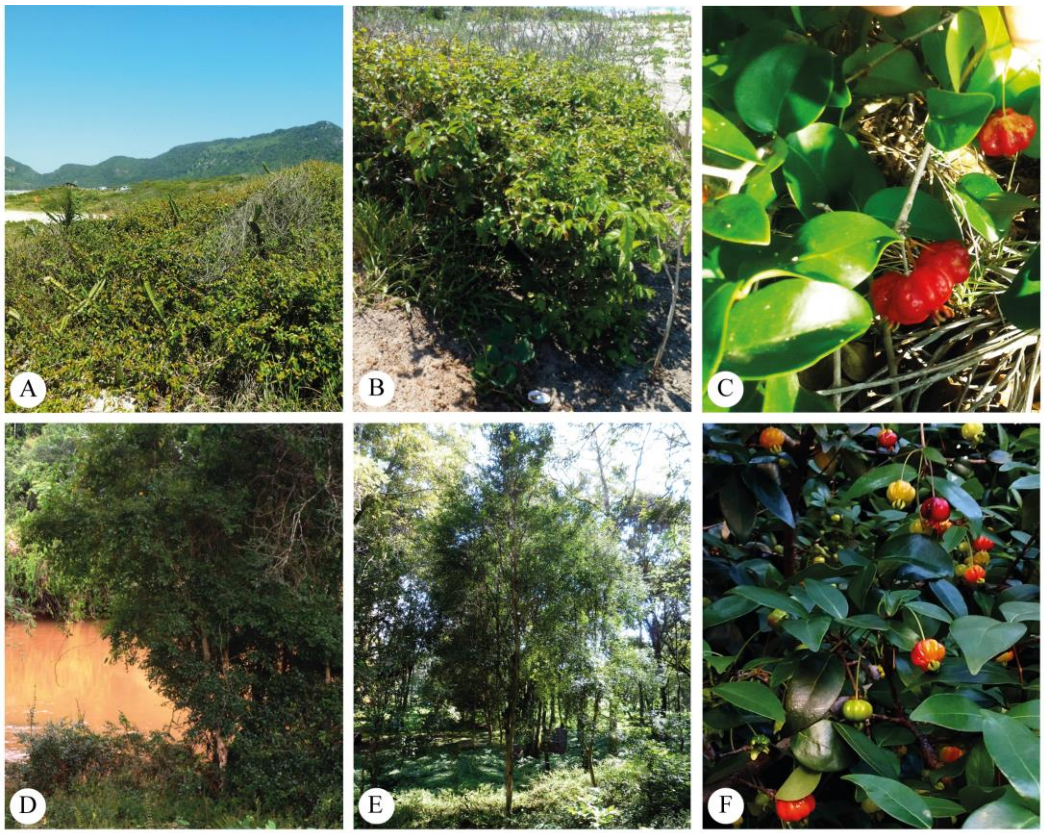

Figure 1. *Eugenia uniflora* L environment, architecture plant and fruit of two populations of Brazil. (**A-C**) Rio de Janeiro, (**D-F**) Rio Grande do Sul

Total RNA isolation

Total RNA was isolated from the mature leaves of the *E. uniflora* plants of each environment for RT-qPCR analysis. The leaves were collected in the greenhouse of the UFRGS manipulated with liquid nitrogen and stored at -80 °C until the extraction of RNA. For extraction process, was used a combination of CTAB method (Gambino, Perrone & Gribaudo, 2008) and Direct-zolTM commercial kit (Zimo Research). From each of the samples macerated in liquid nitrogen, approximately 350 mg were used for extraction. The extraction buffer was composed of 2% CTAB, 2.0% PVP-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 2% β-mercaptoethanol heated in a water bath at 65 ° C. Then, for each sample, 700µL of the extraction buffer was added and the tubes were incubated at 65 ° C for 10 minutes. In the next step, same volume of chloroform: isoamyl alcohol (24: 1) was added to each tube and centrifuged at 7000 xg for 20 minutes at 4 ° C. The supernatant was removed and transferred to a new tube, where a similar volume was obtained. Subsequently, 96% ethanol was added,

and the protocol steps of the extraction kit were carried out (following the manufacturer's instructions). Finally, to evaluate the integrity of the RNA, a 1% agarose gel electrophoresis was implemented, and the quantification was done in the Nanodrop TM 1000 spectrophotometer.

Primers design and PCR amplification

According to the literature reports and the result of the annotation, we selected those candidate genes with 80% similarity to the databases and then the design of primers was made (Table S2), these primers were designed using the Primer3 Input site (Koressaar & Remm, 2007). The regions of seven candidate genes were amplified using Real-time polymerase chain reaction (PCR) in the 10 cDNA samples (5 from each population) samples. The PCR reaction were conducted using 5 µL cDNA, 1 µL magnesium chloride ($MgCl_2$) 50 mM, 1 µL deoxyribonucleotide triphosphates (dNTP) mix 5 mM, 2 µL PCR buffer 10x, 0.08 of Platinum Taq DNA polymerase (Invitrogen) and 2 µL of each primer 5 µM, for a total of 20 µL. After initial tests the following PCR conditions were able to amplify all fragments: an initial hot-start step at 95 °C for 5 min, followed by 35 cycles with denaturation at 94 °C for 30 s, an annealing temperature of 60 °C for 30 s, and 3 m of elongation at 72 °C. To check the amplification of the products we used the 7500 fast v2.0.3 software.

