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Biogeografia e diversificação do gênero
Stizophyllum (Bignoniaceae)

Biogeography and diversification of the genus
Stizophyllum (Bignoniaceae)

São Paulo

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*Aos meus pais, Karl e Regina, por me incentivarem a ir atrás da felicidade
Ao Robson, por sempre cuidar de mim*

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*"The man who is blind to the beauties of nature has missed
half the pleasure of life."*

Baden Powell

RESUMO

A região Neotropical é uma das regiões com maior biodiversidade no planeta. Essa região apresenta uma complexa história geológica que iniciou-se com a quebra da Gondwana, separando os continentes sul-americano e africano, durante o Mesozóico, há ca. de 150 milhões. Todas as mudanças geológicas que seguiram influenciaram a diversificação da fauna e flora dessa região. No entanto, ainda não sabemos quais processos levaram à alta diversidade encontrada nesta região. Esse estudo, foca em *Stizophyllum* (Bignoniaceae), um pequeno gênero de lianas Neotropicais, que ocorre desde o México até o sul do Brasil. Apesar da circunscrição de *Stizophyllum* ser clara, limites específicos permanecem complicados neste grupo e pouco se sabe sobre sua história evolutiva. Este estudo visa: (i) reconstruir a filogenia do gênero e utilizá-la como base para inferir a história biogeográfica do grupo, e (ii) desenvolver marcadores de microssatélites (SSRs) nucleares e plastidiais, para futuros estudos filogeográficos e de genética de populações. Para tal, reconstruímos a filogenia do gênero com base em três marcadores moleculares (*ndhF*, *rpl32-trnL* e *pepC*) e uma ampla amostragem de indivíduos, utilizando inferências bayesiana e de máxima verossimilhança. Em seguida estimamos as idades de divergência das diversas linhagens e reconstruímos a história biogeográfica do grupo. Por fim, desenvolvemos marcadores de microssatélite nucleares (nSSRs) e de cloroplasto (cpSSRs) para o grupo. Ao todo, desenvolvemos trinta e sete SSRs, nove nucleares e vinte oito de cloroplasto. Todos marcadores foram polimórficos em *S. riparium* e apresentaram sucesso de transferabilidade para *S. inaequilaterum* e *S. perforatum*. Cinco clados principais foram reconstruídos na filogenia molecular do gênero, os quais são caracterizados por bons caracteres morfológicos e aqui

reconhecidos como espécies em uma nova sinopse apresentada para o grupo: (i) *S. inaequilaterum* Bureau & K. Schum., distribuído pela Amazônia e América Central; (ii) *S. perforatum* (Cham.) Miers, distribuído pela Mata Atlântica e Áreas Secas do Brasil Central, (iii) *S. riparium* (Kunth) Sandwith, distribuído por toda Bacia Amazônia; (iv) *S. flos-ardeae* (Pitter) Beyer & L.G. Lohmann, distribuído pela América Central, e (v) *S. coriaceum* Beyer & L.G. Lohmann, restrito ao Estado do Pará, na Amazônia Oriental. O estudo biogeográfico indicou que o ancestral de *Stizophyllum* e seu grupo-irmão *Martinella*, estava distribuído pela Amazônia. A divergência destas linhagens ocorreu durante o Eoceno, enquanto a diversificação das espécies de *Stizophyllum* ocorreu durante o Mioceno, a partir de um ancestral amplamente distribuído pela região Neotropical. Essa dissertação traz novos dados para um melhor entendimento dos processos que levaram à estruturação da biota Neotropical e contribui dados importantes para um projeto multidisciplinar amplo neste tópico (FAPESP 2012/50260-6).

ABSTRACT

The Neotropics is one of the most biodiverse regions in the planet. This region has a complex geological history that began during the break of Gondwana, which separated the South American and African continents in the Mesozoic, at ca. 150 million years ago. All the geological changes that followed greatly impacted the diversification of the fauna and flora of this region. However, it is still not clear what processes led to the high diversity found in this region. This study focuses on *Stizophyllum* (Bignoniaceae, Bignoniaceae), a small genus of Neotropical lianas, distributed from Mexico to southern Brazil. Although *Stizophyllum* is well circumscribed, species limits remain complicated in this group and little is known about its evolutionary history. This study aims to: (i) reconstruct the phylogeny of the genus and use it as a basis to infer the biogeographical history of this group, and (ii) develop nuclear and plastid microsatellite markers (SSRs) for future studies on the phylogeography and population genetics of this group. To this end, we reconstructed the phylogeny of the genus based on three molecular markers (*ndhF*, *rpl32-trnL* and *pepC*) and a broad sample of individuals, using Bayesian and Maximum Likelihood approaches. We then estimated divergence times of the various lineages and reconstructed the biogeographical history of the group. Lastly, we developed nuclear microsatellite markers (nSSRs) and chloroplast microsatellite markers (cpSSRs) for the group. In total, we developed thirty-seven SSRs, nine from the nucleus and twenty-eight from the chloroplast. All markers amplified successfully in *S. riparium* and transferability was successful to *S. inaequilaterum* and *S. perforatum*. Five main clades were reconstructed in the molecular phylogeny of the group, all of which are characterized by good

morphological markers and here recognized as species in an updated synopsis of the group: (i) *S. inaequilaterum* Bureau & K. Schum., distributed through the Amazon and Central America; (ii) *S. perforatum* (Cham.) Miers, distributed through the Atlantic Forest and the Dry Areas from Central Brasil; (iii) *S. riparium* (Kunth) Sandwith, distributed throughout the Amazon Basin; (iv) *S. flos-ardeae* (Pitter) Beyer & L.G. Lohmann, distributed through Central America; and, (v) *S. coriaceum* Beyer & L.G. Lohmann, restricted to the state of Pará, in Eastern Amazonia. The biogeographic study indicated that the ancestor of *Stizophyllum* and its sister-group *Martinella* was broadly distributed through Amazônia. These lineages diversified during the Eocene, while the diversification of *Stizophyllum* species occurred during the Miocene, from an ancestor that was broadly distributed through the Neotropics. This dissertation brings new information for the assembly of the Neotropical biota and contributes important data for a broader multidisciplinary project on this topic (FAPESP 2012 / 50260-6).

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Introdução Geral

Diversificação na região Neotropical

A região Neotropical concentra a maior diversidade de espécies de plantas do globo (Thomas, 1999; Kreft e Jetz, 2007; Brown, 2014). De fato, cerca de 37% da diversidade vegetal está na região Neotropical em contraste com outras áreas tropicais, como África ou Ásia (Antonelli e Sanmartín, 2011). Apesar do avanço no conhecimento taxonômico, filogenético, biogeográfico e paleoecológico, ainda não está claro o que levou a alta diversidade nesta região (Rull, 2011a). Esse fato tem intrigado vários pesquisadores há muitos anos, desde o século XIX, o que levou à formulação de muitas hipóteses que buscam compreender os processos envolvidos na alta diversidade encontrada nesta região.

Podemos separar essas hipóteses em duas categorias, a primeira voltada para explicações históricas e geográficas, e outra incluindo explicações ecológicas. Dentro da primeira categoria, temos as hipóteses do refúgio e especiação por isolamento devido à fragmentação da floresta (Haffer, 1969; Hooghiemstra e van der Hammen, 1998; Van Der Hammen e Hooghiemstra, 2000). Apesar desta hipótese ter ganhado popularidade, a mesma foi bastante criticada pois as idades de linhagens bióticas estimadas a partir de dados genéticos diferem significativamente das idades propostas para os refúgios (Moritz et al., 2000). Além disso, dados paleontológicos não corroboram a ideia de que as formações florestais tenham se fragmentado durante o período proposto para os refúgios (Colinvaux et al. 2000; Colinvaux e De Oliveira, 2001).

Outra hipótese incluída na primeira categoria (i.e., explicações históricas e geográficas) é a ideia de que eventos geológicos que ocorreram durante o Neógeno tenham modulado a biota local. Entre os eventos que trouxeram as maiores

alterações na região da Amazônica estão a elevação dos Andes (Hoorn e Wesselingh, 2010), formação hidrológica na bacia do Amazonas (Hoorn et al. 2010), e fechamento do Istmo de Panamá (Hoorn e Flantua, 2015). Estes eventos coincidem com a diversificação de plantas durante o Mioceno médio–tardio (Arakaki et al. 2011; Hughes et al. 2013; Perret et al. 2013).

Por outro lado, as hipóteses incluídas na segunda categoria (i.e., explicações ecológicas) enfatizam a importância do conservantismo e evolução do nicho ecológico, com altas taxas de diversificação associadas a alta capacidade adaptativa (Wiens e Donoghue, 2004; Pennington et al. 2009; Perret et al. 2013). Nesta categoria, temos também as interações ecológicas, como a relação planta polinizador (Kay et al. 2005), as quais também apresentam um papel fundamental na diversificação de taxa.

Esta claro que a alta biodiversidade encontrada na região Neotropical não resulta de um único fator mas sim uma complexa rede multifatorial (Gentry, 1982; Rull, 2011b; Hughes et al. 2013). Apesar dos esforços para um melhor entendimento da alta biodiversidade encontrada nesta região, ainda precisamos de mais estudos, incluindo diversos grupos taxonômicos e uma perspectiva ecológico-evolutiva para que possamos obter um panorama completo dos processos responsáveis pela estruturação da biota Neotropical. Estudos filogenéticos têm contribuído muito, uma vez que trazem informações sobre as relações entre espécies (Moritz et al. 2000) e idade de divergência entre taxa (e.g., Perret et al. 2013).

Grupo de Estudo

A família Bignoniaceae (Lamiales) apresenta distribuição Pantropical (Gentry, 1980), com aproximadamente 80 gêneros e 840 espécies (Lohmann e Ulloa, 2006 em diante). A família é composta por espécies arbóreas, arbustivas, lianescentes e até mesmo herbáceas. Representantes da família Bignoniaceae são caracterizados pela presença de folhas compostas e opostas. As flores são gamossépalas e gamopétalas, geralmente vistosas, com corola tubular, com epipetalia, formada por quatro estames didínamos e um estaminódio. Apresentam um fruto do tipo cápsula, com sementes geralmente aladas. As Bignoniaceae constituem um grupo monofilético, sustentado por caracteres morfológicos e dados moleculares (Olmstead et al., 2009). São reconhecidos oito grandes clados no grupo: (i) Tribo Bignonieae; (ii) Tribo Catalpeae; (iii) Aliança *Tabebuia*; (iv) Clado Paleotropical; (v) Tribo Oroxyleae; (vi) Tribo Tecomeae; (vii) Tribo Tourrettieae; e, (viii) Tribo Jacarandeae (Olmstead et al., 2009).

A tribo Bignonieae, com 21 gêneros e 393 espécies (Lohmann, 2006; Lohmann e Taylor, 2014). A tribo é claramente monofilética (Lohmann, 2006) e apresenta distribuição Neotropical, sendo composta principalmente por lianas, mas também incluindo alguns arbustos (Lohmann e Taylor, 2014). Podemos reconhecer membros de Bignonieae por suas folhas 2-3 folioladas ou 2-3 pinadas com folíolo terminal modificado em gavinha e pela anatomia do caule com variação cambial, formando 4-32 cunhas de floema (Pace et al., 2009). Além disso, o fruto é do tipo cápsula e apresenta deiscência septicida, paralela ao septo. (Lohmann e Taylor 2014).

Espécies de Bignonieae são amplamente distribuídas pela região Neotropical, ocorrendo desde as florestas úmidas da América Central, Amazônia e

Mata Atlântica brasileira, até áreas secas de cerrado da Argentina, Brasil e Paraguai (Lohmann e Taylor, 2014). Dados de filogenia molecular acompanhados da datação da idade de divergência de clados nesta tribo indicam que a mesma surgiu no Leste da Costa Brasileira, há cerca de 54,2 – 45,6 milhões de anos, onde atualmente temos a Mata Atlântica (Lohmann et al., 2013). A origem do grupo foi acompanhada de uma expansão para o noroeste da América do Sul e ocupação da Amazônia há cerca de 30 milhões de anos, onde ocorreu um grande evento de diversificação do grupo (Lohmann et al. 2013). Subsequentes colonizações das áreas secas do Brasil Central, da América Central e até mesmo re-colonizações da Mata Atlântica estão associadas à diversificação de diferentes linhagens (Lohmann et al. 2013).

Stizophyllum Miers é um pequeno gênero de lianas Neotropicais pertencente à tribo Bignonieae (Bignoniaceae). A circunscrição de *Stizophyllum* permaneceu estável desde que o gênero foi descrito por Miers (1863), em contraste com os demais gêneros de Bignonieae cujas circunscrições mudaram bastante nos últimos 200 anos (para uma revisão, ver Lohmann e Taylor 2014). O gênero é monofilético com base em dados moleculares (Lohmann 2006) e bem caracterizado pelos ramos cilíndricos e fistulosos, folíolos 2-3 foliolados, com o folíolo terminal modificado em gavinha simples ou trifida, presença de pontuações nos folíolos, cálice uroceolado e inflado, corolas tubulares e infundibuliformes, e cápsulas lineares achatadas (Fischer et al., 2004; Lohmann e Taylor, 2014). A presença de caules fistulosos e presença de pontuações nos folíolos são sinapomorfias de *Stizophyllum* (Lohmann e Taylor, 2014).

Treze espécies de *Stizophyllum* foram descritas até o momento (Lohmann e Ulloa, 2006 em diante), embora apenas três espécies sejam reconhecidas na sinopse mais recente da tribo Bignonieae (Lohmann e Taylor, 2014): (i) *Stizophyllum*

inaequilaterum Bureau e K. Schum., (ii) *Stizophyllum perforatum* (Cham.) Miers, e (iii) *Stizophyllum riparium* (Kunth.) Sandwith. Segundo a sinopse mais recente do grupo, *Stizophyllum inaequilaterum* e *S. riparium* ocorrem em florestas úmidas e perturbadas da América Central, Guianas, Amazônia e Chaco (Lohmann e Taylor, 2014). *Stizophyllum perforatum*, por outro lado, é restrito à Mata Atlântica e Áreas Secas do Brasil Central (Meyer, Diniz-Filho e Lohmann, 2018).

Apesar do gênero ser facilmente reconhecido e ter uma clara circunscrição, a delimitação de espécies permanece bastante complicada. Caracteres diagnósticos das espécies como a forma dos folíolos, tipo de indumento, cor da corola e morfologia dos frutos, geralmente se sobrepõem entre táxons, impedindo sua identificação. Esta dificuldade para identificar espécies no grupo indicam a necessidade de estudos adicionais no grupo. Além disso, estudos filogenéticos e biogeográficos detalhados também não foram realizados com o grupo. O único estudo filogenético que amostrou membros de *Stizophyllum* até hoje visou reconstruir as relações filogenéticas entre membros de toda a tribo Bignonieae e apenas amostrou duas das três espécies atualmente reconhecidas (Lohmann 2006).

Objetivos do presente estudo

Este estudo visou:

1. Reconstruir a filogenia de *Stizophyllum* com base em caracteres moleculares e uma ampla amostragem de indivíduos dentro de cada espécie, visando testar o monofiletismo do gênero e espécies (Capítulo 1);
2. Estudar a história biogeográfica do grupo (Capítulo 1);
3. Definir limites específicos e apresentar uma sinopse taxonômica para o grupo (Capítulo 1);

4. Desenvolver marcadores de microssatélite de cloroplasto (cpSSR) e nucleares (nSSR) para *Stizophyllum* como um primeiro passo para estudos futuros que visam compreender a diversidade genética desse gênero (Capítulo 2 e 3).

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Figura 1. Árvore de consenso estrito oriunda da análise de máxima verossimilhança do conjunto de dados combinado (*ndhF* e *PepC*). Esta filogenia foi utilizada como base para a atual classificação genérica da tribo (Lohmann and Taylor, 2014). Seta indica o posicionamento de *Stizophyllum*. Árvore retirada de Lohmann (2006).

Stizophyllum

Figura 2. Distribuição de *Stizophyllum*, indicando as localidades de ocorrência para todo o gênero. Mapa produzido por Juan Pablo Narváez (Universidade de São Paulo) a partir do banco de dados de Lohmann (dados não publicados).

Figura 3. Sinapomorfias de *Stizophyllum*. **A.** Caule fistoloso em *Stizophyllum riparium*. **B.** Pontuações na face abaxial dos folíolos em *Stizophyllum* sp. **C.** Flor tubular de *Stizophyllum riparium* **D.** Fruto linear e achatado de *Stizophyllum* sp. nov.

Capítulo 1: Phylogeny and biogeography of *Stizophyllum*
(Bignoniaceae, Bignoniaceae)

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Phylogeny and biogeography of *Stizophyllum*
(Bignoniaceae, Bignoniaceae)

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Abstract— Species are distributed unevenly around the globe, with the highest diversity concentrated in the New World Tropics. Many hypotheses have been proposed to explain the high diversity found in the Neotropics. However, we still do not have a clear picture of the factors that have led to such high diversity in this region. This study focuses on *Stizophyllum* Miers (Bignoniaceae), a small genus of Neotropical lianas. We reconstructed the molecular phylogeny of *Stizophyllum* using chloroplast (i.e., *ndhF* and *rpl32-trnL*) and nuclear (i.e., *pepC*) markers, as well as a broad sampling of taxa. Overall, we sampled 33 individuals representing the breadth of morphological variation and geographical distribution of the three species recognized to date, i.e., *S. inaequilaterum* Bureau & K. Schum., *S. perforatum* (Cham.) Miers, and *S. riparium* (Kunth) Sandwith. Bayesian and Maximum Likelihood approaches reconstructed congruent topologies. Furthermore, a putative new species from Pará was recovered as sister to the remaining species of the genus, while all *S. riparium* from Central America emerged as a second clade that is sister to a polytomy that includes three additional clades: (i) a monophyletic *S. inaequilaterum*, (ii) a monophyletic *S. perforatum*, and (iii) a clade including all *S. riparium* from Amazonia. These findings corroborate earlier circumscriptions of *S. perforatum* (including all *Stizophyllum* from the Atlantic Forest and Brazilian dry areas), and *S. inaequilaterum* (restricted to Western Amazonia and Central America). However, our molecular phylogeny supports the recognition of a new species from Pará, while indicates that *S. riparium* is best divided into two taxa, a narrower *S. riparium* restricted to Amazonia, and a separate species from Central America. These findings are further supported by morphology and the appropriate taxonomic changes are proposed. As such, a broader *Stizophyllum*, including five species is now recognized and an updated synopsis of the genus is presented. We

use our phylogenetic framework to get initial insights into the biogeographic history of the genus. Our results indicate that *Stizophyllum* originated during the Eocene, while most of the diversification within the genus occurred during the Miocene.

Keywords— lianas, molecular phylogenetics, Neotropical flora.

INTRODUCTION

Species are not uniformly distributed around the globe and tend to be more concentrated on Tropical regions (Mckenna and Farrell 2006; Pennington and Dick 2010; Antonelli and Sanmartín 2011). Even within the Tropics, a species gradient can be observed with a higher number of species found in the Neotropics (Govaerts 2001). Indeed, ca. 100.000 plant species are found in this region, while ca. 35.000 are found in África, and between 40.000 – 82.000 in Ásia and Oceania (Antonelli and Sanmartín 2011). This discrepancy has led researchers to search for mechanisms that may help explain these diversity patterns (Jablonski 1993; Qian and Ricklefs 2008; Moreau and Bell 2013; Crame 2016).

Multiple hypotheses have been proposed to explain the extremely high biodiversity found in the Neotropics. For instance, geological changes that occurred in association with the orogeny of the Andes, closure of the Isthmus of Panamá, and formation of the Amazon basin, among others, are thought to have greatly impacted the diversity patterns observed today (Antonelli et al. 2009; Hoorn et al. 2010). On the other hand, ecological changes that resulted from changes in climate during the Cenozoic (Colinvaux et al. 2000; Colinvaux and de Oliveira 2001) are also thought to have greatly impacted the high diversity found in this region. Despite the large number of hypotheses that aim to explain diversity patterns in the tropics, we still do not have a clear understanding of how diversification occurred in this region. It is clear though that a multitude of factors have played important roles in the diversification of Neotropical organisms (Hoorn and Wesselingh 2010; Rull 2011).

Case studies of Neotropical lineages, based on robust phylogenies and divergence time estimates can greatly help to elucidate the processes that may have

contributed to the high diversity found in this region. This study focuses on *Stizophyllum* Miers, a small genus of lianas that is distributed from southern Mexico to southeastern Brazil, where it occurs in the Amazon Forest, Atlantic Forest and Brazilian Dry Areas (Lohmann and Taylor 2014).

The genus belongs to tribe Bignonieae, the largest clade in the plant family Bignoniaceae (Lohmann and Taylor 2014). The tribe as a whole is Neotropical and includes 21 genera and 393 species of shrubs and lianas (Lohmann and Taylor 2014). The generic delimitation of Bignonieae was very problematic in the past, with generic circumscriptions changing dramatically between classification systems during the last 200 years (for a review see Lohmann and Taylor 2014). Despite that, the circumscription of *Stizophyllum* has remained stable since the description of the genus by Miers (1863). The genus is well characterized by the hollow cylindrical branches, leaflets 2-3 foliolate with the terminal leaflet replaced by a tendril, pellucid glands on leaflets, calyces uroceolate and inflated, trumped-shaped corollas, and linear capsules (Fischer et al. 2004; Lohmann and Taylor 2014). The monophyly of the genus has also received support from molecular phylogenetic data (Lohmann 2006).

Even though the circumscription of *Stizophyllum* has been very clear since the genus was described, species limits are complicated. Species diagnostic characters (e.g., leaflet shape and indument, corolla shape and color, and fruit morphology) often overlap among taxa, preventing their identification with certainty. As such, additional morphological and molecular phylogenetic studies based on a comprehensive sampling of taxa, are greatly needed so that clear taxa can be delimited. To date, members of *Stizophyllum* have only been sampled in a study that aimed to reconstruct phylogenetic relationships among members of the whole tribe

Bignonieae (Lohmann 2006). In this study, one individual of two of the three species recognized at that time were sampled.

Thirteen species of *Stizophyllum* were described to date (Lohmann and Ulloa 2006 onwards), although only three are recognized in the most recent synopsis of tribe Bignonieae (Lohmann and Taylor 2014): *Stizophyllum inaequilaterum* Bureau & K. Schum., *Stizophyllum perforatum* (Cham.) Miers and *Stizophyllum riparium* (Kunth.) Sandwith. While *Stizophyllum inaequilaterum* and *S. riparium* occur in humid and disturbed forests from Central America, the Guianas, the Amazon rainforest and the South American Chaco, *S. perforatum* is restricted to the Brazilian Atlantic Forest and the Dry Areas of Central Brazil. Even though *S. riparium* was thought to also reach the Atlantic Forest of Eastern Brazil (Lohmann and Taylor 2014), more recent studies of its distribution range (Meyer, Diniz-Filho and Lohmann 2018), accompanied of morphological studies (Lohmann pers. obs.) indicated that the Atlantic Forest populations of *S. riparium* actually correspond to *S. perforatum*. On the other hand, studies of *S. perforatum*, including detailed analyses of its distribution range (Meyer, Diniz-Filho and Lohmann 2018) and morphology (Lohmann pers. obs.) indicated that the populations from the Amazon and Guiana actually correspond to *S. riparium*.

Given the morphological complexity and broad geographic distribution of *Stizophyllum*, we reconstruct the phylogeny of the genus based on molecular markers and use this information as basis to: (i) redefine species limits in the group; and (ii) examine biogeographical patterns within the genus.

MATERIALS AND METHODS

Taxon sampling— We sampled a total of 35 accessions including two outgroups and 33 members from the ingroup, representing multiple individuals of all three species currently recognized in *Stizophyllum*, i.e., *S. inaequilaterum*, *S. perforatum* and *S. riparium* (Lohmann and Taylor 2014). For each species, we sampled the breadth of morphological variation and geographical distribution. Our final sampling included six specimens of *S. inaequilaterum* (all from Western Amazonia), 11 specimens of *S. perforatum* (seven from the Atlantic Forest and four from the Brazilian Dry Areas), 15 specimens of *S. riparium* (12 from different portions of the Amazon and three from Central America), and one specimen of a putative new species of *Stizophyllum* (from Eastern Amazonia). Additionally, we also included two outgroups, selected based on the phylogeny of Lohmann et al. (2013): *Adenocalymma moringifolium* (DC.) L.G. Lohmann, and *Martinella obovata* (Kunth) Bureau & K. Schum. These samples were obtained from Fonseca and Lohmann (in press), Kataoka and Lohmann (in prep.), and Lohmann (2006).

DNA extraction, amplification and sequencing— We extracted genomic DNA from leaf tissues stored in silica gel and from herbarium specimens. For each material, we pulverized 60 ng of plant material with TissueLyzer (Qiagen, Düsseldorf, Germany) and extracted DNA using the Invisorb Spin Plant Mini Kit (Invitex, Berlin, Germany) following the manufacturer's instructions. For specimens with less than 60 ng of leaf tissue, the CTAB protocol of Doyle and Doyle (1987) was used.

For each sample, we amplified two cpDNA markers, *ndhF* and *rp/32-trnL*, and the nuclear marker *pepC*. These markers have been shown to present adequate levels of variation to resolve relationships between species within tribe Bignonieae

as a whole (Lohmann 2006), and also within individual Bignoniaceae genera such as *Dolichandra* (Fonseca and Lohmann 2015), *Lundia* (Kaehler et al. 2012), *Pachyptera* (Francisco and Lohmann 2017), and *Tynanthus* (Medeiros and Lohmann 2015), among others. We amplified chloroplast markers following the protocol of Lohmann (2006), with adjustments suggested by Zuntini et al. (2013). For the nuclear markers, we used the nested PCR strategy described by Francisco and Lohmann (2017). All samples were purified and sequenced by Macrogen Inc. (Seoul, Korea).

For each sample, we obtained forward and reverse sequences and built contigs by inspecting all chromatographs visually using Geneious 9.1.8 (Kearse et al. 2012). We evaluated sequence quality through Phred scores (Ewing et al. 1998). We aligned contig sequences using MAFFT 7.22 (Katoh et al. 2002), implemented in Geneious 9.1.8 (Kearse et al. 2012), using default parameters (i.e., auto algorithm, scoring matrix: 200PAM/k = 2, gap open penalty: 1.53, offset value 0.123), followed by visual inspection and manual adjustments.

Phylogeny reconstruction— We constructed five datasets: (i) *ndhF* dataset, 31 individuals; (ii) *rp/32-trnL* dataset, 24 individuals; (iii) cpDNA dataset (*ndhF* and *rp/32-trnL*), 34 individuals; (iv) *pepC* dataset, 19 individuals; and, (v) combined dataset (*ndhF*, *rp/32-trnL*, and *pepC*), 35 individuals. For each data partition, we only used complete sequences with high quality Phred scores (> 90%). In the combined datasets, all sequences available for each marker were included; whenever sequences were lacking for a particular marker, those sequences were scored as missing.

Phylogenetic analyses were carried out using Bayesian Inference (BI) and Maximum Likelihood (ML). For both approaches, we determined the best-fit model of

DNA substitution for each partition using the Akaike information criterion implemented in JModelTest 2.1.4 (Darriba et al. 2015). The best models of DNA substitution recovered were the GTR+G for the *ndhF* and *rp/32-trnL* datasets, and HKY for the *pepC* dataset. A mixed model of DNA substitution was used in the analysis of the combined dataset.

Bayesian inference analyses were conducted in MrBayes 3.2.2 (Ronquist et al. 2012). For each analysis, we used four Markov Chain Monte Carlo (MCMC) runs for 10 million generations, sampling every 1000 generations to minimize autocorrelation among samples. Likelihood values were monitored graphically to detect stationarity with Tracer 1.5 (Rambaut and Drummond 2007). Trees were summarized into a consensus tree discarding 25% of burn-in using TreeAnnotator 1.7.5. The Maximum Likelihood analyses were conducted in RaxML (Stamatakis 2014) using the graphical user interface RaxMLGUI 1.3 (Silvestro and Michalak 2012). Tree support was evaluated through ML bootstrap, using 1000 rapid bootstrap replicates. Posterior probabilities (PP) and bootstrap (BS) were used to estimate node support. Results from both analyses were visualized using FigTree 1.4. Nodes with $PP \geq 0.9$ and $BS \geq 75$ were considered well supported.

Congruence testing— We used the ‘Congruence among distance matrices’ test (CADM) (Campbell et al. 2011) to verify whether the chloroplast data partition is congruent with the nuclear data partition. We performed this test using the function ‘CADM.global’ implemented in the *ape* package (Paradis et al. 2004) in an R environment (R Core Team 2016). Only the ingroup data was included in the comparison. Kendall’s concordance statistic *W* was used to compare matrices with a distance value. In this matrix, 0 indicates complete incongruence, while 1 indicates

complete concordance (Campbell et al. 2011). The null hypothesis of complete incongruence was tested with 999 permutations.

Divergence time estimates— We used a reduced version of the combined dataset to estimate divergence times within *Stizophyllum* using BEAST 2.4.3 (Drummond and Rambaut 2007). The reduced dataset included a single individual of each of the five evolutionary lineages identified in the topology that resulted from the analysis of the complete combined dataset (35 individuals). The five individuals were randomly chosen among individuals with complete sequences for all molecular markers. The same two outgroups used in earlier analyses, were included in this analysis. Our final dataset included seven samples, as follows: *Adenocalymma moringifolium*, *Martinella obovata*, *S. inaequilaterum* 1, *S. perforatum* 5, *S. riparium* 5 (Amazonia), *S. riparium* 14 (Central America) [= *S. flos-ardeae* (Pittier) Beyer & L.G. Lohmann], and *Stizophyllum sp. nov.* (= *S. coriaceum* Beyer & L.G. Lohmann).

BEAST uses a Bayesian approach to estimate the phylogeny and molecular rates using Markov Chain Monte Carlo (MCMC). We used mixed evolutionary models, which allowed us to implement the best-fit model of DNA substitution for each data partition. We used a relaxed clock lognormal modal and three ages from Lohmann et al. (2013) as calibration points. The first calibration point was constrained at the root node represented by the *Adenocalymma-Neojobertia* clade, applying a normal prior to a mean 48.0 and a sigma of 2.53. The second calibration point was applied to the node associated with the split of *Martinella* and *Stizophyllum*, also applying a normal prior to a mean 41.3 and a sigma of 3.35. The third calibration point was applied to the node associated with the divergence between *S. perforatum* and *S. inaequilaterum*, also using a normal prior, a mean of

8.4 and a sigma of 2.89. Since the split between *Stizophyllum* and the other genera of Bignoniaceae is known to be relatively old (> 40 Mya) when compared to the diversification within the genus (ca. 10 Mya) (Lohmann et al. 2013), it is plausible that both speciation and extinction may have occurred during those 30 Mya, even though fossil evidence is not available to corroborate this hypothesis. As such, we choose the birth and death model as a tree prior.

Ancestral area reconstructions— We used the time-calibrated phylogeny of *Stizophyllum* as basis for ancestral area reconstructions using the four biogeographic regions of Lohmann et al. (2013): (A) Eastern South America; (B) South American Dry Areas; (C) Lowland Amazonia; and, (D) Western South America and Central America. These regions were defined based on the distribution of various Bignoniaceae taxa, paleogeological data, and major geographical barriers. Ancestral areas were reconstructed using the Dispersal-Extinction-Cladogenesis model (DEC; Ree and Smith 2008), using the Bayes-Lagrange (S-DEC; Smith 2009) approach implemented in RASP 3.2 (Yu et al. 2015), without any area or age restriction.

RESULTS

Phylogenetic analyses of *ndhF*— Complete sequences of *ndhF* were obtained for 27 accessions of *Stizophyllum*. In addition, two ingroup sequences (*S. inaequilaterum* 1 and *S. perforatum* 4) and two outgroup sequences (*A. moringifolium* and *M. obovata*) from a previous study (Lohmann 2006) were added to our data matrix. Our final *ndhF* dataset included 29 ingroup sequences, as follows: *S. inaequilaterum* (six individuals), *S. perforatum* (eight individuals), *S. riparium* (14

individuals), and *Stizophyllum sp. nov.* (one individual). Sequences range in length from 1721 bp to 1730 bp. The aligned *ndhF* matrix includes 1730 bp, including 86 variable and 24 parsimony informative positions. The single optimal ML tree is $-\ln L = 2969.181$. The tree reconstructed using BI was similar to that reconstructed with ML. In both analyses, *Stizophyllum* emerges as monophyletic (PP = 1.0, BS = 97). Four major clades emerged within *Stizophyllum*: (i) *S. riparium* (Central America) (PP = 1.0, BS = 98), (ii) *S. perforatum* (*S. perforatum* 1, *S. perforatum* 3 and *S. perforatum* 5) (PP = 1.0, BS = 98); (iii) *S. inaequilaterum* (PP = 1.0, BS = 89); and (iv) *S. riparium* (Amazonia) (PP = 1.0, BS = 80). Five additional specimens of *S. perforatum* were recovered in a polytomy while the single individual of *Stizophyllum sp. nov.* sampled was recovered in a polytomy with *S. riparium* (Central America) (Figure S1). This clade is sister to a polytomy that includes the remaining three clades and the additional *S. perforatum* specimens (PP = 1.0, BS = 99).

Phylogenetic analyses of *rpl32-trnL*— Complete sequences of *rpl32-trnL* were obtained for 22 accessions of *Stizophyllum*, as follows: *S. inaequilaterum* (four individuals), *S. perforatum* (eight individuals), *S. riparium* (nine individuals), and *Stizophyllum sp. nov.* (one individual). In addition, two outgroups (*A. moringifolium* and *M. obovata*), were obtained from Fonseca and Lohmann (in press) and Kataoka and Lohmann (in prep.), respectively. Sequences range in length from 853 bp to 962 bp. The aligned *rpl32-trnL* matrix includes 966 bp, including 84 variable and 37 parsimony informative positions. The single optimal ML tree is $-\ln L = 1902.322$. The tree reconstructed using BI was similar to that reconstructed with ML. In both analyses, *Stizophyllum* emerges as monophyletic (PP = 1.0, BS = 100). Furthermore, four major clades are recovered: (i) *Stizophyllum sp. nov.* (PP = 1.0, BS = 100); (ii) *S. riparium* (Central America) (PP = 1.0, BS = 100); (iii) *S. perforatum*

(PP = 0.70, BS = 49); and (iv) *S. riparium* (Amazonia) (PP = 0.99, BS = 69). Overall *Stizophyllum* *sp. nov.* is sister to a large clade containing the remaining species of *Stizophyllum*. Within this large clade, *S. riparium* (Central America) is sister to a clade that includes a polytomy including all specimens of *S. inaequilaterum* and two clades, i.e., *S. riparium* (Amazonia) and *S. perforatum*. A clade composed of individuals of *S. perforatum* from the Dry Areas (i.e., *S. perforatum* 8, *S. perforatum* 10 and *S. perforatum* 11) (PP = 0.80, BS = 44), emerged within *S. perforatum*.

Phylogenetic analyses of the combined cpDNA dataset— Visual inspection indicates that the topologies derived from the individual analyses of the *ndhF* and *rpl32-trnL* datasets are largely congruent. Whenever incongruences were detected, these were poorly supported on either topology indicating lack of phylogenetic resolution. Because both markers are derived from the plastid genome, and no “hard” incongruences were detected, both data partitions were combined into a single dataset.

The cpDNA combined data set (*ndhF* and *rpl32-trnL*) included 32 sequences of *Stizophyllum* for both chloroplast markers, as follows: *S. inaequilaterum* (six individuals), *S. perforatum* (ten individuals), *S. riparium* (15 individuals), and *Stizophyllum* *sp. nov.* (one individual). In addition, the two outgroups (i.e., *A. moringifolium* and *M. obovata*) were also included, leading to a final dataset with 34 individuals. Sequences range in length from 958 bp to 2692 bp. The aligned cpDNA matrix included 2696 bp, including 170 variable and 62 parsimony informative positions. The single optimal ML tree is $-\ln L = 4874.062$. The tree reconstructed using BI was similar to that reconstructed with ML. In the trees that resulted from both analyses, *Stizophyllum* emerges as monophyletic (PP = 1.0, BS = 100).