GPATs expression analysis by RT-qPCR

The cDNA synthesis was carried out from approximately 1 µg of total RNA. Each reaction was primed with 1 µM oligonucleotide dT24V (Alpha DNA, Montreal, QC, Canada). Before transcription, RNA and primer oligo dT24V were mixed with RNase-free water to a total volume of 10 µL and incubated at 70°C for 5 minutes followed by ice-cooling. The reactions were reverse transcribed with 1X M-MLV RT buffer, 0.5 mM dNTP (Ludwig, Porto Alegre, RS, Brazil) and 200U of M-MLV RT Enzyme (Promega, Madison, WI, USA) in a final volume of 30 µL. The synthesis was performed at 40° C for 60 minutes on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). All cDNA samples were diluted 100-fold with RNase-free water before use as a template in RT-qPCR analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) amplification was performed to validate and investigate the expression of GPAT genes in leaves tissue. The RT-qPCR reactions were performed in a Bio-Rad CFX384 real-time PCR detection

system (Bio-Rad, Hercules, CA, USA) using SYBR Green I (Invitrogen, Carlsbad, CA, USA) to detect double-stranded cDNA synthesis. Reactions were conducted in a volume of 10 µL containing 5 µL of diluted cDNA (1:100), 0.2X SYBR Green I, 0.1 mM dNTP, 1X PCR buffer, 3 mM MgCl2, 0.25 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 200 nM of each forward and reverse primer. Samples were analyzed as biological quintuplicate and technical quadruplicates in a 384-well plate. A non-template control was also included. The PCR reactions were run as follows: an initial polymerase hot start step for 5 min at 94° C and 40 cycles of 15 seconds at 94° C, 15 seconds at 60° C and 10 seconds at 72° C. A melting curve analysis was programmed at the end of the PCR run over the range of 65 to 99° C, and the temperature increased stepwise by 0.5° C. The threshold and baselines were manually determined using the Bio-Rad CFX manager. We employed E3 ubiquitin ligase (E3) and histone H2A (H2A) as reference genes, which were observed to be optimal normalizers for different tissues in *E. uniflora* by geNorm analysis (Anton *et al*., unpublished; Guzmán *et al.*, 2014; Vandesompele *et al.*, 2002). To calculate the relative expression of the genes, we used the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Student's t-test was performed to compare pair-wise differences in expression. The parameters of two-tailed distribution and two samples assuming unequal variances were established. The means were considered significantly different when $p < 0.05$.

Anatomical and histochemical analysis of leaves of *E. uniflora*.

After one year, 12 different plants were selected (6 from each environment) to perform anatomical and histochemical analysis of their leaves. The cross sections of the leaf blades were obtained by hand, using steel blades under magnifying glass. Then the cuts were sent to a solution of sodium hypochlorite (50%) for discoloration (Kraus & Arduin, 1997). After washing in distilled water, the cross sections were stained according to a technique described by Bukatsch (1972), with safranin and Astra blue. Subsequently, all sections were mounted on semi-permanent slides, following the usual procedures in plant anatomy (Johansen, 1940, Sass, 1951).

The sections obtained manually were subjected to three histochemical tests to detect lipids, Nile Blue (Cain, 1947), Sudan III and Black Sudan IV (Pearse, 1980). The appropriate controls were run simultaneously, and the material analysis was performed by using Leica M165FC stereomicroscope and the images were taken by Leica DFC 500 digital camera.

RESULTS

Selection, annotation and identification of *GPAT* homologous sequences in *E. uniflora* and its closest homologous.

Initially, for the identification and characterization of the *GPAT* homologues in *E. uniflora*, we performed BLAST searches against the transcriptome data of this species using the GPATs sequences of *Arabidopsis thaliana* and *Eucalyptus grandis*, available in the Phytozome database, as queries.

A total of seven candidate genes were identified: Euni_k21131380, Euni_k25280104, Euni_k21137491, Euni_k21135418, Euni_k21284220, Euni_k25121268, Euni_k25344309, which presented an identity greater than 80% with their respective homologous of *A. thaliana*, and nucleotide sequence sizes ranging from 1092 to 1785 nucleotides (**Table 1**). A phylogenetic analysis was performed with GPAT sequences of *A. thaliana* and *E. grandis* and including these seven sequences obtained for *E. uniflora* (**Fig. 2**), in order to identify the homologous.

Table 1. Identity of candidate genes of *E. uniflora* homologs to GPAT.

ID-SEQ	SIZE TO CLC	LENGTH nt\aa	Identity with *A. thaliana*	GPAT associated
Eun_k21_137491	1132	1092 - 363	93%	GPAT1
Eun_k21_131380	2379	1584 - 527	84%	GPAT 2/3
Eun_k25_280104	2844	1785 - 594	83%	GPAT 2/3
Eun_k21_135418	1846	1506 - 501	88%	GPAT6
Eun_k21_284220	1837	1509 - 502	92%	GPAT 4/8
Eun_k25_344309	1644	1107 - 368	86%	GPAT9
Eun_k25_121268	1590	1128 - 375	94%	GPAT9

The seven *E. uniflora* sequences identified by BLAST grouped in clades I and III of the *GPAT* gene family, which include GPAT9 and GPAT1-8 genes, respectively (Washburger *et al.,* 2018). No sequences of *E. uniflora* grouped inside the clade II, which includes the plastidial soluble GPAT.

In clade I where AtGPAT9 is located, two genes of *E. uniflora* (Euni_k25121268, Euni_k25344309) and seven of *E. grandis* were added.