Furthermore, five major clades are recovered within the genus: (i) *Stizophyllum sp. nov.* (PP = 1.0, BS = 100); (ii) *S. riparium* (Central America) (PP = 1.0, BS = 99); (iii) *S. perforatum* (PP = 0.61, BS = 41); (iv) *S. inaequilaterum* (PP = 1.0, BS = 77); and (v) *S. riparium* (Amazonia) (PP = 1.0, BS = 71) (Figure 1). *Stizophyllum sp. nov.* is the first lineage to diverge within the genus, while *S. riparium* (Central America) is the second. *Stizophyllum riparium* (Central America) is sister to a large clade that includes a polytomy that, in turn, includes three sub-clades: (i). *S. inaequilaterum*; (ii) *S. perforatum*; and (iii) *S. riparium* (Amazonia) (PP = 1.0, BS = 100).

Phylogenetic analyses of *pepC*— Complete sequences of *pepC* were obtained for 15 accessions of *Stizophyllum*. In addition, two ingroup sequences (*S. inaequilaterum* 1 and *S. perforatum* 4) and two outgroup sequences (*A. moringifolium* and *M. obovata*), were obtained from a previous study (Lohmann 2006). As such, our final *pepC* dataset included 17 ingroup sequences, as follows: *S. inaequilaterum* (four individuals), *S. perforatum* (five individuals), *S. riparium* (seven individuals), and *Stizophyllum sp. nov.* (one individual). Sequences range in length from 331 bp to 399 bp. The aligned *pepC* matrix included 400 bp, including 91 variable and 35 parsimony informative positions. The single optimal ML tree is -lnL = 1065.186. The tree reconstructed using BI was similar to that reconstructed with ML, although more poorly resolved. In both analyses, *Stizophyllum* emerges as monophyletic (PP = 1.0, BS = 100). Furthermore, five major clades were recovered within the genus: (i) *Stizophyllum sp. nov.* (BS = 39); (ii) *S. riparium* (Central America) (PP = 1.0, BS = 99), (iii) *S. inaequilaterum* (PP = 1.0, BS = 97) (iv) *S. riparium* clade 1 (Amazonia) (*S. riparium* 5, *S. riparium* 6 and *S. riparium* 12) (PP = 1.0, BS = 90), and (v) *S. riparium* clade 2 (Amazonia) (*S. riparium* 2 and *S. riparium* 3) (PP = 0.98, BS = 89). Five additional specimens of *S. perforatum* fall in a

polytomy (Figure S3). Overall *Stizophyllum sp. nov.* and *S. riparium* (Central America) emerges in a polytomy in the tree that resulted from the Bayesian inference. This clade is sister to a clade that includes all specimens of *S. perforatum*, plus two clades with all specimens of *S. riparium* (Amazonia), and a monophyletic *S. inaequilaterum* (PP = 1.0, BS = 93).

Congruence among cpDNA and nDNA datasets— Visual inspection indicates that the topology that resulted from the analysis of the combined chloroplast dataset (cpDNA) is congruent to the topology that resulted from the analysis of the nuclear dataset (*pepC*), with a few exceptions. Whenever incongruences were detected, these were poorly supported on either topology indicating a lack of “hard” incongruences. Furthermore, the CADM test (Campbell et al. 2011) also indicated that both datasets are congruent and can be combined.

Phylogenetic analyses of the combined dataset— The combined data set (cpDNA + *pepC*) included 33 sequences of *Stizophyllum*, as follows: *S. inaequilaterum* (six individuals), *S. perforatum* (11 individuals), *S. riparium* (15 individuals), and *Stizophyllum sp. nov.* (one individual). In addition, the two outgroups (i.e., *A. moringifolium* and *M. obovata*) were also added to our data matrix, leading to a final combined dataset with 35 individuals. Sequences range in length from 398 bp to 3090 bp. The aligned combined matrix includes 3096 bp, including 261 variable and 97 parsimony informative positions. The single optimal ML tree is -lnL = 5948.693. The tree reconstructed using BI is similar to that reconstructed with ML. In both analyses, *Stizophyllum* emerges as monophyletic (PP = 1.0, BS = 100). Furthermore, six major clades emerge within the genus: (i) *Stizophyllum sp. nov.* (PP = 1.0, BS = 100); (ii) *S. riparium* (Central America) (PP = 1.0, BS = 99); (iii) *S.*

perforatum clade 1 (*S. perforatum* 1, *S. perforatum* 3 and *S. perforatum* 5) (PP = 0.93, BS = 98), (iv) *S. perforatum* clade 2 (*S. perforatum* 6 and *S. perforatum* 7) (PP = 0.88, BS = 83); (v) *S. inaequilaterum* (PP = 1.0, BS = 89), and (vi) *S. riparium* (Amazonia) (PP = 0.82, BS = 57). Six additional specimens of *S. perforatum* fall in a polytomy (Figure 2). Overall *Stizophyllum* sp. nov. is sister to a large clade that includes the remaining species of *Stizophyllum*. Within this large clade, *S. riparium* (Central America) is sister to a clade that includes the two clades of *S. perforatum*, plus six specimens of *S. perforatum* in a polytomy, one clade with all specimens of *S. riparium* (Amazonia), and a clade with a monophyletic *S. inaequilaterum* (PP = 1.0, BS = 99).

Biogeography — Divergence time estimates indicate that the stem *Stizophyllum* likely originated during the late Eocene, at around 40.5 Mya (35 – 46.4 Mya) (Figure 3, Table 2). On the other hand, most of the diversification within the genus seems to have occurred during the Miocene, at around 17.5 Mya (11.2 – 24.8 Mya) (Figure 3, Table 2). The most likely scenario reconstructed using the S-DEC model implemented in RASP, reconstructs a MRCA (i.e., Most Recent Common Ancestor) for the stem *Stizophyllum* that is most likely restricted to lowland Amazonia (Area C, 62.7%). Three main biogeographical areas were occupied subsequently: Eastern South America (Area A); Southern America Dry Areas (Area B), and Western South America and Central America (Area D) (Figure 4, Table 2).

The S-DEC analysis inferred dispersal and peripatric speciation ($P = 0.0997$) as key drivers of Lowland Amazonian diversification (Figure 4, Table 2). The Central American lineage of *Stizophyllum riparium* (= *S. flos-ardeae*) diverged from the MRCA of the remaining species of the genus, at around 12.3 Mya (7.6 -17.9 Mya). A

vicariance event was inferred at this split ($P = 0.1244$), separating the Central American populations from their sister populations from Lowland Amazonia, the South American Dry Areas, and Eastern South America (Figure 4, Table 2). Subsequently, *S. riparium* (Amazon) diverged from *S. perforatum* and *S. inaequilaterum* at approximately to 5.5 Mya (2.9 – 8.4 Mya). While *S. inaequilaterum* remained in Lowland Amazonia, *S. perforatum* dispersed into Eastern South America, and the Southern American Dry Areas (Figure 4, Table 2). The divergence between *S. perforatum* and *S. inaequilaterum* is estimated to have occurred at around 4.9 Mya (2.5 – 7.7 Mya) (Figure 4, Table 2). The S-DEC analysis supports a dispersal event from the South American Dry Areas (Area B), Lowland Amazonia (Area C) and Western South Amazonia and Central America (Area D) to Eastern South America, followed by a vicariance event, in which *S. inaequilaterum* remained in Lowland Amazonia and Western South America and Central America, while *S. perforatum* reached the South American Dry Areas and Eastern South America ($P = 0.4158$) (Figure 4, Table 2). Dispersal seems to have predominated in the diversification history of the genus.

DISCUSSION

In this study, we used molecular data derived from the plastid and nuclear genomes to investigate phylogenetic relationship within *Stizophyllum*, a small genus of Neotropical lianas. Our analyses included a broad sampling of individuals, representing the breath of morphological variation and geographical distribution of the taxa recognized. Five major clades consistently emerged, three of which correspond to the three currently recognized species in the genus, i.e., *S.*

inaequilaterum, *S. perforatum* and *S. riparium*. The remaining two lineages represent new taxa. These taxonomic novelties are also supported by ongoing morphological studies (Beyer and Lohmann in prep) and the necessary taxonomic changes are here proposed.

Despite the significant improvements on the phylogeny of *Stizophyllum*, and the recovery of five-well supported clades, relationships among species remained poorly supported indicating the need for a more comprehensive phylogenetic study for the group, including an even higher number of markers and taxa. Given the taxonomic updates and confusing patterns of morphological variation in this group, a revised monograph for the whole genus is greatly needed (Beyer and Lohmann in prep.). Divergence time estimates indicate that the stem group of *Stizophyllum* originated during the late Eocene, while most of the diversification within the genus occurred during the Miocene, as demonstrated in previous studies (Lohmann et al. 2013). Ancestral area reconstructions reconstructed Lowland Amazonia as the most likely distribution of the MRCA of the stem group of *Stizophyllum*. Subsequent diversification events and occupation of new areas led to more broadly distributed taxa.

Phylogeny of *Stizophyllum*—The phylogeny of *Stizophyllum* reconstructed here corroborates the monophyly of the genus (Lohmann 2006). The five datasets analyzed (i.e., *ndhF*, *rpl32-trnL*, cpDNA, *pepC*, and combined dataset) consistently recover five clades that provide further support for the recognition of the three species currently recognized, i.e., *S. inaequilaterum*, *S. perforatum*, and *S. riparium* (Lohmann and Taylor 2014), plus two additional taxa. Even though the relationships among the three currently recognized species is not completely resolved, the

monophyly of two of these taxa (i.e., *S. inaequilaterum*, *S. perforatum*) is supported. *Stizophyllum riparium* consistently appears as polyphyletic, divided into two main clades, one including all the specimens from Central America and another including all Amazonian specimens, indicating the need of taxonomic adjustments.

Biogeography— Our results indicate that the stem *Stizophyllum* likely originated during the Eocene, at approximately 40.5 Mya (35 – 46.4 Mya) (Table 2). Lowland Amazonia represents the most likely distribution for the MRCA of the genus ($P = 0,0997$). Despite the low support for this inference, this result is in agreement with those from an earlier study (Lohmann et al. 2013). A broadly distributed ancestor was recovered for the crown group, although the ancestral area of this lineage was ambiguous. During the Miocene, several organisms diversified extensively in the Neotropics, especially primates (Buckner et al. 2015), amphibians (Castroviejo-Fisher et al. 2014) and plants, including other genera of tribe Bignonieae (de Medeiros and Lohmann 2015; Fonseca and Lohmann 2015), and other plant groups (Fine et al. 2014; Santos et al. 2017). These events are thought to have resulted from the intense geological activity of South America, especially in the Amazon region (Hoorn et al. 2010).

The MRCA of *Stizophyllum* was likely distributed within four main areas: Eastern South America (Area A); Southern American Dry Areas (Area B); Lowland Amazonia (Area C); and Western South and Central America (Area D) (Area ABCD, 27.53%). However, our RASP analysis indicates that the second most likely area are Lowland Amazonia (Area C) and Western South Amazonia and Central America (Area D) (Area CD, 25,17%), which explains the current distribution of the two first lineages to diverge within the genus. *Stizophyllum* sp. nov. (= *S. coriaceum*) was the

first lineage to diverge. No extinction was inferred in the 20 Mya gap between the origin of *Stizophyllum* and its diversification.

The next lineage to diverge within *Stizophyllum* was the Central American lineage of *S. riparium* (= *S. flos-ardeae*), which also diverged in the Miocene ~12.3 Mya (7.6- 17.9 Mya). The vicariant event inferred to explain the divergence between this lineage and the rest of the genus is congruent with the recently proposed older connection between South and Central America through the Panamanian Isthmus, at around 15-12 Mya (Farris et al. 2011; Bacon et al. 2013; Coates and Stallard 2013; Wilson et al. 2014; Hoorn and Flantua 2015), although considerable debate still exists regarding the actual age of closure of the Isthmus (see O'Dea et al. 2016).

The last two diversification events within *Stizophyllum* are even more recent, dating back to ~5.5 Mya (2.9- 8.4 Mya) for the divergence between *S. riparium* (Amazonia) and the remaining species of the genus, and ~4.9 Mya (2.5-7.6 Mya) for the divergence between *S. perforatum* (Eastern South America) and *S. inaequilaterum* (Lowland Amazonia). Sister-group relationships between Atlantic Forest and Amazonian taxa have been documented for several taxa (Batalha-Filho et al. 2013; Ledo and Colli 2017). The ages proposed here are more consistent with a connection through ancient forests in Northern South America (Costa 2003; Batalha-Filho et al. 2013), than through connections through Southern South America. On the other hand, the distribution of *S. perforatum* through the Dry Areas suggests a connection through the Central Brazilian Dry Areas.

Taxonomic treatment— The phylogeny of *Stizophyllum* brought new insights into the classification of the genus. More specifically, it suggests that five species should be recognized, instead of the three species recognized in the most recent

synopsis of the group (Lohmann and Taylor 2014). While our data supports the recognition of *S. inaequilaterum* as circumscribed in the most recent treatment of the tribe (Lohmann and Taylor 2014), our results provide further support for the recently proposed adjustments in the distribution of *S. riparium* and *S. perforatum* (Meyer, Diniz-Filho and Lohmann 2018). More specifically, our molecular phylogenetic data corroborates the recognition of a *S. perforatum* that is restricted to the Atlantic Forest and Brazilian Dry Areas, while provides additional support for the recognition of a *S. riparium* that is restricted to Amazonia. In addition, our molecular results and ongoing morphological studies (Beyer and Lohmann in prep.) provide strong support for the recognition of *S. riparium* (Central America) as a separate species. Our molecular phylogenetic study also provides strong support for the recognition of our putative new species as a separate taxon. We present a revised synopsis for the genus, based on our molecular phylogenetic results and ongoing morphological studies (Bayer and Lohmann in prep.).

***Stizophyllum* Miers**, Proc. Roy. Hort. Soc. London 3: 197. 1863. TYPE:

Stizophyllum perforatum (Cham.) Miers (lectotype, designed by Ballion [1888:30]).

Lianas, without strong odor; stems with 4 phloem wedges in cross-section, with hollow piths; branchlets cylindrical to sub tetragonal, striated, with pubescent or puberulous indument (hispid in *S. inaequilaterum* and sometimes glabrous in *S. riparium*), without lenticels or interpetiolar ridge; prophylls of axillary buds foliaceous and stipitate, without glands. *Leaves* 3-foliolate or 2-foliolate with terminal leaflet often replaced by a simple or trifid tendril; leaflets membranaceous (chartaceous in *S. coriaceum*), with pellucid punctations distributed over the lower surface, without

domatia. *Inflorescence* axillary and/or terminal, a lax to congested, few-flowered raceme, with 1 to 4 flowers (6 to 10 flowers in *S. coriaceum*). *Flowers* zygomorphic, pentamerous; calyx green (pinkish in *S. coriaceum*), urceolate, inflated, 5-lobed, membranaceous, puberulent or villose externally; corolla white, pink, or magenta, infundibuliform, straight in tube, dorso-ventrally compressed, membranaceous, pubescent externally, without glands; stamens with well-developed filaments, included, anthers glabrous, thecae straight, pollen in monads, colpate, with exine reticulate; ovary sessile, smooth and lepidote externally, ovules in two series on each placenta, stigmas elliptic, glabrous. *Fruits* capsule, linear, narrow, flattened, straight, coriaceous, with two valves, smooth, with calyx persistent; seeds winged, wings hyaline and linear.

Number of species, distribution and habitat. *Stizophyllum* includes five species, distributed from Mexico to southeastern of Brazil (Paraná), occurring in wet to dry forest and disturbed vegetation. Species of *Stizophyllum* are often pioneers, occupying borders of forest and secondary vegetation (Lohmann and Taylor 2014).

The circumscription of *Stizophyllum* remained stable since its description by Miers (1863). The genus is easily recognized by the hollow stems and branchlets, as well as the pellucid punctuation on leaflets. Pellucid punctations are also found in *Pyrostegia* (Lohmann and Taylor 2014), and the hollow stems and branchlets are also found in some species of *Pleonotoma* (Gomes 2006), and *Pachyptera* (Francisco and Lohmann 2018). In addition, the urceolate and inflated calyx, racemose few-flowered inflorescences, linear and narrow capsules are also helpful in its identification. The main features that distinguish each species are described below and summarized in the key to species.

Key to species of *Stizophyllum*

1. Branches, petioles and leaflet veins hispid, with reddish indument; leaflets asymmetrical ..... ***S. inaequilaterum***
- 1'. Branches, petioles and leaflet veins pubescent, puberulous or glabrous, with white, gray, yellowish or purplish indument; leaflets symmetrical **2**
2. Branches, petioles and leaflet veins puberulous; leaflets ovate, base rounded or obtuse ..... ***S. riparium***
- 2'. Branches, petioles and leaflet veins pubescent; leaflets elliptic or oblong-elliptic, base cunate **3**
3. Branches, petioles, leaflet margins and veins covered by yellowish indument; corolla tube White or cream and lobes pink; calyx longer than broad, 11.0-16.0 mm long ..... ***S. perforatum***
- 3'. Branches, petioles, leaflet margins and veins covered by greyish or purplish indument; corolla tube and lobes white or cream; calyx length similar to width, 6.0-9.0 mm long **4**
4. Branches, petioles and leaflet veins with greyish indument; leaflet membranaceous, pellucid punctuations sparsely distributed on the abaxial surface; tendrils simple; inflorescences with 1-4 flowers; calyx green ..... ***S. flos-ardeae***
- 4'. Branches, petioles and leaflet veins with purplish indument; leaflet chartaceous, pellucid punctation densely distributed on the abaxial surface; tendrils trifid; inflorescences with 6-10 flowers; calyx pinkish ..... ***S. coriaceum***

1. *Stizophyllum coriaceum*. Beyer & L.G. Lohmann, *sp. nov.* TYPE: Brazil, Pará, Altamira, beira de estrada BR-320, 03°12'20.4"S, 51°14'56.1"W, 07 December 2014, M. Beyer & L.H.M. Fonseca 317 (SPF).

Diagnosis. *Stizophyllum coriaceum* is morphologically similar to *S. perforatum* and *S. flos-ardeae* but can be separated from these taxa by the striking purplish indument on branches, petioles, and leaflet veins (vs. whitish indument), trifid tendrils (vs. simple tendrils), chartaceous leaflets (vs. membranaceous leaflets), inflorescences with 6-10 flowers (vs. inflorescences with 1-4 flowers) and pinkish calyces (vs. green calyces). *Stizophyllum coriaceum* can be differentiated from *S. perforatum* by a smaller calyx, up to 0.6 mm (vs. up to 16.0 mm) and white corolla tube and lobes (vs. white or cream corolla tube and pinkish lobes).

Habitat and distribution. This species is only known from disturbed vegetation from the Brazilian state of Pará.

2. *Stizophyllum flos-ardeae* (Pittier) Beyer & L.G. Lohmann, *comb. nov.* Basionym:

Adenocalymma flos-ardeae Pittier, Contr. U.S. Natl. Herb. 18: 256. 1917.

TYPE: Panamá, Cón, along Rio Fató, 9 July 1911, H. Pittier 3898 (holotype, US-000081 image!).

Diagnostic features. This species is characterized by dense and greyish indument on branches, petioles, and leaflet veins (Pittier 1917; Gentry 1937), tendrils simple, calyces green and short (up to 9.0 mm), and corolla tube and lobes white or cream (Pittier 1917; Blake 1922, Gentry 1937).

Habitat and distribution. This species occurs in humid forests and disturbed vegetation of Central America (Belize, Costa Rica, Guatemala, Mexico and Panama).

3. *Stizophyllum inaequilaterum* Bureau & K. Schum. Fl. Bras. 8(2): 221. 1896.

TYPE: Peru. Loreto: Maynas, 1831, *E.F. Poeppig 1827* (holotype, WI; isotype F-0042165 image!).

Diagnostic features. This species is characterized by a hispid and reddish indument that covers the branches, petioles and leaflets, by the leaflets asymmetrical, with sparse pellucid punctations, and tendrils trifid.

Habitat and distribution. This species occurs in humid to lowland premontane vegetation and is distributed through Central America (Costa Rica, Nicaragua, and Panama), the Guianas (French Guiana and Suriname), the Amazon rainforest (Brazil, Colombia Ecuador, Peru and Venezuela) and the South American Chaco (Bolivia) (Lohmann and Taylor 2014).

4. *Stizophyllum perforatum* (Cham.) Miers. Roy. Hort. Soc. London 3: 198. 1863.

Bignonia perforata Cham. Linnean 7: 667. 1832[1833]. TYPE: Brazil. S. loc., 1840, *F. Sellow s.n.* (holotype, LE image!; isotypes, K!, HAL-0098688 image!, NY00313145 image!, US-00125839 image!).

Diagnostic features. This species is characterized by a yellowish pubescence on branches, leaflet margins and veins, as well as dense pellucid-punctuation in the abaxial surface of leaflets, calyx longer than broad, with 11.0 -16.0 mm long and 0.8-0.9 mm width, tendrils simple.

Habitat and distribution. This species occurs in humid forests, disturbed vegetation and dry areas from the Atlantic Forest from Brazil (Bahia, Ceará, Espírito Santo, Rio de Janeiro, Maranhão, Minas Gerais, Piauí, Rio de Janeiro, São Paulo and Paraná) South American savannas (Brasília, Goiás and Mata

Grosso) and South American Chaco (Bolívia) (Meyer, Diniz-Filho and Lohmann 2018).

5. *Stizophyllum riparium* Kunth Lilloa 3: 462. 1939. *Bignonia riparia* Kunth, Nov.

Gen. Sp. (quarto ed.) 3: 138 1818 [1819]. TYPE: Colombia. Bolívar: Rio Magdalena, near Mompox, May 1801, *F.W.H.A. von Humboldt & A.J.A. Bonpland s.n.* (holotype, P-00670815 image!)

Diagnostic features. This species is characterized by the puberulous branches and leaflets, leaflets ovate, base obtuse or rounded, and sparsely distributed pellucid-punctations in the abaxial side of leaflets.

Habitat and distribution. This species occurs in humid forest and disturbed vegetation from the Amazon (Brazil, Colombia, Ecuador, Peru and Venezuela), the Guianas (French Guiana) and South American Chaco (Bolivia) (Meyer, Diniz-Filho and Lohmann 2018).

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Table 1. Taxa, vouchers, localities and biogeographical areas (sensu Lohmann et al. 2013) for sampled *Stizophyllum* and outgroups. Samples in bold were obtained from earlier studies.

Taxa^a	Vouchers	Locality	Biogeographical Areas (Lohmann et al. 2013)
<i>Stizophyllum inaequilaterum</i> 1	L.G. Lohmann 454	Brazil, Acre, Marechal Taumaturgo	Amazon
<i>Stizophyllum inaequilaterum</i> 2	E. Ferreira 2917	Brazil, Acre, Rio Branco	Amazon
<i>Stizophyllum inaequilaterum</i> 3	M. Beyer 376	Brazil, Acre, Senador Guilmard	Amazon
<i>Stizophyllum inaequilaterum</i> 4	J. Schunke-Vigo 14609	Peru, San Martín, Tocache	Amazon
<i>Stizophyllum inaequilaterum</i> 5	H. Romero-Saltos 2831	Ecuador, Amazonia, Orellana	Amazon
<i>Stizophyllum inaequilaterum</i> 6	D.A. Giraldo-Cañas 1028	Colombia, Antioquia, Cocoroná	Amazon
<i>Stizophyllum perforatum</i> 1	L.H.M. Fonseca 105	Brazil, Paraná, Londrina	Atlantic Forest
<i>Stizophyllum perforatum</i> 2	M. Groppo 1199	Brazil, São Paulo, Ribeirão Preto	Atlantic Forest
<i>Stizophyllum perforatum</i> 3	V. Souza, 9703	Brazil, São Paulo, Candido Mota	Atlantic Forest
<i>Stizophyllum perforatum</i> 4	J. Lombardi 2431	Brazil, Minas Gerais, Belo Horizonte	Atlantic Forest
<i>Stizophyllum perforatum</i> 5	L.H.M. Fonseca 103	Brazil, Espírito Santo, Linhares	Atlantic Forest
<i>Stizophyllum perforatum</i> 6	A.R. Zuntini 12	Brazil, Espírito Santo, Sooretama	Atlantic Forest
<i>Stizophyllum perforatum</i> 7	P. Fiaschi 1864	Brazil, Bahia, Juçari	Atlantic Forest
<i>Stizophyllum perforatum</i> 8	C. Proença 1978	Brazil, Goiás, Morrinhos	Dry Areas
<i>Stizophyllum perforatum</i> 9	E.P. Heringer 5704	Brazil, Distrito Federal, Brasília	Dry Areas
<i>Stizophyllum perforatum</i> 10	H.S. Irwin 8884	Brazil, Distrito Federal, Brasília	Dry Areas
<i>Stizophyllum perforatum</i> 11	M. Beyer 371	Brazil, Mato Grosso, Peixoto de Azevedo	Dry Areas
<i>Stizophyllum riparium</i> 1	M. Beyer 295	Brazil, Pará, Parauapebas	Amazon
<i>Stizophyllum riparium</i> 2	M. Beyer 302	Brazil, Pará, Parauapebas	Amazon
<i>Stizophyllum riparium</i> 3	M. Beyer 315	Brazil, Pará, Novo Repartimento	Amazon
<i>Stizophyllum riparium</i> 4	R. Secco 325	Brazil, Pará, Marabá	Amazon
<i>Stizophyllum riparium</i> 5	B. Gomes 685	Brazil, Amazonas, Manaus	Amazon

<i>Stizophyllum riparium</i> 6	B. Gomes 683	Brazil, Amazonas, Manaus	Amazon
<i>Stizophyllum riparium</i> 7	L.G. Lohmann 632	Peru, Madre de Dios, Manu	Amazon
<i>Stizophyllum riparium</i> 8	H. Romero-Saltos 2533	Ecuador, Amazonia, Orellana	Amazon
<i>Stizophyllum riparium</i> 9	A.H. Gentry 60204	Ecuador, Napo, Dureno	Amazon
<i>Stizophyllum riparium</i> 10	C.E. Ceron 4105	Ecuador, Napo, Parque Nacional Yasuni	Amazon
<i>Stizophyllum riparium</i> 11	J. Zarucchi 4909	Colombia, Antioquia, Caucasia	Amazon
<i>Stizophyllum riparium</i> 12	G. Cremers 8127	Guiana Francesa,	Amazon
<i>Stizophyllum riparium</i> 13	Alvarez 5027	Mexico, Campache, Calakmul	Central America
<i>Stizophyllum riparium</i> 14	D. Atha 1040	Belize, Cayo	Central America
<i>Stizophyllum riparium</i> 15	J.P. Abascal 79	Mexico, Chipas, Ocosingo	Central America
<i>Stizophyllum</i> sp.nov.	M. Beyer 317	Brazil, Pará, Altamira	Amazon
<i>Adenocalymma moringifolium</i>	L. G. Lohmann 19	Brazil, Amazonas, Manaus	Amazon
<i>Martinella obovata</i>	L. G. Lohmann 126	Surinam, Sipaliwini, Tepui	Amazon

Note: ^aGenBank accession numbers, *ndhF* and *pepC* respectively: *A. moringifolium*, DQ222613, DQ222773; *M. obovata*, DQ222606, DQ222763; *S. inaequilaterum*, DQ222638, DQ222808, and *S. perforatum*, DQ222639, DQ222809. GenBank accession numbers are not yet available for the remaining sequences.

Table 2. Divergence time estimates and ancestral areas* for nodes indicated in Fig. 4.

Ages (Mya)			Ancestral Areas		
Node	Mean	95 % CI	Area*	Event Routes	Probabilities (%)
13	48	43.9-52.2	C (0.70)	C->C^C->C C	0.44
12	40.5	35-46.5	C (0.63)	C->C^C^D->ABCD^C^D->CD ABCD	0.17
11	17.5	11.1-24.6	ABCD (0.27)	ABCD->ABCD^C->C ABCD	0.10
10	12.3	7.6-17.9	ABCD (0.36)	ABCD->D ABC	0.12
9	5.9	3.2-9.1	ABC (0.34)	ABC->BC->BC^C->BCD^C->C BCD	0.14
8	5.2	2.7-8.4	BCD (0.42)	BCD->ABCD->CD AB	0.42

Note: CI = Confidence Interval; A = Eastern South America; B = South American Dry Areas; C = Lowland Amazonia; and (D) Western South America and Central America.

*Probabilities are shown in parentheses.

Figure 1. Morphological features of *Stizophyllum*. **A.** Lianescent habit. **B-G.** *S. riparium*. **B.** Opposite and compound leaves. **C.** 2-3 foliolate leaves. **D.** Stipitated and elongated prophylls of the axillary buds. **E.** Flower. **F.** Branches with hollow pith. **G.** Flower bud. **H-I.** *S. perforatum*. **H-I.** Flowers, showing color variation. **J.** *S. coriaceum*, branch with terminal inflorescence. **K.** *S. inaequilaterum*, branch with yellowish pubescence.

Figure 2. Consensus tree derived from the Bayesian analysis of the *cpDNA* dataset (*ndhF* + *rpl32-trnL*) indicate phylogenetic relationships within *Stizophyllum*. Posterior probability and Bootstrap values are shown above the branches, respectively.

Figure 3. Consensus tree derived from the Bayesian analysis of the combined dataset (*ndhF* + *rpl32-trnL* + *pepC*) indicate phylogenetic relationships within *Stizophyllum*. Posterior probability and Bootstrap values are shown above the branches, respectively.

Figure 4. Maximum clade credibility tree for *Stizophyllum* derived from BEAST. Branch lengths are proportional to time and blue bars indicate 95% of highest posterior density (HPD).

Figure 5. A. Biogeographical areas proposed by Lohmann et al. (2013). **B.** Ancestral area reconstructions within *Stizophyllum* inferred with RASP, using the dispersal, extinction and cladogenesis model (DEC, Bayesian-Lagrange S-DEC) and the combined dataset (i.e., *ndhF*, *rpl32-trnL*, and *pepC*), including a single individual per species. Branches are proportional to time. Distributions assigned to each species are indicated by the colors next to the species names. Pie charts describe the relative probabilities of putative ancestral areas. Further details on the ancestral areas of the individual nodes are shown in Table 2.

Supplemental Material

Figure S1. Majority rule consensus tree derived from the Bayesian analysis of the *ndhF* dataset indicating phylogenetic relationships within *Stizophyllum*. Posterior probabilities and Bootstrap values are shown above branches, respectively.

Figure S2. Majority rule consensus tree derived from the Bayesian analysis of the *rp/32-trnL* indicating phylogenetic relationships within *Stizophyllum*. Posterior probabilities and Bootstrap values are shown above branches, respectively.

Figure S3. Majority rule consensus tree derived from the Bayesian analysis of the *pepC* dataset indicating phylogenetic relationships within *Stizophyllum*. Posterior probabilities and Bootstrap values are shown above branches, respectively.

Capítulo 2: Using genomic data to develop chloroplast DNA SSRs for the Neotropical liana *Stizophyllum riparium* (Bignoniaceae, Bignoniaceae)

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**USING GENOMIC DATA TO DEVELOP CHLOROPLAST DNA SSRs FOR THE
NEOTROPICAL LIANA *STIZOPHYLLUM RIPARIUM*
(BIGNONIEAE, BIGNONIACEAE)¹**

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Abstract

Premise of the study: We developed chloroplast microsatellite markers (cpSSRs) to be used as basis to understand the patterns of genetic structure and genetic diversity of populations of *Stizophyllum riparium* (Bignoniaceae, Bignoniaceae).

Methods and results: We used genomic data obtained through an Illumina HiSeq sequencing platform to develop a set of chloroplast microsatellite markers (cpSSRs) for *S. riparium*. A total of 36 primer pairs were developed, of which 28 displayed polymorphisms across 59 individuals from three populations. Two to 12 alleles were recorded and the unbiased haploid diversity per locus ranged from 0.037 to 0.905. All 28 cpSSRs presented transferability to two closely related species, *S. inaequilaterum* and *S. perforatum*.

Conclusions: We report a set of 28 cpSSRs for *S. riparium*. All markers were shown to be variable in *S. riparium* indicating that these markers will be valuable for population genetic studies across *S. riparium* and congeneric species

Key words: chloroplast microsatellites; cross-amplification; neotropical flora; *Stizophyllum inaequilaterum*; *Stizophyllum perforatum*.

Introduction

Stizophyllum Miers (Bignoniaceae) is a small genus of Bignoniaceae, the largest tribe in the Bignoniaceae (Lohmann and Taylor, 2014). The genus is clearly monophyletic (Lohmann, 2006), and includes three species, i.e., *S. inaequilaterum* Bureau & K. Schum., *S. perforatum* (Cham.) Miers and *S. riparium* (Kunth) Sandwith (Lohmann and Taylor, 2014). All species of *Stizophyllum* are vines and have trumpet-shaped flowers that are pollinated by medium sized bees (Gentry, 1974). Their winged seeds are dispersed by wind (Gentry, 1974). Members of *Stizophyllum* are easily recognized by their hollow stems, pellucid-punctate leaflets, urceolate calyces and linear fruits (Lohmann and Taylor, 2014). The genus as a whole is broadly distributed, occurring from Southern Mexico to the Atlantic Forest in Southern Brazil (Lohmann and Taylor, 2014). While the generic circumscription is clear, the three species of *Stizophyllum* currently recognized are morphologically similar and can occur sympatrically in Amazonia.

Nuclear DNA polymorphisms based on microsatellites (Simple Sequence Repeats–SSR) are powerful sources for population genetics studies (Kalia et al., 2011). These molecular markers, present also in the genomes of organelles (e.g., chloroplast), allow us to access genetic information that facilitates genotype identification, mainly due to their multi-allelic nature (Masi et al., 2003). The chloroplast microsatellite and nuclear microsatellite markers (cpSSR and nSSR, respectively) have been widely used to study phylogenetic and genetic diversity in plants (Kalia et al., 2011). Unlike the nSSRs which are highly polymorphic, codominant and biparentally inherited, the cpSSRs are a non-recombinant molecule and uniparentally inherited, allowing us to trace the history of populations through

their haplotype diversity (Ebert and Peakall, 2009). Despite these difference, the two markers are complements for understanding the genetic structure in natural population (Ebert and Peakall, 2009; Kalia et al., 2011).

Beyond that, the advent of high-throughput sequencing technologies (Metzker, 2010) has allow the development of SSR markers for multiple taxonomic groups (Zalapa et al., 2012; Francisco et al., 2016). Here, we used chloroplast genome sequence data obtained by *de novo* and reference guided assembly to develop and characterize chloroplast microsatellite markers for *Stizophyllum riparium*. The cross-amplification of the cpSSR markers developed for *S. riparium* was tested in all congeneric species in order to evaluate the utility of those markers for population genetics studies in *Stizophyllum* as a whole.