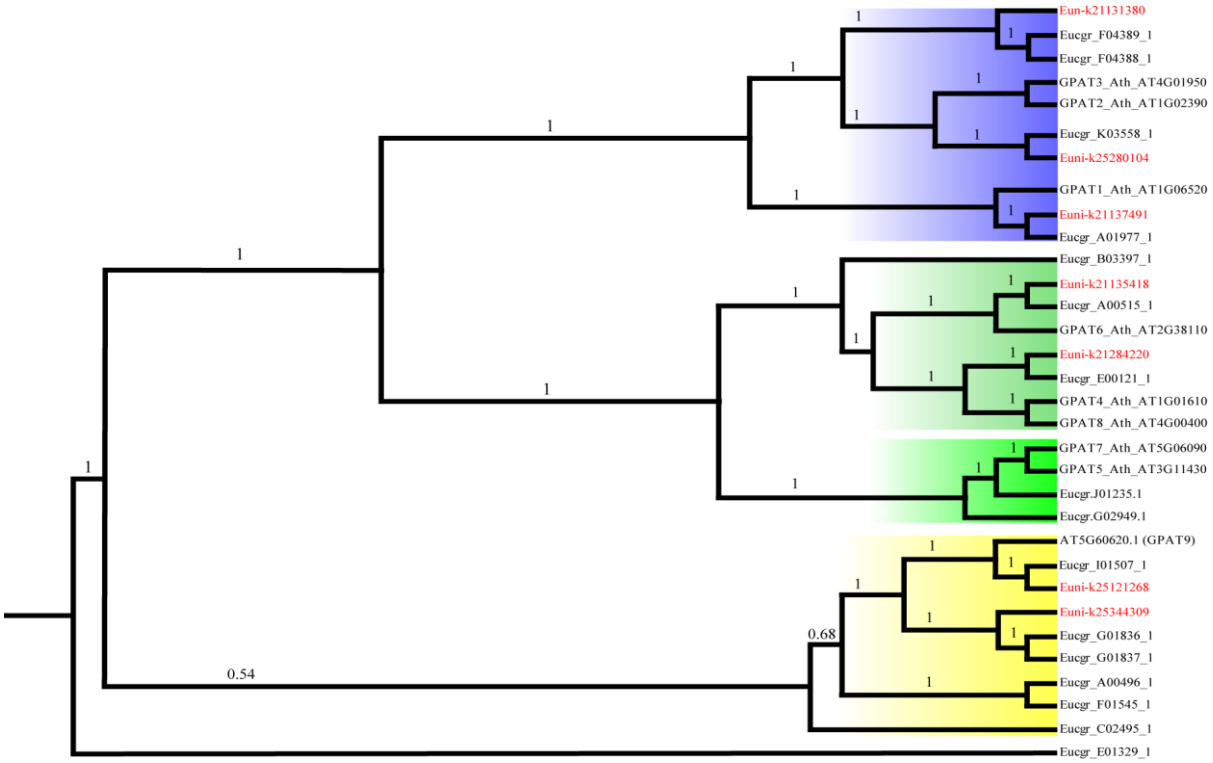

Figure 2. Evolutionary relationships among *GPAT* genes of *Arabidopsis thaliana* (Ath), *Eucalyptus grandis* (Eucgr) and *Eugenia uniflora* (Euni).

Inside the Clade III (GPAT1-8) we could identify homologous of GPAT1, GPAT2, GPAT3, GPAT6, GPAT4 and GPAT8 (**Figure 2**). Three *E. uniflora* sequences (Euni_k21137491, Euni_k21131380 and Euni_k25280104) grouped with GPAT1, GPAT2 and GPAT3 of *A. thaliana* and *E. grandis* (**Figure 2**). In the case of GPATs 2/3, little is known about its physiological function, however it is known that they present 2 intermembrane domains and interact with the extracellular medium respectively. The orthologous of GPAT2 and GPAT3 could not be distinguished because the duplication event that originated these two paralogous occurred after the divergence of Brassicaceae species (Washburger *et al.*, 2018). In the subclade including GPAT6, and GPAT4 and GPAT8 (Clado III) we identified two *E. uniflora* genes (Euni_k21135418 and Euni_k21284220). One of them (Euni_k21135418) was closely related to GPAT6 of *A. thaliana* and *E. grandis*, while the other one grouped with GPAT4 and GPAT8. Once the duplication event that originated GPAT4 and GPAT8 occurred after the Brassicaceae divergence, it was not possible to identify the orthologous in *E. uniflora*. In this analysis, using transcriptome data from leaf, we were note able to identify homologous of GPAT5 and GPAT7.

Evolutionary relationships between GPAT1-8 and GPAT9 genes of *E. uniflora* in the Rosidae clade.

To discern the evolutionary relationships of the GPATs genes of *E. uniflora*, we increased the number of species to be evaluated, attracting attention to the Rosidae clade species (APG IV, 2016). We conducted a search in the Phytozome database, using BLASTX with the total length of predicted amino acid sequences for the GPATs of *A. thaliana* as a query of the available species belonging to the Rosidae. A total of 115 sequences (including *A. thaliana*) and other 12 species of the following botanical families were recovered: Brassicaceae (3 spp), Fabaceae (3), Euphorbiaceae (2), Myrtaceae (2), Salicaceae (1) and Ranunculaceae (1) as an external group (Table S1). Then we carried out a phylogenetic analysis and the phylogenetic tree was reconstructed (**Fig. 3**) that represents the evolutionary relationships that exist between the different species of the clade. In that order, the groupings of the species in clades I and III were recovered as shown in the identification of *E. uniflora* homologs.

In this phylogenetic reconstruction, different events that contributed to the evolution and variability of terrestrial plants can be evidenced, especially in the eudicotyledonous group, where the duplication and diversification events of the different *GPAT* orthologs of *E. uniflora* are appreciated. In subclade 1 (AtGPAT1 and AtGPAT2/3) it is observed that the duplications that originated this group occurred at different times, for example, *GPAT1* presented duplication events before the origin of *GPAT* 2/3, the latter from a group where probably the species of the

Myrtaceae family lost one gene for each species; in **Figure 3** it is observed that the species of the other families have two genes for each of the species.