Methods and Results

We first obtained the chloroplast genome sequence of *Stizophyllum riparium* using an Illumina platform. For that, we extracted the genomic DNA from fresh leaf material dried in silica gel, from a single individual of *S. riparium* (Voucher: Nogueira 170) collected in Manaus (Amazonas State, Brazil) using the Invisorb Plant Mini Kit (Invitek, Berlin Germany) and following the manufacturer's instructions. About 5 µg of total DNA was fragmented using a Covaris S-series sonicator (Covaris, Woburn, Massachusetts, USA) and a short-insert (300 bp) library was constructed with NEBNext DNA Library Prep Master Mix Set and NEBNext Multiplex oligos for Illumina (New England BioLabs Inc., Ipswich, MA), following the manufacturer's protocol. The library concentration was diluted to 10 mM, and sequenced (single end) on an Illumina HiSeq 2000 system (Illumina Inc., San Diego, CA) at the

University of São Paulo (ESALq, Escola Superior de Agricultura Luiz de Queiroz da Universidade de São Paulo) in Piracicaba, Brazil. A Perl script was used to filter for quality using a Phred score of 20 or more for the cleaned reads, with the exclusion of reads with more than three uncalled bases, or shorter than 40 bp. We used a combination of reference-guided and *de novo* assembly to construct the chloroplast genome of *S. riparium* following Nazareno et al. (2015). The chloroplast genome for *S. riparium* was annotated using DOGMA (Dual Organellar GenoMe Annotator, <http://dogma.cccb.utexas.edu/>) (Wyman et al., 2004). Start and stop codons were inspected and adjusted manually.

The Imperfect Microsatellites Extractor (IMEX) interface (Mudunuri and Nagarajaram, 2007) was used to detect perfect microsatellites, with threshold of ten repeat units for mononucleotide, and six repeats for di-, tri-, tetra-, penta- and hexanucleotide. The annotated chloroplast genome was used to select markers located in non-coding regions exclusively. Primer pairs were designed from microsatellite sequence sites using Primer3web 4.0 (Rozen and Skaletsky, 1999), with the following parameters: primer size of 18—23 bp, temperature of 50—62°C with maximal difference between Forward and Reverse primers of 1°C, and GC content of 40—80%.

In total, 36 primer pairs were designed. The PCR amplifications were performed in a final volume of 10 µL and contained 15 ng of genomic DNA, 0.5 µL 10 mM of each primer with forward primers labeled with 6-FAM or JOE fluorescent dyes (Macrogen, Seoul, South Korea, Table 1), 0.6 µL 25 mM MgCl₂ (Promega Corporation, Madison, Wisconsin, USA), and 5 µL 1x of KAPA2G® Fast RedyMix (Kapa Biosystems, Wilmington, Massachusetts, USA). The cycling conditions were as follows: an initial denaturation step of 3 min at 94°C followed by 30 cycles of 30 s

at 94°C for denaturation, 30 s at the specific annealing temperature for each primer pair (Table 1), and 72°C for 60 s; and a final extension of 5 min at 72°C. To test the utility of the individual primers, PCR products were detected using a 1.0% agarose gel electrophoresis with a 100-bp range DNA ladder (Promega Corporation).

Of the 36 primer pairs tested, 28 successfully amplified (Table 1), and were checked for polymorphism in 59 individuals from three *S. riparium* populations (ranging from 18 to 22 individuals; Appendix 1). All samples tested were also extracted using fresh leaf material dried in silica gel or from herbarium specimens using the Invisorb Plant Mini Kit, following the manufacturer's instructions. The amplicons with fluorescent labels were resolved to genotype on an automated DNA sequencer, ABI 3500 XL with GeneScan 500 Rox Size Standard (Applied Biosystems, Foster City, California, USA). The microsatellite marker profiles were analyzed using GeneMarker (Holland and Parson, 2011). For each cpSSR, the number of alleles (A) and unbiased haploid diversity index (h) were obtained using GenAlEx 6.41 (Peakall and Smouse, 2006). In addition, we calculated chloroplast haplotype variation within populations by estimating the total number of haplotypes and the unbiased haplotype diversity as $He = [n/(n-1)](1 - \sum p_i^2)$, where n is the number of individuals analyzed in each population and p is the frequency of the i th haplotype in the respective population (Nei, 1978). Cross-amplification of polymorphic cpSSRs was tested in six individuals of *S. inaequilaterum* and *S. perforatum* using the same PCR conditions described above.

A partial chloroplast genome (128,239 bp) was obtained and used to develop a set of 28 polymorphic cpSSRs (Table 2). The number of alleles ranged from two to 12, and unbiased haplotype diversity varied from 0.037 (*Stiz40*) to 0.905 (*Stiz45*) (Table 2). When alleles at each of the 28 loci were jointly analyzed, we observed 18, 19 and

22 haplotypes for EAM (i.e., East of Amazonia), CAM (i.e., Central America) and SAM (i.e., South of Amazonia) populations, respectively. Furthermore, the maximum unbiased haplotype diversity ($H_e = 1.00$) was observed for all *S. riparium* populations. All polymorphic cpSSR markers successfully amplified for *S. inaequilaterum* and *S. perforatum* (Table 3).

Conclusion

We developed and characterized 28 chloroplast microsatellite markers for *S. riparium*. Due to the high rate of cross-amplification (100%), these chloroplast microsatellite markers will be useful for genetic studies involving *Stizophyllum* as a whole.

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TABLE 1. Characteristics of 28 intergenic chloroplast microsatellite markers developed for *Stizophyllum riparium*.

Locus	Repeat Motif	Primer Sequences (5'–3')	Allele size range (bp)	Position	T (°C)	GenBank accession no.
Stiz2	(A) ₆ (G) ₁₀	F: JOE - CCTTGTTGGTTAGTTGAGTTCC R: GGGGTAGCGACTTGTATATAACT	211-216	<i>trnK-UUU/rps16</i>	49.9	KP863512
Stiz3	(T) ₁₀	F: JOE - AGGAAGTTTTCTCCTCGTAC R: CTTGAGTTATGAGTACGAATGG	170 -176	<i>rps16/rps16</i>	54	KP863513
Stiz4	(A) ₁₀	F: 6-FAM - ATCTAATGATCCGGGGCG R: TTCCCTTTCCGTTGATGACT	160-169	<i>psbI/trns-GCU</i>	55.8	KP863514
Stiz5	(A) ₁₁	F: JOE - CCCTTCCCGAACCAACATG R: TTGTCAGAAGAGTCCTCCAAGT	176-181	<i>atpF/atpF</i>	57.4	KP863515
Stiz6	(A) ₁₂	F: JOE - GCCACACATACATTGCTTTGC R: TCCCTGTCATGTTCTTGGA	143-150	<i>atpH/atpI</i>	57.4	KP863516
Stiz7	(A) ₁₀	F: JOE - TCCTAGTATGCTGGCCAACA R: AGAAGAGAAGGTTCCATCGGA	164-171	<i>rps2/rpsC2</i>	57.4	KP863517
Stiz10	(T) ₁₀	F: 6-FAM -CCAACCTAAAATTCTTCCGA R: CTTATAGAAAGTGCGGTGC	165-173	<i>petN/psbM</i>	57.4	KP863519
Stiz11	(A) ₁₀	F: JOE - CAGATTGCCTTTCCACTTCGA R: TCGAAGAAATCCCCAACCCT	190-199	<i>psbM/trnD-GUC</i>	54	KP863520
Stiz12	(A) ₁₁	F: 6-FAM -GGATTTCTTCGATGGGCCCT R: GCCAGTCCCGACAGATCC	167-180	<i>psbM/trnD-GUC</i>	56.9	KP863521

Stiz13	(T) ₁₀	F: JOE - CCTGTTCTTTCCATGACCCC R: AAAGAAAGGGGAATGGCTCG	201-210	<i>psbC/trnS-UGA</i>	54	KP863522
Stiz14	(A) ₁₃	F: JOE - GCATAGCTAGCAATCCATTCT R: GGAACAATTGGAATAGAATGCC	136-149	<i>psaA/ycf3</i>	54	KP863523
Stiz15	(A) ₁₀	F: 6-FAM -CATTCTCGGCTTTTCATTCGT R: TCTTCCTGCCATTTCTCCCA	90-96	<i>trnT-UGU/trnL-UAA</i>	51.7	KP863525
Stiz18	(T) ₁₀	F: JOE - CATTCGTTCTTCAGCCTCA R: TTGAGTTGCATGGATTTGGA	179-184	<i>ycf4/cemA</i>	54.7	KP863527
Stiz20	(T) ₁₂	F: 6-FAM -TTCTAGGAGGCTTGTCTTCC R: TGTCCACTACTTTACTGTACGT	183-191	<i>petA/psbJ</i>	56.9	KP863528
Stiz21	(T) ₁₀	F: 6-FAM -TACTGCGTCAATTGCCAATT R: TCACCACGCCTGTATTGTAA	186-197	<i>petL/petG</i>	48.6	KP863529
Stiz22	(T) ₁₀	F: JOE - ACCCACCTATACAGTAACGGT R: TCTCCATTGGTAGCAAATGGT	161-169	<i>rpl20/rpl1s</i>	56.9	KP863531
Stiz23	(A) ₁₀	F: 6-FAM -GATTCTCTATCTGCTGTGTG R: TCTTGAATGGAAAGTAAGGG	153-162	<i>ClpP/psi_psbT</i>	56.9	KP863532
Stiz24	(A) ₁₁	F: 6-FAM -AGTCGAGTATCTGAAACACGA R: AGAACCATTGGATCATCACGT	179-183	<i>psbH/petB</i>	61.4	KP863534
Stiz25	(T) ₁₁	F: JOE - TGGAGGAGAAACGATATTAG R: CTTCTCGAGGTATAATGACA	168-175	<i>infA/rps8</i>	61.4	KP863535
Stiz26	(T) ₁₀	F: 6-FAM -TCTAGCCATATCAGCATTTTCGT	168-170	<i>rps8/rpl14</i>	61.4	KP863536

		R: GTGTCTCACGGCATATACCT				
Stiz27	(T) ₁₀	F: JOE - TCTTCTCATCCAGCTCCTCG R: CGAATAAGCGCTACGACTGA	243-248	<i>rpl16/rps3</i>	53.2	KP863537
Stiz28	(A) ₁₀	F: JOE - GTCCTTTATCCAAGTTTACC R: TCTTACTGATTCACTAGTCG	168-169	<i>rpl16/rps3</i>	55.8	KP863538
Stiz33	(T) ₁₃	F: 6-FAM -TGGATCTCTCAGTCTAAGCAGG R: CCACAAGCCGAGGAGATCTT	230-238	<i>ycf1/ycf1</i>	49.9	KP863539
Stiz34	(T) ₁₀	F: JOE - GACACGCAGGATAATTCATA R: TCCCAGAATGAATACAGAAC	182-206	<i>ycf1/ycf1</i>	56.9	KP863540
Stiz36	(TA) ₆	F: 6-FAM -ACATCCCTATTTCTCCATTG R: TCTTAGCGAGTAGTTCCGA	168-170	<i>trnP-UGG/psaJ</i>	56.9	KP863530
Stiz39	(ATA) ₄	F: JOE - CTCTAACCTCTGAGCTAAGC R: CGAAATTCTATATCGCTGCA	175-176	<i>trnT-UGU/trnL-UAA</i>	58	KP863524
Stiz40	(TAA) ₄	F: JOE - GATCCAAGAAATTACAGGAC R: TGAGCTATCCTGACCATT	130-132	<i>trnL-UAA/trnF-GAA</i>	53.2	KP863526
Stiz45	(TCTCTT) ₇	F: JOE - GAAATCCCATATGACCCA R: CCCCAAAGAAAGAGAAAG	156-171	<i>ClpP/psi_psbT</i>	53.2	KP863533

TABLE 2. Characteristics of 28 polymorphic chloroplast microsatellite markers in three populations of *Stizophyllum riparium*.^a

Locus	CAM (n=19)		SAM (n=22)		EAM (n=18)		All (n=59)	
	A	h	A	h	A	h	A	h
Stiz2	3	0.368	4	0.614	4	0.654	6	0.660
Stiz3	4	0.551	2	0.524	4	0.575	6	0.671
Stiz4	5	0.719	3	0.626	3	0.451	9	0.773
Stiz5	3	0.550	2	0.095	2	0.125	4	0.533
Stiz6	3	0.433	4	0.726	2	0.209	6	0.668
Stiz7	4	0.618	3	0.511	2	0.529	6	0.719
Stiz10	5	0.693	2	0.505	2	0.118	6	0.633
Stiz11	2	0.529	3	0.426	4	0.802	5	0.653
Stiz12	5	0.683	4	0.671	4	0.712	6	0.760
Stiz13	4	0.419	6	0.766	3	0.385	7	0.584
Stiz14	7	0.850	8	0.884	4	0.600	10	0.893
Stiz15	4	0.575	2	0.467	4	0.582	6	0.627
Stiz18	3	0.608	4	0.633	3	0.228	6	0.588
Stiz20	3	0.342	8	0.833	2	0.294	9	0.780
Stiz21	7	0.596	5	0.745	3	0.601	8	0.717
Stiz22	1	0.000	2	0.416	4	0.467	5	0.703
Stiz23	4	0.642	4	0.676	3	0.582	6	0.698
Stiz24	2	0.441	3	0.706	2	0.111	4	0.665
Stiz25	2	0.525	3	0.552	2	0.523	4	0.732
Stiz26	3	0.632	2	0.395	1	0.000	3	0.420
Stiz27	4	0.608	3	0.386	3	0.600	4	0.625
Stiz28	2	0.125	2	0.233	1	0.000	2	0.458
Stiz33	4	0.419	5	0.737	2	0.309	9	0.796
Stiz34	2	0.485	3	0.338	2	0.476	5	0.725
Stiz36	2	0.264	3	0.451	2	0.111	3	0.516
Stiz39	3	0.542	2	0.521	1	0.000	3	0.493
Stiz40	1	0.000	2	0.100	1	0.000	2	0.037
Stiz45	5	0.788	9	0.900	3	0.621	12	0.905
Mean	3.4	0.500	3.6	0.551	2.6	0.381	5.7	0.644

Note: A = number of alleles; h = unbiased haploid diversity.

^a Voucher and locality information are provided in Appendix 1.

TABLE 3. Transferability of 28 chloroplast microsatellite markers developed for *Stizophyllum riparium* across two species of *Stizophyllum*.

Locus	Repeat motif	<i>S. perforatum</i>	<i>S. inaequilaterum</i>
Stiz2	(A) ₆ (G) ₁₀	+	+
Stiz3	(T) ₁₀	+	+
Stiz4	(A) ₁₀	+	+
Stiz5	(A) ₁₁	+	+
Stiz6	(A) ₁₂	+	+
Stiz7	(A) ₁₀	+	+
Stiz10	(T) ₁₀	+	+
Stiz11	(A) ₁₀	+	+
Stiz12	(A) ₁₁	+	+
Stiz13	(T) ₁₀	+	+
Stiz14	(A) ₁₃	+	+
Stiz15	(A) ₁₀	+	+
Stiz18	(T) ₁₀	+	+
Stiz20	(T) ₁₂	+	+
Stiz21	(T) ₁₀	+	+
Stiz22	(T) ₁₀	+	+
Stiz23	(A) ₁₀	+	+
Stiz24	(A) ₁₁	+	+
Stiz25	(T) ₁₁	+	+
Stiz26	(T) ₁₀	+	+
Stiz28	(T) ₁₀	+	+
Stiz27	(A) ₁₀	+	+
Stiz33	(T) ₁₃	+	+
Stiz34	(T) ₁₀	+	+
Stiz36	(TA) ₆	+	+
Stiz39	(ATA) ₄	+	+
Stiz40	(TAA) ₄	+	+
Stiz45	(TCTCTT) ₇	+	+

Note: successful amplification (+) evidenced by the occurrence of distinct single band on sequencing gel.

APPENDIX 1. Voucher and locality information for all individuals of *Stizophyllum* sampled.

Species	Population Code	Locality	Geographic coordinates	Voucher no. ^a
<i>Stizophyllum riparium</i> (Kunth) Sandwith	CAZ	Brazil, Amazonas, Manaus	3°00'14"N, 59°55'07"W	A. Nogueira 170*
	CAM	Mexico, Campache, Calakmul	18°48'39"N, 89°18'28"W	D. Álvarez, 5027 ¹
	CAM	Mexico, Quintana Roo, Playa del Carmen	20°36'58"N, 87°03'58"W	E. Cabrera ,6450 ¹
	CAM	Mexico, Yucatan, Buctzotz	21°12'60"N, 88°45'60"W	E. Cabrera, 13716 ¹
	CAM	Mexico, Quintana Roo, Adolfo de la Huerta	19°34'45"N, 89°03'31"W	D. Álvarez, 9469 ¹
	CAM	Mexico, Campache, Calakmul	18°32'27"N, 89°54'53"W	D. Álvarez, 5168 ¹
	CAM	Belize, Toledo, Maya Mts.	16°24'25"N, 89°06'07"W	B.K. Holst, 4319 ¹
	CAM	Belize, Cayo	17°21'05"N, 88°55'12"W	D. E. Atha, 1040 ¹
	CAM	Mexico, Chipas, Ocosingo	16°54'18"N, 92°02'17"W	E. Martinez, 12499 ¹
	CAM	Mexico, Chipas, Ocosingo	17°01'39"N, 91°16'31"W	J.P. Abascal, 79 ¹
	CAM	Mexico, Chipas, Ocosingo	17°01'30"N, 91°18'22"W	G. Aguilar, 1688 ¹
	CAM	Mexico, Veracruz, Los Tuxtla	18°34'60"N, 95°05'60"W	C.S. Sinaca, 1561 ¹
	CAM	Mexico, Veracruz, San Andres Tuxtla	18°26'60"N, 95°12'58"W	C.S. Sinaca, 339 ¹
	CAM	Guatemala, Peten, San Jose	17°01'39"N, 89°52'60"W	B. Wallnöfer, 5907 ¹
	CAM	Costa Rica, Puntarenas, Peninsula de Osla	8°24'01"N, 83°17'30"W	R. Aguilar, 1960 ¹
	CAM	Costa Rica, Guanacaste, Nandayure	9°50'57"N, 85°20'26"W	A. Estrada, 87 ¹
	CAM	Costa Rica, Puntareans, Palmar Norte	8°58'60"N, 83°27'60"W	A.H. Gentry, 78803 ¹

CAM	Costa Rica, San José, Tircoles	9°47'29"N, 84°31'37"W	J.F. Morales, 4012 ¹
CAM	Costa Rica, Puntarenas, Golfito	8°33'03"N, 83°20'60"W	A. Azofeifa, 719 ¹
CAM	Costa Rica, Puntarenas, Bueno Aires	9°02'04"N, 83°25'49"W	L. González, 897 ¹
EAM	Brazil, Pará, Parauapebas	06°10'12"S, 50°21'02"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°09'46"S, 50°20'52"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°10'13"S, 50°21'04"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°09'45"S, 50°20'51"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°09'48"S, 50°20'52"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°10'08"S, 50°21'01"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°10'04"S, 50°21'02"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°10'04"S, 50°21'02"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°10'02"S, 50°21'02"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°09'46"S, 50°20'51"W	M. Beyer, 295
EAM	Brazil, Pará, Parauapebas	06°09'48"S, 50°20'53"W	M. Beyer, 301
EAM	Brazil, Pará, Parauapebas	06°09'36"S, 50°25'21"W	M. Beyer, 302
EAM	Brazil, Pará, Parauapebas	06°04'10"S, 50°14'46"W	M. Beyer, 303
EAM	Brazil, Pará, Parauapebas	06°03'55"S, 50°12'57"W	M. Beyer, 303
EAM	Brazil, Pará, Parauapebas	06°04'14"S, 50°14'44"W	M. Beyer, 303
EAM	Brazil, Pará, Novo Repartimento	04°19'57"S, 49°56'57"W	M. Beyer, 315
EAM	Brazil, Pará, Altamira	03°12'20"S, 51°14'56"W	M. Beyer, 317
EAM	Brazil, Pará, Moju	03°50'25"S, 46°06'40"W	M. Beyer, 318

SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer, 353
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer, 354
SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer, 356
SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer, 356
SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer, 356
SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer, 356
SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer, 356
SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer, 356

<i>Stizophyllum perforatum</i> (Cham.) Miers	-	Brazil, São Paulo, Candido Mota	22°45'27"S, 50°22'06"W	JP. Souza, 9703
	-	Brazil, Paraná, Londrina	23° 34' 12"S, 50° 57'42"W	LHM. Fonseca, 105
	-	Brazil Paraná Londrina	23° 34' 12"S, 50° 57'42"W	LHM. Fonseca, 103
	-	Brazil, Minas Gerais, Belo Horizonte	11° 54'S, 71° 22'W	Lombardi, 2431 ¹
	-	Brazil, Rio de Janeiro, São Pedro de Aldeia	22°50'10"S, 42°06'13"W	JA. Kallunki, s.n.
	-	Brazil, Piauí, Eliseu Martins	08°05'27"S, 43°39'42"W	P. Martins & E. Nunes, s.n. ⁴
	-	Brazil, Acre, Marechal Taumaturgo	08°56'29" S, 72°47'33"W	LG. Lohmann, 454
<i>Stizophyllum inaequilaterum</i> Bureau & K Schum	-	Equador, Amazonia, Orellana	0°40'59" S, 76°24' W	H. Romero-Saltos, 2831
	-	Peru, Ucayali, Pedro Abad	09°09'02"S, 75°47'20"W	J. Schunke-Vigo, 15997
	-	Frech Guiana, Saul	03°37'22"N, 53°12'34"W	SA. Mori, 24242 ²
	-	Peru, San Martin, Tocache	08°11'22"S, 76°30'57"W	J. Schunke-Vigo, 14609 ³
	-	Brazil, Amazonas, Letícia	04°12'19"S, 69°55'58" W	A. Gentry, 18302

Note: CAZ = Central Amazonia; EAM = East of Amazonia; SAM = South of Amazonia; SWA = South Western Amazonia.

^aMost specimens are deposited at the University of São Paulo Herbarium (SPF), São Paulo, Brazil, except from 20 samples (¹) that are deposited at the Missouri Botanical Garden Herbarium (MO), St. Louis, USA; one sample (²) that is deposited at the Muséum National d'Histoire Naturelle (P), Paris, France; one sample (³) that is deposited at the New York Botanical Garden Herbarium (NY), New York, USA; and, one sample (⁴) that is deposited at the Herbarium of the Federal University of Ceará (EAC), Fortaleza, Brasil.

Capítulo 3: Development and characterization of nuclear microsatellite markers for the Neotropical liana *Stizophyllum riparium* (Bignoniaceae, Bignoniaceae)

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**DEVELOPMENT AND CHARACTERIZATION OF NUCLEAR MICROSATELLITE
MARKERS FOR THE NEOTROPICAL LIANA *STIZOPHYLLUM RIPARIUM*
(BIGNONIEAE, BIGNONIACEAE)¹**

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ABSTRACT

Premise of the study: We developed nuclear microsatellite markers (nSSRs), as a complement to previously developed chloroplast markers, to be used as basis to study the genetic structure and diversity of *Stizophyllum riparium* (Bignoniaceae, Bignoniaceae).

Methods and results: We used genomic data obtained through an Illumina HiSeq platform to develop a set of nuclear microsatellite markers for *S. riparium*. A total of 34 primer pairs were isolated, of which nine displayed polymorphisms across 57 individuals from three populations. Eight to 29 alleles were recorded and the unbiased expected heterozygosity per locus ranged from 0.724 to 0.952. All nine nSSRs presented transferability to two closely related species, *S. inaequilaterum* and *S. perforatum*.

Conclusions: We report a set of nine polymorphic nSSRs for *S. riparium*. All markers are variable in *S. riparium* indicating that these markers will be useful for population genetic studies across this species and its congeneric taxa.

Key words: cross-amplification; genetic diversity; microsatellites; *Stizophyllum inaequilaterum*; *Stizophyllum perforatum*.

INTRODUCTION

Stizophyllum Miers (Bignoniaceae, Bignoniaceae) is a small genus of Neotropical lianas that occurs in humid and disturbed forests (Lohmann and Taylor, 2014). The genus is clearly monophyletic (Lohmann, 2006) and currently includes five species (Beyer and Lohmann, in prep.). These five species correspond to the three species recognized in the most recent treatment synopsis the whole tribe Bignoniaceae (Lohmann and Taylor, 2014), i.e., *S. inaequilaterum* Bureau & K. Schum., *S. perforatum* (Cham.) Miers and *S. riparium* (Kunth) Sandwith (Lohmann and Taylor, 2014), plus two newly recognized taxa (Beyer and Lohmann, in prep.), i.e., *S. coriaceum* Beyer & L.G. Lohmann and *S. flos-arede* (Pittier) Beyer & L.G. Lohmann. *Stizophyllum* is characterized by hollow stems and pellucid-punctate leaflets, which are easily recognized in the field (Lohmann and Taylor, 2014). All species of *Stizophyllum* are vines and have trumpet-shaped flowers that are pollinated by medium sized bees (Gentry, 1974). The winged seeds of *Stizophyllum* species are dispersed by wind (Gentry, 1974).

Polymorphic molecular markers such as the nuclear and chloroplast microsatellites (i.e., Simple Sequence Repeats, SSRs) are widely used to access contemporary gene flow, mating system and population genetic structure (Palma-Silva et al., 2009; Kalia et al., 2011; Khadivi-Khub et al., 2014; Hmeljevski et al., 2017). Unlike cpSSRs markers, nSSR markers show high levels of polymorphism, are codominant, and present biparental inheritance (Navascués and Emerson, 2005; Ebert and Peakall, 2009). While cpSSRs were recently characterized for *S. riparium* (Beyer et al., 2017), no nSSRs are available for the genus. Here, we developed and characterized nSSR markers for *Stizophyllum riparium* using genomic data obtained through an Illumina HiSeq platform. Additionally, the cross-amplification of the nSSR

markers developed for *S. riparium* was tested for two related species, *S. inaequilaterum* and *S. perforatum*, in order to evaluate the utility of those markers for population genetics studies in the genus.

METHODS AND RESULTS

We obtained sequences of the nuclear genome of *Stizophyllum riparium* using an Illumina platform, following Nazareno *et al.* (2015). Genomic DNA was extracted from fresh leaf dried in silica gel of a single individual of *S. riparium* (Voucher information: Nogueira 170) using the Invisorb Plant Mini Kit (Invitex, Berlin Germany) according to the manufacturer's instructions. Briefly, the purified DNA was fragmented using a Covaris S-series sonicator, and short-insert (300 bp) libraries were constructed with the NEBNext DNA Library Prep Master Mix Set and NEBNext Multiplex oligos for Illumina (New England BioLabs Inc., Ipswich, MA) following the manufacturer's protocol. The library concentration was determined using the Kapa Library Quantification Kit (Kapa Biosystems Inc., Wilmington, MA) on an Applied Biosystems 7500 Real-Time PCR System. We diluted the concentration of the library to 10 mM and sequenced our library (single end) on an Illumina HiSeq 2000 system (Illumina Inc., San Diego, CA) at the University of São Paulo (Escola Superior de Agricultura Luiz de Queiroz da Universidade de São Paulo, Piracicaba, Brazil).

We used a Perl script to clean the raw reads and excluded all reads with a Phred score lower than 20, as well as reads with more than three uncalled bases, or reads shorter than 40 bp, following Nazareno *et al.* (2015). The nuclear genome was separated from the chloroplast genome using *de novo* assembly. The genome was filtered based on sequence coverage. Contigs with coverage lower than 10x

correspond to contigs of the nuclear genome, while the higher coverage contigs correspond to those from the chloroplast genome. The remaining *de novo* contigs were assembled into larger contigs in Sequencher 5.3.2 (Gene Codes Inc., Ann Arbor, MI) based on at least 20bps overlap and 98% similarity. The UNIX 'grep' function was used to find reads and assemble them. We used a *perl* script to look for repeated regions, searching for possible motifs. We used a threshold of ten repeats for dinucleotide, eight repeats for trinucleotide and four repeats for tetra-, penta- and hexanucleotides (i.e. AG₁₀, CGC₈, AGAC₄, etc). Finally, we used the Blast function on NCBI, to check for chloroplast and mitochondria sequences, and only kept the sequences from the nuclear genome while designing primers.

Primers were designed using Primer3web 4.0.0 (Rozen and Skaletsky, 1999), with the following parameters: primer size with a minimum of 18 and a maximum of 23 bp, annealing temperatures of 50°C to 62°C, with maximal difference between Forward and Reverse primers of 1°C and GC content varying from 40 to 80%. A total of 213 nSSRs were designed (Appendix 1).

A set of 32 nSSRs was chosen and synthesized. PCR amplifications were performed in a final volume of 10 µL that contained 15 ng of genomic DNA, 10 mM of each primer with forward primers labeled with 6-FAM or JOE fluorescent dyes (Macrogen, Seoul, South Koreae, Table 1), 25 mM MgCl₂ (Promega Corporation, Madison, Winsconsin, USA), and 5 µL 1x of KAPA2G® Fast RedyMix (Kapa Biossytems, Wilmington, Massachusetts, USA). The cycling conditions were as follows: an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C for denaturation, 30 s at the specific annealing temperature for each primer pair (Table 1), 72°C for 60 s, and a final extension of 5 min at 72°C. To test the utility of

the individual primers, PCR products were detected using a 1.0% agarose gel electrophoresis with a 100-bp range DNA ladder (Promega Corporation).

Of the 32 primer pairs tested, nine amplified successfully (Table 1) and were checked for polymorphisms in 57 individuals from three *S. riparium* populations (individual population sizes ranged from 17 to 22 individuals, Appendix 2). DNA was extracted from leaf material dried in silica gel or herbarium specimens using the Invisorb Plant Mini Kit, following the manufacturer's instructions. The amplicons with fluorescent labels were resolved to genotype on an automated DNA sequencer, ABI 3500 XL (Applied Biosystems, Foster City, California, USA) with GeneScan 500 Rox Size Standard (Applied Biosystems). The microsatellite marker profiles were analyzed using GeneMarker (Holland and Parson, 2011). For each nSSR, the number of alleles (A), the number of effective alleles (A_e), the observed heterozygosity, unbiased expected heterozygosity (H_e), and Hardy-Weinberg equilibrium (HWE) were estimated using GenAlEx 6.41 (Peakall and Smouse, 2006). In addition, gametic disequilibrium was tested for each *S. riparium* population using FSTAT (Goudet, 1995). In order to avoid false-positives, P-values (= 0.05) were adjusted by applying a sequential Bonferroni correction for multiple comparisons (Rice, 1989). To check the presence of null-alleles, we used Micro-Checker 2.2.3 (Van Oosterhout et al., 2004).

The number of alleles ranged from eight to 29, the unbiased expected heterozygosity varied from 0.724 (*Stiz135*) to 0.952 (*Stiz133*) for all populations, except from one population (EAM) that only presented one monomorphic loci (*Stiz135*) (Table 2). In the three populations analyzed, no significant deviation of HWE in terms of heterozygosity deficiency was detected for any loci, only when populations were analyzed individually (i.e., *Stiz123* – SWA, *Stiz112* and *Stiz154* –

SAM and *Stiz112*, *Stiz123* and *Stiz138* – EAM) (Table 2). For the gametic disequilibrium, no pairs of nSSR loci were found to be in significant genotypic disequilibrium after Bonferroni correction ($p < 0.0001$). The presence of null alleles was detected in at least one locus of all nSSRs and null allele frequency values ranged from -0.4731 (*Stiz112* – EAM) to 0.2674 (*Stiz135* – SWA) (Appendix 3). Cross-amplification of polymorphic nSSRs was tested in six individuals of *S. inaequilaterum* and *S. perforatum* using the same PCR conditions described above. All nSSR markers successfully amplified for *S. inaequilaterum* and *S. perforatum* (Table 3).

CONCLUSIONS

We developed and characterized nine nuclear microsatellite markers for *S. riparium* that showed high polymorphism. These molecular markers will be useful for further investigations of the population genetic structure and mating system patterns within the genus. Due to the high rate of cross-amplification (100%), these nuclear microsatellite markers will be useful for genetic studies involving *Stizophyllum* as a whole.

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TABLE 1. Characteristics of the nine nuclear microsatellite loci developed for *Stizophyllum riparium*.

Locus	Repeat Motif	Primer Sequences (5'-3')	T_a (°C)	Allele size range (bp)	GenBank accession no.
<i>stiz63</i>	(AG) ₁₄	F: FAM - CGTGAGGAAAAGGGCTGTTT R: CTTGTGGCGTGATATGGTC	48	92 - 119	KP863502
<i>stiz104</i>	(AC) ₁₇	F: JOE - GGTATTACTCATGCCATGC R: TGCGCTAAAATCTAGGATGCT	51	109 - 129	KP863504
<i>stiz112</i>	(TC) ₁₆	F: JOE - TCTGCTTGAACCTGTGACTCA R: AGTACTTAAATCGAGCGAGCG	48	79 - 90	KP863505
<i>stiz123</i>	(TC) ₁₆	F: JOE - GCCAAAGCCCTTTCTACA R: ACAAACATAGCACACACAAG	51	94 - 116	KP863506
<i>stiz133</i>	(CTT) ₁₁	F: FAM - ACAGATGGTTGTGGGTTTGA R: GGTGATGAGACCCCTTTTCT	51	88 - 126	KP863507
<i>stiz135</i>	(CTT) ₉	F: FAM - TCAATTTCTCCACACCTCGC R: CAGGAATTGGTGCATGTCATG	51	91 - 102	KP863508
<i>stiz138</i>	(CTT) ₈	F: FAM - ACCCTCGACATGTTCTCTCC R: GTGACAGCAAGTGAAGAGCA	51	93 - 124	KP863509
<i>stiz154</i>	(CTC) ₁₁	F: FAM - CCATTGCTGTTCAATTCTAC R: CATTCTTTTACGTTCTTGGC	48	99 - 117	KP863510
<i>stiz156</i>	(CTC) ₈	F: JOE - TGCTGCTCTTCACTTCAGGA R: CTCATGTGCGGTGGTTGC	48	88 - 111	KP863511

Note: T_a = annealing temperature.

TABLE 2. Genetic variation of the nine polymorphic nuclear microsatellite markers in three populations of *Stizophyllum riparium*^a.

	SWA (n= 17)					SAM (n= 22)					EAM(n=18)					ALL (n=57)			
Locus	A	Ae	Ho	He		A	Ae	Ho	He		A	Ae	Ho	He		A	Ae	Ho	He
Stiz63	1		0.37	0.54			13.63	0.72	0.94	**	1		1.00	0.87		2		0.71	0.93
	0	2.949	5	4	***	17	4	7	8	*	3	6.680	0	5	***	9	13.370	9	3
Stiz10			0.68	0.80			10.37	1.00	0.92	**			0.61	0.73		1		0.79	0.90
4	5	3.125	8	8	***	15	6	0	6	*	8	3.522	1	7	***	7	9.242	6	0
Stiz11			0.75	0.78				0.90	0.78				0.80	0.55				0.80	0.80
2	4	2.338	0	2	**	6	4.324	0	8	ns	3	2.174	0	9	ns	8	4.812	4	0
Stiz12	1	10.88	0.56	0.88				1.00	0.91	**	1		1.00	0.89		2		0.95	0.95
3	4	9	3	3	ns	15	9.025	0	3	*	1	6.857	0	1	ns	2	16.667	6	1
Stiz13			1.00	0.90			13.16	0.90	0.94	**	1	14.45	1.00	0.95		2		0.83	0.95
3	9	4.983	0	2	***	18	4	5	7	*	9	0	0	9	*	7	17.742	6	2
Stiz13			0.94	0.91				0.36	0.74	**			0.00	0.00		1		0.17	0.72
5	3	1.446	4	6	***	10	3.639	4	2	*	1	1.000	0	0		1	3.536	0	4
Stiz13			0.88	0.63				1.00	0.89	**	1		0.94	0.88		2		0.85	0.89
8	9	5.885	2	6	**	14	8.018	0	7	*	0	6.964	1	2	ns	2	8.986	2	7
Stiz15			0.88	0.80				1.00	0.90				0.41	0.72		1		0.79	0.88
4	7	4.091	9	6	***	12	8.417	0	2	ns	7	3.400	2	7	***	5	8.379	6	9
Stiz15	1		0.68	0.82				1.00	0.81	**			0.88	0.80		1		0.88	0.88
6	1	8.127	8	9	***	8	4.819	0	3	*	9	4.500	9	0	***	5	8.191	9	6
Mean	8	4.870	0.75	0.79	-	12.	8.380	0.87	0.87	-	9	5.505	0.73	0.71	-	1	10.103	0.75	0.88
			3	0		8		7	5				9	4		8		8	1

Note: A = number of alleles; Ae = number effective alleles; Ho = observed heterozygosity; He = unbiased expected heterozygosity; n= sample size for each population,

^a Voucher and locality information are provided in Appendix 2.