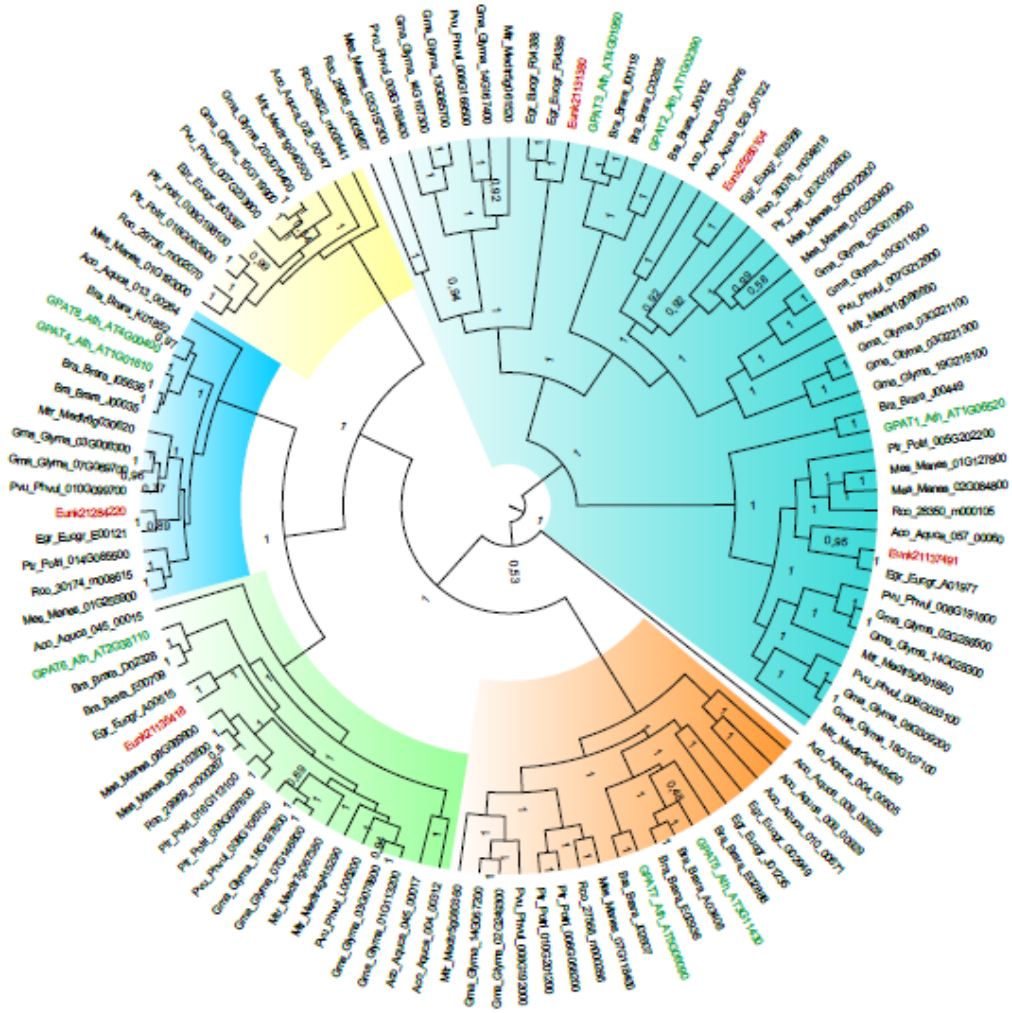

Figure 3. Evolutionary relationships among GPATs 1-8 in Rosidae clade. Green tips represent the well characterized GPATs from *Arabidopsis thaliana* and the red tips represent the GPATs from *E. uniflora* identified in this study. Posterior probability values are given below the branches.

In the second subclade including AtGPAT6 and AtGPAT4/8 (Clado III) Studies have revealed that it is the oldest subset within the group of GPATs of terrestrial plants. There were grouped two genes of *E. uniflora*, which is important, since this subclass is directly related to the production of cutin and suberin (Beisson *et al*., 2012). Finally, in relation to the clade I (GPAT9), it is observed that these genes have evolved in a divergent way (**Fig. 4**), which leads to diversified and independent functions, probably guaranteeing the adaptation of the species to the different regimes across the centuries of the earth, as proposed by Gidda *et al*., 2009 & Yang *et al*., 2012 where they link this clade with the production of different membrane lipids and plants protection.

Figure 4. Evolutionary relationships among GPAT9 in Rosidae clade. Green tips represent the well characterized GPAT9 from *Arabidopsis thaliana* and the red tips represent the GPAT from *E. uniflora* identified in this study. Posterior probability values are given below the branches.

Detection of protein conserved domains

According to the results shown in **Table 2**, we showed that the *GPAT* genes identified in *E. uniflora* are membrane associated proteins, as well as the well characterized GPAT proteins of *A. thaliana*. It is known that in *A. thaliana* the GPATs present different transmembrane domains and act according to their cellular localization, for example, for GPAT1/2/3 are present in mitochondria membrane. The prediction of transmembrane domains revealed that GPATs of *E. uniflora* have between two and four membrane domains (**Table 2**), thus probably the structure of the *E. uniflora* GPAT proteins also present characteristic domains similar to those previously characterized in other species.

Gene expression profile of GPATs of *Eugenia uniflora* by RT-qPCR

The expression of the genes Euni_k21131380, Euni_k25280104, Euni_k21137491 and Euni_k21135418, were validated and measured by RT-qPCR. The expression of these seven genes was analyzed in mature leaves of *E. uniflora* from two populations of Brazil (Restinga and Riparian Forest).

Table 2. Prediction of transmembrane domains of the GPATs of *E. uniflora*.

Eugenia uniflora			*Arabidopsis thaliana*		
Code – (GPAT)	Protter	TMHMN	Code – (GPAT)	Protter	TMHMN
Eun_k21_137491 (1)	3	2	Ath_AT1G06520 (1)	3	2
Eun_k21_131380 (2/3)	2	2	Ath_AT1G02390 (2)	4	1
Eun_k25_280104 (2/3)	4	3	Ath_AT4G01950 (3)	4	2
Eun_k21_284220 (4/8)	4	3	Ath_AT1G01610 (4)	3	3
			Ath_AT4G00400 (8)	3	2
-	-	-	Ath_AT3G11430 (5)	2	3
			Ath_AT5G06090 (7)	2	2
Eun_k21_135418 (6)	3	1	Ath_AT2G38110 (6)	3	2
Eun_k25_121268 (9) Eun_k25_344309 (9)	3	3	Ath_AT5G60620 (9)	3	3

For this, seeds of both localities of the species were planted in the greenhouse. Its germination began after day 20 of the sowing and reached a percentage of germination higher than 80%.