Statistically deviation from HWE is indicated as * (P < 0.05), ** (P < 0.01), *** (P < 0.001) and ns= not significant.

TABLE 3. Transferability of the nine nuclear microsatellite loci developed for *Stizophyllum riparium* across two species of the genus.

Locus	Repeat motif	<i>S. perforatum</i>	<i>S. inaequilaterum</i>
<i>stiz63</i>	(AG) ₁₄	+	+
<i>stiz104</i>	(AC) ₁₇	+	+
<i>stiz112</i>	(TC) ₁₆	+	+
<i>stiz123</i>	(TC) ₁₆	+	+
<i>stiz133</i>	(TC) ₁₆	+	+
<i>stiz135</i>	(CTT) ₁₁	+	+
<i>stiz138</i>	(CTT) ₉	+	+
<i>stiz154</i>	(CTT) ₈	+	+
<i>stiz156</i>	(CTC) ₈	+	+

Note: successful amplification (+) evidenced by the occurrence of distinct single band on sequencing gel.

APPENDIX 1. Characteristics of 213 nuclear microsatellites markers designed for *Stizophyllum riparium* including sequence name, repeat motif, primer sequence, estimated annealing temperature, and fragment size

Sequence name	Repeat Motif	Primer sequence (5'–3')	T_a (°C)	Fragment size (bp)
<i>stiz46</i>	(GT) ₁₄	F: GCGATACGAGAGAGAAAACT R: TCCTCTTAACCTAGAAAACTCC	55.05 54.57	150
<i>stiz48</i>	(GT) ₁₅	F: ACATCATGGGCTGGATCTA R: TGAACCTCCGTTGTACTTCTC	55.09 54.78	156
<i>stiz50</i>	(GT) ₁₆	F: GCCTAAAACCCAACAAAC R: TGACAATTCAACTACAGAGG	51.82 52.55	124
<i>stiz51</i>	(GT) ₁₇	F: GATTATGGGCAATTAATCGC R: GTGTTTTCAGGTTCTCTCTC	52.85 53.59	132
<i>stiz52</i>	(GT) ₁₅	F: AGTTGGGTTACAGTTTGTT R: ACTCCGTTGTACTTCTCAAGA	55.6 56.35	144
<i>stiz54</i>	(GT) ₁₁ (GA) ₁₀ (GT) ₁₁ (GA) ₇	F: GGCTCCCTCACGCATATACA R: AGACGCTCGCTCACTGAAAT	59.32 59.76	166
<i>stiz55</i>	(GT) ₁₃	F: CCTGCTCTTTCTACTACAACA R: CTCGTGCAGTGATCGTAG	54.99 55.03	158
<i>stiz56</i>	(GT) ₂₀ (GA) ₁₂	F: TCAGTTCATGTTTGCCTGTG R: CAACCCCTTTGCTTCAACT	56.55 55.91	121
<i>stiz58</i>	(GT) ₁₅	F: TCTACAATTGCAGGTACAG R: GATGATGAGGAAATAAGGGG	51.93 52.78	150
<i>stiz60</i>	(AG) ₁₂	F: AACTCAACTCAACTGGCCCA R: TGCATGGGGTTAGACGAATAGT	59.45 59.23	139
<i>stiz61</i>	(AG) ₁₁	F: TCATGGTACTTCTTGTCTGA R: TATGATTTAGCTGCCCCTC	53.31 53.96	150
<i>stiz62</i>	(AG) ₁₁	F: GCATAAAGTAGCAAAGGAAAGG R: TATCCACCACTCGTTATCCC	55.69 56.12	132
<i>stiz63</i>	(AG) ₁₄	F: CGTGAGGAAAAGGGCTGTTT R: CTTGTGGCGTGGATATGGTC	58.68 58.7	120
<i>stiz67</i>	(AG) ₁₃	F: CCGTGCAATGTTCACTGA R: TGGAGCAATGGTTTCTTTCT	55.61 55.14	156
<i>stiz69</i>	(AG) ₁₃	F: TTACAATGAGTGGAGTGTC R: GCTACGAAGATAGGATTTTC	51.85 51.22	130
<i>stiz70</i>	(AG) ₁₈	F: TTTGTGTGAAATCGGAGCA R: CTTTTATTTCTTTCCCCTCCGT	55.35 56.33	104
<i>stiz72</i>	(AG) ₁₅	F: CAATAAGTTGCCATGATACACCC R: GACACATTCCTCCCAACC	57.81 57.73	154
<i>stiz73</i>	(AG) ₁₇	F: GAGAAAGCAAGAAAAGATGGC R: CTTGACAATCTCACACATTCTC	55.76 55.14	105
<i>stiz75</i>	(AG) ₁₄	F: AGAACTTTAGAACACAAGGGG R: TCATATTGCACGAGTCAAGG	55.42 55.57	104
<i>stiz76</i>	(AG) ₁₅	F: TTAGTTGCAATTGGATGGGA R: TCGTTGTCTATCTCCCCAC	54.89 55.84	116

<i>stiz80</i>	(AG) ₂₅	F: GTGAAGATGGACAAAAGAAG R: CATATTGTCTCTCCCTGTTG	52.1 53.1	168
<i>stiz85</i>	(AC) ₁₁	F: TGTGCAACCTTGTCTATG R: TGTGCAACCTTGTCTATG	52.14 51.21	104
<i>stiz86</i>	(AC) ₁₅	F: CCATGTTTCAGAGTCCAGCA R: GAAGTCATACTACTGCGCGT	57.81 57.81	102
<i>stiz89</i>	(AC) ₁₀	F: TGATTCTGAATGTGTAGGCCA R: ATCCATGCAAGACTGACCAC	56.93 57.88	128
<i>stiz90</i>	(AC) ₁₈	F: TCTCTTCCTTTTCTCTAAGCTC R: GTGCATTTTCACCATTACCTCT	56.65 56.95	100
<i>stiz91</i>	(AC) ₁₇	F: CTTTCATGGAAGTCAACAATTGCA R: TGTGGGGTTTAATGTTTGTGTC	57.49 58.2	146
<i>stiz92</i>	(AC) ₁₅	F: CGCACACAACACAAGTCA R: AGAGAGAGAGATGAAAGCTTTG	56.21 55.57	157
<i>stiz97</i>	(AC) ₁₂	F: GGATAGCTCTTAAATTCGATG R: GGCAGTATCTTCAAGTCCAA	54.52 55.06	114
<i>stiz98</i>	(AC) ₁₁	F: GCAATAAATGGTCCCCGAA R: TCATGGACAAAGGCTGCTTT	58.23 58	125
<i>stiz99</i>	(AC) ₁₂	F: CCTTCCCGTCGTCTCTGTT R: AGGAGAGAAAATGGGGAGTAGAA	59.03 58.3	114
<i>stiz100</i>	(AC) ₁₅	F: TGGATTGTGGTTTTATGCGC R: GGGGAATGAATTTGCTTGGT	57.35 56.55	109
<i>stiz101</i>	(AC) ₁₃	F: TGGGTCTCATGTATCGCTT R: GTACAAACCCTCACCATAATCT	55.81 55.52	150
<i>stiz102</i>	(AC) ₁₅	F: TGGTCAAAAGTGCGAACAAG R: AAGTTGAATCTTCGAGGGCT	57.16 56.84	150
<i>stiz104</i>	(AC) ₁₇	F: GGTTCATCACTCATGCCATGC R: TGCCTTAAATCTAGGATGCT	58.29 57.52	135
<i>stiz105</i>	(AC) ₁₅	F: CAACGAGTTTCAGAGGCACA R: GAAATGGGTGGCAGTGTGTA	58.42 58.1	129
<i>stiz108</i>	(TC) ₁₅ (AC) ₈ (TC) ₁₂	F: CTCTTGATAATACCAGCAA R: ACACCGAGATAGAGAGAGA	53.36 53.48	158
<i>stiz109</i>	(TC) ₁₁	F: TCTGGCATATTTTGGTTCTGGT R: TATGTGGGGCGGAAAGATCT	57.95 58.49	106
<i>stiz112</i>	(TC) ₁₆	F: TCTGCTTGAAGTTGTGACTCA R: AGTACTTAAATCGAGCGAGCG	57.46 58.28	114
<i>stiz113</i>	(TC) ₁₄	F: TTCTTACGCAGGCATATACG R: AACCAAAGGAAACCAGAGAA	55.47 54.66	105
<i>stiz114</i>	(TC) ₁₇	F: GCAGCATAGCATACCGTACG R: GGAAGTTGTAATAGTTCATGCGCT	58.94 59.13	110
<i>stiz115</i>	(TC) ₁₃	F: GCTGTCCCAAATTTCTAACA R: ATTGCACTCATACACGCA	53.78 54.61	161
<i>stiz117</i>	(TC) ₁₇	F: CTTTCGGTGGGCCTATCGAT R: GGCGAGCGAGTGAGAGAA	59.61 59.12	108

<i>stiz119</i>	(TC) ₁₆	F: CTTTGCTCAGGAAAGGTGTGA R: AGTACTTAAATCGAGCGAGCG	58.42 58.28	116
<i>stiz120</i>	(CT) ₁₅	F: TCTGATTTTCTTTGGTGTGTCG R: TTCCACAAGCAAAGAGAGATTC	57.19 56.63	102
<i>stiz121</i>	(TC) ₁₄	F: CCCCAACTCAAACCTTCCTCA R: ACCAAAGGAAACCAGAGAATGA	57.34 57.55	100
<i>stiz122</i>	(TC) ₂₁	F: ACAAACCTCCACTTCACCCCA R: TCCGAGCTAAATCTTTTGTGCA	59.08 58.59	122
<i>stiz123</i>	(TC) ₁₆	F: GCCAAAGCCCTTTCTACA R: ACAAACATAGCACACACAAG	54.78 54.44	105
<i>stiz127</i>	(AGG) ₈	F: GGAGTCAAATTCAGCGGAGGA R: CACCAACCCCTCCACCAC	60.07 59.88	105
<i>stiz128</i>	(AGG) ₈	F: GGCACCCAGTTCATCCCAT R: CACTTGCAAGGCCACTGAG	59.69 59.05	108
<i>stiz131</i>	(CTT) ₈	F: TCCTCAATTTCTCTACTCCTC R: AGGTTGTGTTTCTTGACGGC	58.71 58.98	155
<i>stiz133</i>	(CTT) ₁₁	F: ACAGATGGTTGTGGGTTTGA R: GGTGATGAGACCCCTTTTCT	57.25 56.82	117
<i>stiz134</i>	(CTT) ₁₂	F: GAGATGTTCTTGAGATTTGCCTC R: GGAGGAGGAGGAGGAGGA	57.52 58.22	104
<i>stiz135</i>	(CTT) ₉	F: TCAATTTCTCCACACCTCGC R: CAGGAATTGGTGCATGTCATG	58.19 57.84	100
<i>stiz138</i>	(CTT) ₈	F: ACCCTCGACATGTTCTCTCC R: GTGACAGCAAGTGAAGAGCA	58.81 58.41	114
<i>stiz140</i>	(CTT) ₁₀	F: ATCGCAACAACACAACACA R: ACACAACCACACACCATAC	56.29 55.34	116
<i>stiz143</i>	(ACA) ₉	F: TGA CTACCGCCACTACTACC R: AGCATGTTTACAGTGGTGGTG	58.24 58.77	118
<i>stiz144</i>	(ACA) ₈	F: TTGGTTCCGAGAAAACACGA R: CGTGTTGTTTCATGTCATGCT	57.69 57.02	105
<i>stiz148</i>	(AGA) ₈	F: TCTTGGCTGGCTTATGTACAA R: ACTGCTTCTTCCTCCACTTTC	57.29 57.87	101
<i>stiz150</i>	(AGA) ₁₀	F: CCAATGCTGTATTCTTCTCTT R: TCCCTTTTGTGGCTTCTTTG	56.13 56.43	100
<i>stiz151</i>	(AGA) ₉	F: TAGTTCCTAGCCTCCTGAAC R: ATTACCTTTGTCACTAACTCCC	55.66 55.45	100
<i>stiz152</i>	(AGA) ₁₀	F: GACATGAGGCAGAAAACCGA R: GCATAGCGGCTGTATGTTGT	58.19 58.7	115
<i>stiz154</i>	(CTC) ₁₁	F: CCATTGCTGTTCAATTCTAC R: CATTCTTTTACGTTCTTGGC	52.55 53.2	161
<i>stiz155</i>	(CTC) ₈	F: TCCTACTACTGCTCATATTGCT R: GAGTAGCAGGAGGAGCAG	56.16 56.11	136
<i>stiz156</i>	(CTC) ₈	F: TGCTGCTCTTCACTTCAGGA R: CTCATGTGCGGTGGTTGC	58.95 59.44	114
<i>stiz157</i>	(CGC) ₈	F: CCTTCCTCCTCGTCCTCCT	59.69	100

<i>stiz158</i>	(CGC) ₈	R: CGGAGGTGGTGGGAAGAGG F: GCCGCTAACACAACCATCG	59.02 59.58	113
<i>stiz159</i>	(TGT) ₈	R: CTCGGGTCAACTCGGGAAG F: TGTTGTTGTTGTCATCGTCGTC	59.78 59.72	107
<i>stiz162</i>	(ACT) ₉	R: TTGAATGAATTGGCGGGCTC F: ACTACATGACCACTACTACA	59.18 52.42	110
<i>stiz163</i>	(ATG) ₈	R: GGTGTAGCGATAATTATCCC F: ATGGACGGCTGAGATGCTT	53.06 59.09	110
<i>stiz164</i>	(CAT) ₈	R: GTCTGTCTTTCAACGCTGCA F: TCTTGTTTAGCATCACCAGCTG	59.06 58.92	105
<i>stiz165</i>	(CAT) ₁₀	R: GGAGGAGGAGATCAGGAAGC F: CTGCCTCCGAAGACTCTCAA	58.95 59.11	114
<i>stiz166</i>	(AAAG) ₇	R: CTGTTGCTCTAGTACTCTTCCCT F: AGAGGCATCACTTGGAGTATGA	58.98 58.62	127
<i>stiz167</i>	(AAAG) ₄	R: GGGAAATAAAGGAGGGAGTAGGT F: TGAGCTACAAACCCTTTACCA	58.33 56.85	101
<i>stiz168</i>	(AAAG) ₄	R: AGTGTATGTAGTGTCTGTGAGA F: GAATGACATGGCTGCTCA	55.99 54.99	111
<i>stiz169</i>	(AAAG) ₅	R: CGAAGCTTAGAGAGTGGAG F: GTGTATGCATTTTGGAGTGT	54.46 54.18	101
<i>stiz170</i>	(AAAG) ₅	R: CCAAAGCAAGAAGAAAGGAG F: TACTTGGGCACTACTTTGT	54.49 53.55	119
<i>stiz171</i>	(AAAG) ₄	R: GGCAAGCTATCAACTTCTTT F: GAATGACATGGCTGCTCA	53.87 54.99	111
<i>stiz172</i>	(AAAG) ₄	R: CGAAGCTTAGAGAGTGGAG F: CCTTAAGCTCTTTCTATTGTCC	54.46 54.28	132
<i>stiz173</i>	(AAAG) ₅	R: GAATTTGGTGTTATTGCGGA F: CCCCTGTCAGAATATCGGGT	54.57 58.58	134
<i>stiz174</i>	(AAAG) ₆	R: CAACACCCTCAGTTTCAGTCT F: GTGCATGAGTTTCAATGAAGCA	57.8 58.1	106
<i>stiz175</i>	(AAAG) ₄	R: GCCACAAGTGTCTCCATTT F: TGCAAACCAGCCATGAACAA	58.38 58.88	104
<i>stiz176</i>	(AAAG) ₄	R: GTTCATCAGTCACTTTGTTGACC F: GACATGGCTGCTCAAGAA	58.15 54.92	107
<i>stiz177</i>	(AAAG) ₄	R: CGAAGCTTAGAGAGTGGAG F: AAGTGGAAGTGGAAGATAAAGG	54.46 55.71	113
<i>stiz178</i>	(AAAG) ₄	R: CGGGTTATTAACATTTTGCACC F: AGCTGCCTGATACACTCCAT	56.56 58.5	100
<i>stiz181</i>	(AAAT) ₄	R: TTAGGACTAGTTGAAGGTTGCT F: CGGAGTTTCAGTCGTAGG	59.17 54.5	117
<i>stiz182</i>	(AAAT) ₄	R: GTAGTCACGACAAGCTCTAG F: AAATAGAAACATCACGGCGGT	55.08 58.29	103
<i>stiz183</i>	(AAAT) ₅	R: CCGCCGCGATATTTTCTGTAT F: ATAGAGTTTGTTAAGGGCAC	58.93 52.88	102
		R: AAGTTCTTCTGCAATGTCC	53.15	