It is worth noting that the plants expressed the same phenotype of the ecosystems where the seeds came from, being more creeping or shrubby, with larger leaves in comparison with the plants of the other population and having a tree-like structure. In addition, measures of the first count were taken, the emergency rate, Germination percentage (GP) and the emergency rate index (EVI) and did not show significant differences between the populations (Table S3). On the other hand, Ubiquitin ligase E3 (E3) and histone H2A (H2A) were used as reference genes, which were suitable normalizers for different tissues in *E. uniflora* by geNorm analysis (Anton *et al*., unpublished; Guzmán *et al.,* 2014; Vandesompele *et al.,* 2002). They were designed at least one set of primers with their respective forward and reverse for each gene (Table S2) identified an expression pattern for the seven genes. The validation of these primers is still in execution, it is expected that when obtaining the results of the seven genes, their products will be implemented in an agarose gel. This step is carried out with the purpose of verifying the size of the amplicon predicted after the transcriptome assembly. The results will be visualized and confirmed by agarose gel analysis.

Morphological and histochemical analysis of leaves of *Eugenia uniflora*

Cross sections were made in mature leaves of *Eugenia uniflora* from both populations and it was observed that the central nerve presents a biconvex contour, uniseriate epidermis and is covered by an unadorned and thick cuticle. The collenchyma is formed by at least 3 strands of cells in the adaxial phase and the vascular axis is covered by sclerenchyma fibers in the different individuals analyzed, regardless of the population of origin (**Fig. 5**).

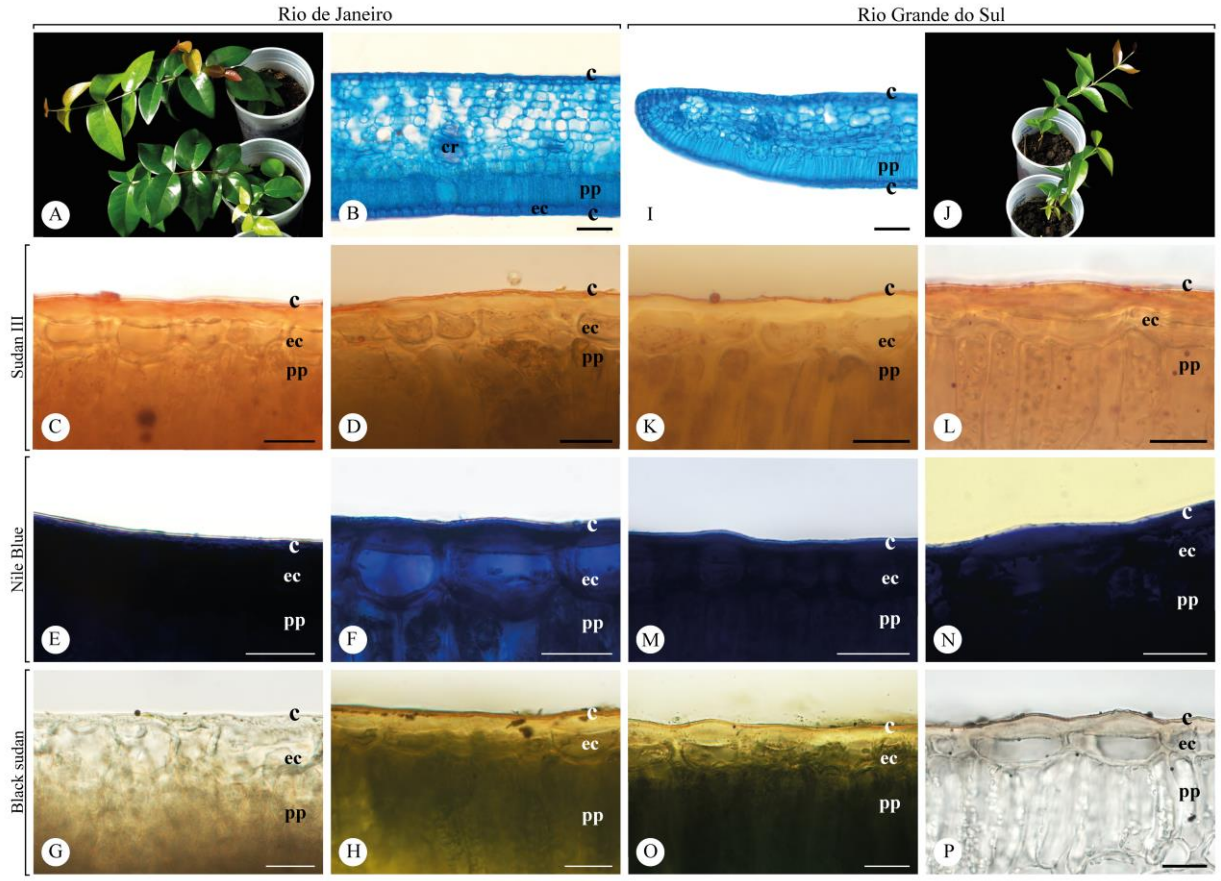

Figure 5. Structural and histochemical characteristics of two populations of *E. uniflora***.** (A-H) Rio de Janeiro and (I-P) Rio Grande do Sul. (B, I) **Astra Blue and Safranin** test, cross section. **Sudan III** (C, L) Control (D, K) Samples. **Nile Blue** (E, N) Control, (F, M) samples. **Black Sudan** (G, P) Control, (H, O) samples. C (cuticle), (cr) crystal, (ec) epidermis cell, (pp) palisade parenchyma. Scale of all the images correspond to 20µm.

The mesophyll contains palisade parenchyma in two series and a spongy parenchyma organized by layers of cells of different thickness, as well as in the analyzed samples, elements such as drusen and prismatic crystals (Fiuza *et al*., 2008) in different regions were visualized, as well as secretory cavities in the two sheets of the leaf (**Figure 5 B, I**). The results of the Safranin and Astra Blue tests showed that there are no morphological differences between the populations, except for the size of the epidermal cells or the presence and absence of drusen.

In relation to the histochemical tests performed (Sudan III, Sudan Black and Nile Blue) the presence of lipid compounds (probably essential oils, characteristic of the Myrtaceae family) was evidenced in secretory cavities, parenchyma cells and especially in the cuticle of the individuals of *E. uniflora* (**Figure 5 D, F, H, K, M, O**). According to the observations made, differences were found in the thickness of the cuticle, with the population of Rio Grande do Sul having a highest volume. This structure, constitutes an essential structural element of importance functional and ecological, because it is the outermost layer of plant cells that interacts with the environment (Kunst & Samuels, 2003; Jeffree, 2006). These structural differences are certainly involved with the adaptation of the species to different environments and it has managed to partially penetrate its phenotype. Each of the tests was carried out with the development of controls of the samples for each of the populations.

DISCUSSION

Knowledge about the evolution of plants on Earth has allowed us to generate various hypotheses about the establishment of organisms in certain areas and what are the possible mechanisms that have allowed them to adapt and survive in a given environment. This is how studies on native species have taken the lead in the advances of science around evolutionary patterns and mechanisms of adaptation, providing the option for reconstructing a clear history and with solid arguments of how, when, why, where and from where they have built their way. In this sense, we highlight the role of *Eugenia uniflora* and we suggest, according previous studies (Turchetto-Zolet *et al.*, 2016, Veto *et al.*, unpublished) that this species is a good model to study evolutionary adaptations in the Atlantic Forest. *E. uniflora* stands out for being a species with a diversity of uses, both natural and industrial, in addition to having its distribution in distinct ecosystems within Atlantic Forest (Santos *et al*., 2012; Costa *et al.*, 2009; Sobral *et al.*, 2015; Turchetto *et al.*, 2016). Along its distribution, this species can occupy heterogeneous environments, like Restinga and Riparian Forests where it manages to present different phenotypes. This capacity to growth and stablishes in such distinct environment may be related to its adaptability. Thus, to know the molecular and evolutionary mechanism involved in *E. uniflora* adaptation to distinct environments along with its distribution is very relevant.

The adaptation of an organism in a certain environmental condition may be a result of selection acting on a portion of genome responsible for a specific phenotype. There are many molecular aspects that could be involved in response to abiotic factors. The internal mechanisms

such as lipid synthesis, which, by participating in various metabolic pathways, could promote resistance to stress caused by biotic and abiotic factors, among others (Buchanan *et al.,* 2015; Chen *et al.,* 2011). These mechanisms could facilitate the development of different responses to the conditions offered by the environment. From this perspective, lipid synthesis involves a series of enzymes encoding by genes or gene families such as GPAT, LPAAT, PAP, and DGAT, guaranteeing the optimal development of this pathway. The first gene family to participate in this pathway is GPATs, which interact in different physiological processes and are relevant to recognize the history and diversity of organisms (Maraschin *et al.,* 2019). In the plant kingdom, GPATs are present in all divisions, highlighting that, in each of these divisions, even among species, the number of members that are part of the gene family may vary. In the case of *Eugenia uniflora*, the genes were recovered in two of the three clades (I and III respectively) proposed by Washburger *et al*., 2018, which was expected since it responds to the evolutionary line of this family within the plants, since there are exclusive GPATs of terrestrial plants, as in the case of the group conformed by GPATs 1-8 (Yang *et al.,* 2012). Most of the genes identified in *E. uniflora* belongs to GPAT1-8 clade.

Studies based on the phylogenetic relationships of the *GPAT* gene family allow us to know that clade I (GPAT9) participated in the biosynthesis and storage of membrane lipids, in addition to allowing the synthesis of nonplastid glycerolipid glycerolipids (Gidda *et al*., 2009, Yang *et al*., 2012). The evolutionary history of GPAT9 suggests that in terrestrial plants it was established and maintained after the divergence of terrestrial plants and algae, favoring the role in the TAG biosynthesis of the seed, and the production of lipids in the leaves (Iskandarov *et al*., 2016; Shockey *et al*., 2015; Singer *et al*., 2016). The protein sequences grouped with GPAT9 present at least one putative TMD, what shows that they are membrane proteins.

For the clade III, GPAT1 has a remarkable activity in the differentiation of the mat and male fertility (Zheng *et al*., 2003). In GPATs 2/3, little is known about its physiological function, however it is known that they present two intermembrane domains and interact with the extracellular medium (Takeuchi & Reue, 2009). For *GPAT* 4/8 and 6 it is known that these genes have a prominent role in the synthesis of extracellular lipids such as cutin and suberin (Li *et al*., 2007; Li-Beisson *et al*., 2009 Beisson *et al*., 2012). The GPAT5/7 genes have not been recovered in *E. uniflora* transcriptome, possible because we used transcriptome of leaves and studies have demonstrated that this subclass is characterized by having greater activity in the roots and seeds, and the induction of its activity is due to the generation of wounds (Yang, *et*

al., 2012, Beisson *et al*., 2007). Finally, in relation to TMD, two and four were identified, which were also identified as membrane proteins according to their location, even being mitochondria. All these elements indicate that the diversity of this group presents a versatile pattern, which is probably due to the fact that the genes belonging to this clade are regulated differently in relation to tissues of the plant.

The phylogenetic analysis showed the clear relationship between the *GPAT* genes of *E. uniflora* and their respective homologous genes with other plant species (Table S2), which allows us to identify orthologous genes that participate in several metabolic, physiological and interacting routes in favor of the organism guaranteeing its adaptation to the environment, providing protection, and developing different mechanisms to survive the conditions imposed by man and the environment.

We highlight that the plants that growth in the greenhouse recovered and expressed the same phenotype of the localities where they were collected, regardless of the controlled edaphoclimatic conditions.

Anatomically the populations are similar, the size of some structures varies (for example: in mesophilic, the layers of palisade parenchyma) or the amount of drusen. There is no difference in their lipids composition, in leaves lipids are accumulated in regions such as the cuticle, where it forms and accumulates cutin and suberin (Srivastava, 2002). These act as extracellular lipid barriers, which are important for controlling the flow of elements. Several authors agree that this lipid barrier is involved in the adaptation of plants to a terrestrial environment (Rensing *et al*., 2008). At one, with that genes encoding lipid in plants are positively selected and are certainly operating in genetic diversification processes to aid in the adaptation of plants (Maraschin *et al.*, 2019).

In the case of *E. uniflora*, these genes are probably being selected as a response to the environment and allowing express morphological differences between populations according to the environment where they develop and guarantee the production and accumulation of substances such as lipids in various tissues throughout the life cycle of plants. From this perspective, the identification of *GPAT* genes in *Eugenia uniflora* opens a door to continue exploring and identifying which are the mechanisms that favor the species establishment in a given ecosystem and which structural, functional, epigenetic elements participate in the processes of adaptation of native species, in order to understand how they (the genes) have behaved as throughout history and among populations.

CONCLUSION

In this study we provide insights on GPATs in native plant, including their gene family members, evolutionary history and gene expression profiles. These results reinforce and offer a further path that allows us to explore more deeply the importance of the lipids in the adaptation processes of native species. Phylogenetic analysis indicates that the seven *GPAT* genes recovered from *E. uniflora* were grouped into several subclades of the gene family. Thus, our study was supported by morphological and histochemical analyzes suggesting that the anatomy of the populations is similar but the accumulation of lipids varies when compared to the dimensions of the leaves. The presence of cutin in leaves in different volumes is a possible mechanism of phenotypic plasticity of the species, as an adaptation response. In this sense the presence of several genes encoding GPATs in terrestrial plants may be related to their adaptation to a terrestrial environment. Despite the knowledge that there is still little about the functions of GPATs in plants and more specifically in native plants, the study allows to obtain a light on those mechanisms that participate in the adaptive evolution.

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FIGURES LEGENDS

TABLES LEGENDS

Supplementary Table 1. Access numbers of Rosidae clade sequences for GPATs, available in Phytozome.

Botany family	Species	Accesion code	
		GPAT 1-8	GPAT9
Brassicaceae	*Arabidopsis thaliana*	Ath_AT1G06520 Ath_AT1G02390 Ath_AT4G01950 Ath_AT1G01610 Ath_AT3G11430 Ath_AT2G38110 Ath_AT5G06090 Ath_AT4G00400	AT3G11325
	Brassica oleracea	-	Bol013729
	Brassica rapa	Brara_J00449 Brara_J00102 Brara_K01852 Brara_A03408 Brara_I00118 Brara_C02835 Brara_I05638 Brara_E02936 Brara_D02328 Brara_J00035 Brara_E00709 Brara_J02607 Brara_E02888	Brara_A00307 Brara_J01416
Fabaceae	*Glycine max*	Glyma_14G028300 Glyma_02G286500 Glyma_18G107100 Glyma_08G309200 Glyma_14G167300 Glyma_13G085700 Glyma_03G221100 Glyma_19G218100 Glyma_02G010600 Glyma_07G146800 Glyma_14G167400 Glyma_03G221300 Glyma_10G011000 Glyma_18G197800 Glyma_01G113200 Glyma_03G078600 Glyma_20G070400 Glyma_10G119900 Glyma_02G249300 Glyma_03G008300 Glyma_07G069700 Glyma_14G067200	Glyma_09G119200 Glyma_08G085800 Glyma_05G131100
	Medicago truncatula	Medtr5g091660 Medtr3g448430 Medtr5g061520 Medtr4g415290 Medtr7g067380 Medtr1g040500 Medtr8g030620 Medtr1g086650	Medtr4g127910 Medtr2g438210

	Phaseolus vulgaris	Medtr5g080360 Phvul_006G033100 Phvul_008G191600 Phvul_008G169500 Phvul_008G106700 Phvul_008G169400 Phvul_007G233600 Phvul_007G212600 Phvul_010G099700 Phvul_L005200 Phvul_008G192000	-
Euphorbiaceae	*Manihot esculenta*	Manes_02G084800 Manes_01G127800 Manes_02G157300 Manes_01G230400 Manes_05G012900 Manes_01G193000 Manes_09G103500 Manes_08G089900 Manes_07G118400 Manes_01G255900	Manes_11G106900 Manes_04G065100
	Ricinus comunis	Rco_28350_m000105 Rco_29736_m002070 Rco_29822_m003441 Rco_29908_m005967 Rco_30076_m004618 Rco_30174_m008615 Rco_27568_m000266 Rco_29969_m000267	Rco_30122_m000357
Myrtaceae	*Eucalyptus grandis*	Eucgr_A01977 Eucgr_F04388 Eucgr_B03397 Eucgr_K03558 Eucgr_F04389 Eucgr_A00515 Eucgr_E00121 Eucgr_J01235 Eucgr_G02949 Eucgr_G01836	Eucgr_I01507 Eucgr_G01836 Eucgr_G01837
	Eugenia uniflora	Euni_k21131380 Euni_k25280104 Euni_k21137491 Euni_k21135418 Euni_k21284220	Euni_k25121268 Euni_k25344309
Salicaceae	*Populus trichocarpa*	Potri_005G202200 Potri_002G192600 Potri_016G063900 Ptr_Potri_006G198100 Ptr_Potri_016G113100 Ptr_Potri_006G097800 Ptr_Potri_010G201200 Ptr_Potri_008G058200 Ptr_Potri_014G085500	Potri_004G183300 Potri_009G143200
Ranunculaceae	*Aquilegia coerulea*	Aquca_057_00060 Aquca_003_00476 Aquca_045_00017 Aquca_029_00122 Aquca_004_00312	Aquca_001_00233 Aquca_047_00038

	Aquca_025_00147
	Aquca_045_00015
	Aquca_004_00505
	Aquca_010_00671
	Aquca_013_00284
	Aquca_009_00928
	Aquca_009_00929

Supplementary Table 2. Primers referring to 7 sequenced genes

GPATs	Primers code	Sense	Sequence	Size fragment	Test result
GPAT 2-3	Eun_k21_131380	Forward	CCTCGTCCCTCTTCCCTTAC	214	Ok
		Reverse	CCCATGTCCTCCAGAAAGAA		
GPAT1	Eun_k21_137491	Forward	TCTTGGTGGGCATCTACCTC	237	Ok
		Reverse	TTATCGGGGCTATGATCTCG		
GPAT 2-3	Eun_k25_280104	Forward	TTGGGAAGGAGACATTTTCG	151	Ok
		Reverse	TTTTCTCTGGGGAGGTGATG		
GPAT6	Eun_k21_135418	Forward	AGCGAGAGTGACCACGAGTT	173	Ok
		Reverse	GGAAGCCACAAGAAGGTGAG		
GPAT4	Eun_k21_284220	Forward	CTTGGGATTCATCCTCTCCA	243	Ok
		Reverse	TTGCTGACGCTGTAGGTGAC		
GPAT9	Eun _k25_121268	Forward	TGGGCTGTTGTTTGTGATGT	156	Ok
		Reverse	AGGGCGGGAGTATTTCAGAT		
	Eun _k25_121268_2	Forward	TCCGAACTGGACCTGGATAG	249	Ok
		Reverse	GATCAAGTACCGAACCACCA		
	Eun _k25_344309	Forward	AACTCGGAGACCAGACGAGA	165	Ok
		Reverse	ATGGACTCGGCATAACTTCG		
	Eun _k2 _344309_2	Forward	TAGGAGAGCTGGAGCTGAGG	282	x
		Reverse	TGGCAAGTATTACGACCCTTG		

Supplementary Table 3. Data on seed germination

Population	**Number seed**	**GP**	**CV**	**EVI**
RJ	274	81,75	3,06	125,75
RS	225	80	3,73	108,05

Germination percentage (GP), Coefficient of variation (CV), Index emergency speed (EVI)

5. CONSIDERAÇÕES FINAIS

Os lipídios interagem em uma ampla gama de reações metabólicas, com papéis fisiológicos relevantes no desenvolvimento das plantas, como componentes das membranas celulares, camadas protetoras extracelulares, moléculas sinalizadoras e de armazenamento. Nesse contexto, genes que codificam enzimas que atuam na rota da biossíntese de lipídios como as da família gênica das GPATs, associadas ao crescimento, desenvolvimento e resistência das plantas a estresses bióticos e abióticos, tem ganhado espaço como genes candidatos em resposta aos mecanismos de adaptação nas plantas. Alguns estudos reportam que genes GPAT estão envolvidos em respostas a diferentes tipos estresse, por serem parte da constituição de membranas. Para complementar este panorama o interesse pelo conhecimento sobre os mecanismos envolvidos na evolução e adaptação de plantas nativas tem crescido por diferentes motivos, como a geração de estratégias que visam o manejo e a conservação da biodiversidade, promovidos principalmente pelas aceleradas mudanças climáticas de nosso planeta e como esses eventos podem refletir nas populações naturais. Assim a *Eugenia uniflora* candidata-se como um modelo no estudo já que possui elementos chaves que podem ajudar a reconstruir os eventos de sua adaptação, devido à sua distribuição em locais de características ambientais e climáticas distintas, além de sua plasticidade fenotípica.

O capítulo que compõe esta dissertação identificou que os genes GPATs da espécie alvo neste estudo estão agrupados em diferentes subclados da família gênica e resultaram de diferentes eventos de duplicação e diversificação permitindo sua participação em diversos processos do desenvolvimento da planta. Além disso, análises morfológicas e histoquímicas evidenciaram a presença de lipídios na cutícula em folhas das duas populações estudadas e que recuperaram a mesma arquitetura morfológica dos indivíduos das populações de origem.

Assim, novas perguntas e perspectivas são formuladas ao redor da exploração mais a fundo dos nossos resultados, como a elaboração de análise de seleção incluindo um maior número de espécies, que nos permite expandir o plano evolutivo da família gênica por exemplo no clado das eudicotiledôneas. Também é necessário obter os dados de seleção positiva, pelo fato de que se espera uma seleção positiva depois da duplicação em genes GPATs, já que estão sendo parte das biossínteses de diferentes tipos de lipídios (por e.g. cutina e suberina) e respostas ao estresse, como reportam vários estudos. Por outro lado, a realização de experimentos de estresse para avaliar o comportamento dos genes, supondo que tenham diferentes níveis de expressão e funcionalidade podem oferecer perspectivas mais claras sobre

associações entre fenótipo e ambiente. Finalmente os resultados dos analises de perfil de expressão em RT-qPCR nas populações naturais de espécies nativas, nos apresentaram dados sobre os quais, provavelmente poderemos fazer inferências sobre que eventos e/ou mecanismos estão agindo na adaptação e evolução da família Myrtaceae e mais especificamente da *Eugenia uniflora* que vem mostrando-se ser interessante modelo para estudos com esta abordagem.

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