<i>stiz184</i>	(AAAT) ₄	F: GCCCAATTAGCCCATCAAAC R: GGAGAAGAAGTCCGGGAGTC	58.89 59.19	161
<i>stiz185</i>	(AAAT) ₄	F: TCTATCCTAACCATGCATCA R: GCACTAATGGCTCTAAGAG	53.18 52	135
<i>stiz186</i>	(AAAT) ₄	F: TCACGATCGAAAATCCATCAAC R: GGATGAAGGAAAGAGCTATGAGA	57.14 57.46	113
<i>stiz188</i>	(AAAC) ₆	F: CGAAACCACAACCTGCAACT R: TGAGTATGAACCTGGGCGTT	59.26 59.02	113
<i>stiz189</i>	(AATG) ₅	F: GAAAGAGGGAAATAAGAGAGAG R: GTGTAATGGTTGATGCTGG	53.39 54.06	108
<i>stiz190</i>	(AACG) ₄	F: GGAGTGATAAATGAATTCCTCC R: GGAATCGGTATGTGTGT	54.31 53.93	125
<i>stiz191</i>	(AACG) ₄	F: GCAGGAGTGATAAATGAATTCC R: GGAATCGGTATGTGTGTAAG	55.22 55.93	127
<i>stiz192</i>	(AACG) ₅	F: AAATGAATTCCTCCTCTTGG R: GGTGTGTGTGTAAGTTTGT	52.8 53.41	114
<i>stiz193</i>	(AACG) ₆	F: TATCAAGGTGAACAGACAGACG R: GGAATCGGTATGTGTGTAAGT	57.83 57.87	100
<i>stiz194</i>	(AACG) ₄	F: GGAATGCAGGAGTGATAAATGA R: TCTGAAATATTTGCCCCGGA	56.21 56.89	171
<i>stiz195</i>	(AACG) ₄	F: GAATGAATTCCTCCTCTTGG R: CGGTATGTGTGTAAGTTTGT	53.34 53.59	111
<i>stiz196</i>	(AACG) ₄	F: GCAGGAGTGATAAATGAATTCC R: GGAATCGGTATGTGTGTAAG	55.22 56.14	128
<i>stiz197</i>	(AATC) ₄	F: CGCATATCGTTAGCTAATG R: TGACCTATATTTGAGTGTGG	51.43 51.98	115
<i>stiz198</i>	(AATC) ₄	F: CACAATCGTTCGAACACT R: CACATCATACGTGGTAATTG	53.04 52.11	141
<i>stiz199</i>	(AATC) ₄	F: CGATATAAATCCTTATCCCTCC R: TAAGATGTGACATGTGGTTG	53.1 53.23	103
<i>stiz200</i>	(AATC) ₄	F: CGGTAGCTAATGAATGACTG R: GATGACCTATATTTGAGTGTGG	53.64 54.08	110
<i>stiz201</i>	(AATC) ₄	F: TTGCTCTTGGGTAAACATTAC R: CGCCCCTATGCCTATGAAC	57.15 57.77	100
<i>stiz202</i>	(AGCT) ₄	F: TTAGTGGAAGCAGAGATG R: CTATAAATACGCAGGCTAAG	51.91 51	109
<i>stiz203</i>	(AGCT) ₄	F: TGTCCCGAGCTAAATTTGAGT R: TGGAGGAAAGTGAGGGGTC	57.3 58.28	136
<i>stiz204</i>	(GGAC) ₄	F: CATTTTCGTTTGTCCGTCCG R: AGCTCCCTACATTCATTGAAGT	58.05 57.15	109
<i>stiz205</i>	(GGAC) ₄	F: ACTGGGTGGCGGACATAAC R: TTATGTCCGTGTCCGTCCG	59.7 59.49	114
<i>stiz206</i>	(GGAC) ₄ ...(GGAC) ₃	F: TCCATCCATATATCCGCATG R: TCGAAACGCATATATCGGG	54.54 55	112

<i>stiz208</i>	(GTTT) ₄ ...(CTTT) ₄	F: TGGCTTTCTTGTCTTAGGTGCA R: AGAGAACGCCCCAACCCA	60.16 60.85	103
<i>stiz209</i>	(GTTT) ₄	F: ACTTGGATTCTCTGTGCTCT R: CAGTTTGTGCTGTTGGGT	56.16 55.7	140
<i>stiz210</i>	(GTTT) ₄	F: TGGAGATAGGATTTTCAAGGGAC R: CCCTCCATTTTCACCCTTGA	57.63 57.4	107
<i>stiz212</i>	(CCCT) ₄	F: TCACAATCCTCTCTCTCTCGTC R: GACGTGCAAGGTTTCGATCC	58.72 58.92	103
<i>stiz213</i>	(CCCT) ₄	F: TGGAAGTTAGCATTAGGAGGG R: CTCAGATTCATATGCACATGGT	56.79 56.29	110
<i>stiz214</i>	(CCCT) ₆	F: TCCAGTGACGTTCAAATTC R: AACTCAACACTGTAAGGAGG	54.16 54.62	100
<i>stiz215</i>	(CCCT) ₄	F: AGCTTGGAGATGGAAGGTTT R: TGCTAACCTAGGACAATAAGCT	56.72 56.62	128
<i>stiz216</i>	(CCCT) ₄	F: ACGAGGGCAAGAACTGAAAG R: AGATACTGGAACCACACACAC	58.78 58.26	108
<i>stiz217</i>	(CCTT) ₄	F: GATTCGGAATTTGAACCATG R: GTTCGTGTCGTGTTATCG	52.92 53.76	123
<i>stiz218</i>	(AGAC) ₄	F: CAGCAGCCAAGAACTTTACTG R: AGCTTCAAAAGGGTCTGTCC	57.45 57.72	101
<i>stiz220</i>	(ATAC) ₅	F: ACGGTCAATAAACACACACACA R: TAGCTCAGGGTGTCGTTGAG	58.73 59.11	116
<i>stiz221</i>	(ATAC) ₄	F: ACGCTTCAATATACACACGGC R: TGCACGAATGAATGTATGCTGT	59.07 58.99	102
<i>stiz222</i>	(ATAC) ₅	F: ACGGTAACAATTCTCAGGATAC R: ATTGACCTTAGGCCACATTC	55.64 55.39	119
<i>stiz223</i>	(GAGT) ₄ (GT) ₁₄ (GA) ₉	F: CGTCTTTGACTTTGTTTTCG R: TCAGGTTTTCTTCATTCAGC	53.79 53.81	112
<i>stiz224</i>	(GAGT) ₄	F: TCGCGTTTGAGTTATCGATTGA R: GCATGTCAATCAATAAGTCCCTG	58.5 57.82	114
<i>stiz225</i>	(GAGT) ₄	F: CTCCACACACACACACACAC R: GCACTGAAGCATTATAGCGC	59.27 58.36	113
<i>stiz226</i>	(TCCC) ₇	F: ACTCTCTTTCTCTTTTCTCCCT R: AGAAATGTTACATGCGGGCA	57.99 58.17	113
<i>stiz227</i>	(TCCC) ₄	F: TGGAAGTTAGCATTAGGAGGG R: CTCAGATTCATATGCACATGGT	56.79 56.29	110
<i>stiz228</i>	(TCCC) ₆	F: CTGTATGTTTCCAGTGACGTTCA R: TCAACACTGTAAGGAGGGGC	58.94 59.31	106
<i>stiz229</i>	(CCCT) ₄	F: TAAGCTTGGAGATGGAAGG R: GCTCATCAATGTTGTCTTGT	53.8 54.2	111
<i>stiz230</i>	(TCCC) ₄ ...(TG) ₁₄	F: CCCCTTGTGTTTCGTAATGAA R: TGAGCTACACAAATGAGAGGT	55.67 56.66	154
<i>stiz231</i>	(CCCT) ₄	F: AGGTCACAATCCTCTCTCTCTC R: GCAAGGTTTCGATCCCCATTC	58.37 58.97	101

<i>stiz232</i>	(CCGT) ₄	F: AAACACACTAGCACCGGAGA R: CAACTGTTGGCACGAGATCC	58.95 59.2	159
<i>stiz233</i>	(CCGT) ₄	F: GGTCCGTCCGTTAGTGTTTT R: ACAACCGAAAATCAATGGCCT	58.13 58.75	105
<i>stiz234</i>	(CCGT) ₄	F: CTGGATGTAGGTTACACACA R: ATCATTTTCGATCCGTCCG	54.09 54.61	103
<i>stiz235</i>	(TTTA) ₄	F: TCAGAGACATAAGCAGAACT R: CAGTGCCAGAATGAGTTAAC	53.41 54.32	129
<i>stiz236</i>	(TTTA) ₄	F: ATCTCGGCCCTCTAAATTGT R: CCTCCCATTCTTACGAAGG	56.3 55.53	113
<i>stiz237</i>	(TTTA) ₅	F: AGGGTTCTTAATTCGGAGTTC R: TGATGGGGAGTACAAGACGA	57.78 57.77	103
<i>stiz238</i>	(TTTA) ₄	F: GTGGACCATTTTGAATTTGCGT R: GGCACAGGGAAAATGACTAACA	58.61 58.85	119
<i>stiz239</i>	(ATTT) ₄	F: TACACAATTAGCAACTTGGC R: AAACATAGGTAGCTAGGTGG	54.2 53.83	110
<i>stiz241</i>	(TTTA) ₄	F: CTTGAGCCAGCTTTCAAAT R: AATGTGAATCTTCCAGGTCT	53.84 53.92	100
<i>stiz242</i>	(TTTA) ₆	F: ACTGATCCTTTGACTGAGAACT R: CTCCTGTCAAGCTCAATCGT	56.76 57.62	126
<i>stiz243</i>	(TTTA) ₄	F: AAATTAGTAAGGAGACCAGC R: CATTGAGAAAGAGGTGATTG	52.27 51.86	155
<i>stiz244</i>	(TTTA) ₄ ...(AAAT) ₄	F: CATCGAAAATGCTGAAAGAC R: TGTGAAAGTTGAGGAGACAT	53.29 54.2	108
<i>stiz245</i>	(TTGA) ₄	F: GTGTAGGTTGCGCGTTGATT R: GTGTAGGTTGCGCGTTGATT	58.56 58.93	123
<i>stiz246</i>	(TTGA) ₄	F: CGCATCTGAGCTACCTATTGAT R: AGTCCCTGTCAATGAATCCCT	57.81 58.43	151
<i>stiz247</i>	(TTGA) ₄	F: GCATCCGAGCTACTTATTGA R: AAGTCCCTGTCAATGAATCC	55.06 55.02	132
<i>stiz248</i>	(TTGA) ₄	F: CCGAGCTACTTATTGATTGA R: TGATCATTAAGTCCCTGTCA	52.53 53.34	122
<i>stiz249</i>	(TTGA) ₄	F: TCTGCCCCATGCCTATGAA R: AGTCCTTGATTCACATGCTCA	57.88 57.29	122
<i>stiz250</i>	(TTGA) ₄	F: GAGTGAGTGTTAATCGAATGAG R: AAGTCCCTGTCAATGCATTT	54.72 55.5	111
<i>stiz251</i>	(TTGA) ₄	F: GGCTGCGTTGATTTTATCCG R: AGTCCCTGTCAATGAATCCCT	57.89 58.43	166
<i>stiz252</i>	(TTGA) ₄	F: ATCGGTTGGTCCCCTGAAG R: GTGACCACTCAGAGGGAACA	59.01 58.95	111
<i>stiz253</i>	(TTGA) ₄	F: GAACGCCACATTGCACGT R: GACATGCCGTAATGCCCAAC	59.36 56.9	116
<i>stiz255</i>	(TTGA) ₄	F: ATCGAACACCACATTGCACG R: CCGTAATGCCCAACCAAGAC	59.48 59.19	109

<i>stiz256</i>	(TTGA) ₄	F: AAATCGCATCTGAGCTACCT R: AGTCCCTGTCAATGAATCCC	56.71 56.89	128
<i>stiz257</i>	(TTGA) ₄	F: GAGTGAGTGTTAATCGAATG R: GTCAATGAATCCCTGATCAT	51.78 52.69	117
<i>stiz258</i>	(TTGA) ₄	F: GAGATCTCGATTGAACGCC R: ATCCTCTAAGAAGTTCGTGGG	55.93 56.83	100
<i>stiz259</i>	(TTGA) ₄	F: TATCCGCCCTATGCCTATG R: GCTCTTGGGTAAACATTCACTCA	58.5 58.93	102
<i>stiz260</i>	(TTGA) ₄ ... (TTGA) ₄	F: GAATGAAGGAGTGTGTGAACC R: AGAATGCATGTCGATCGTTAAG	56.82 56.96	100
<i>stiz261</i>	(TTGA) ₄	F: AGGTTGCGCGTTGATTGTAT R: CTCTCTTGGGTAAACATTCACTC	57.6 56.88	120
<i>stiz262</i>	(TTGA) ₄	F: GATCTCGATTGAACGCCACA R: CCGTAATGCCCAACCAAGAC	58.36 59.19	120
<i>stiz263</i>	(TTGA) ₄	F: GAGATCTCGATTGAACGCC R: ACCCTCTAAGAAGTTCGTGG	55.93 56.58	102
<i>stiz264</i>	(TTCA) ₄	F: CACTCTCAACACACAGAC R: CTGTTCCACTGATTATGAGA	52.61 52.01	101
<i>stiz265</i>	(TGTA) ₇	F: CCAGCAGAGAACATAAGTA R: GAGAGGGATTTAAGTCTTTC	51.29 50.56	117
<i>stiz266</i>	(TATG) ₇	F: CGAACCATGTTTGTGCGGG R: AATGCGGGCGATGATTGTAC	59.13 59.06	113
<i>stiz267</i>	(TATG) ₄	F: TGTATCCACTCACAAGATTG R: CACCAACACACACACATA	52.62 52.41	109
<i>stiz268</i>	(TATG) ₆	F: ATCTTGCTCCAATTCTTCAC R: GATGATGTCAAATGCATGC	53.57 53.03	109
<i>stiz269</i>	(TATG) ₄	F: GTGTCGCCTCATATTTAGTCTC R: GTCCAAAACTCGGTCACAT	56.44 55.45	125
<i>stiz270</i>	(TATC) ₄	F: GTTGTGCTAGCTAATGTCAGTA R: CATGCCGCCATCTATATG	52.69 52.79	128
<i>stiz271</i>	(TCTG) ₄	F: AAGGCCAGTGAGATTGAGGT R: TATCAGGGGAAGAAGGCCTG	58.63 58.49	110
<i>stiz272</i>	(TCTG) ₅	F: CAATCAAATGTCTCTGTCTC R: CTGACCAGTCTTGTCTAAA	51.62 52.48	111
<i>stiz273</i>	(TCTG) ₅	F: TCATCGGACTTTCTGTTCTGT R: GTGTAAAATTGTCAAGTGTCTGTC	56.97 56.2	118
<i>stiz274</i>	(AAAAG) ₄	F: TGAGTGGGAGATTGGAAGGT R: CCACTCTTTACGATGCCTTGT	58.65 58.3	104
<i>stiz275</i>	(AAAAG) ₄	F: CCAATTTCCACAATAGCACA R: CGACTTCCAGATAGTTTTGC	54.15 54.44	141
<i>stiz276</i>	(AAAAG) ₄	F: GTGGATTATCTACTGGGAAA R: GGCTTATGATTTGGGACA	51.63 51.77	115
<i>stiz277</i>	(AAAAG) ₄	F: AAGTCGTCTTTCATTACCAG R: CATTGCATGGGGAAGATT	52.97 52.48	100

<i>stiz278</i>	(AAAAT) ₄	F: TGATGCTTCTGTAGGCAC R: TCCGCCTGACAAATTAAGAT	54.28 54.74	113
<i>stiz279</i>	(AAAAT) ₄	F: GTATTGACATTTCCACTCCA R: TGAAAATGGAGAGAGGGG	52.89 52.97	152
<i>stiz280</i>	(AAAGG) ₄	F: TCGAGGAAGAGGAGATGAAC R: TGGATAGCTGACTTTCTTCCT	56.08 56.07	115
<i>stiz282</i>	(GGGTT) ₄	F: CCATTTTGGCTTTGGGTCCA R: CCGATCTGTGTCGGTTCAG	58.94 57.96	151
<i>stiz283</i>	(GGGTT) ₅	F: AGGTTGGGTTAGGGTTGGAC R: GGTGGGTTTCGTGTCAGGTTA	59.22 59.61	110
<i>stiz284</i>	(AGAAG) ₄	F: CCCACTTTCTACTCATGTTGCC R: TCACACAAACTTGCTCTTGGT	59.25 58.28	113
<i>stiz285</i>	(AGAAG) ₄	F: AGTGTTTCGATTAGGAAACCC R: CAACAACTTGCTCTTGGTT	55.07 54.91	123
<i>stiz286</i>	(AGAAG) ₄	F: CTACTCATGTTGCCTTTAGT R: CTCACACAACTTGCTCTT	52.62 53.43	106
<i>stiz288</i>	(TTTTA) ₄	F: ACACTCTCATAGGAAATTCGGAC R: AAACCAAGACCCACCAAACA	57.99 57.76	150
<i>stiz289</i>	(TTTTA) ₄	F: GGTGTCGCTACTTTGATGA R: GGAATCCCATGTTGATCTCTTCT	57.73 57.95	106
<i>stiz290</i>	(TTTTC) ₄	F: CTATGAACCCTAACTCTTTG R: CGTTCATACACAGTTTGG	50.57 51.1	124
<i>stiz291</i>	(TTTG) ₄ ...(TTTTC) ₄	F: TGGCTTTCTTGTCTTAGGTGCA R: AGAGAACGCCCAACCCA	60.16 60.58	103
<i>stiz292</i>	(TTTCG) ₄	F: CATAAGCAGGAACACGGTGG R: GAGCAAAATACTCTGTCAAGGGA	58.91 58.42	107
<i>stiz293</i>	(CCAAG) ₄	F: TGGGAAGTATTCATCAGAGTTCG R: AGGTTTCGACTCAATTCCACAC	57.99 58.23	150
<i>stiz295</i>	(GTCAA) ₅	F: CGAGTTCTCTATATTCACAGCCC R: GACTTCCCGTAGCATGGAAAC	58.45 58.99	151
<i>stiz297</i>	(CTCTT) ₄	F: TCCTCACACAACTTGCTCT R: GTGCTTGATTATGAAACCCACA	56.99 57.22	127
<i>stiz298</i>	(CTCTT) ₅	F: CCTCACACAACTTGCTCTT R: TGACTTTAGTGCTCGATTAGGA	56.18 56.67	140
<i>stiz299</i>	(CTCTT) ₅	F: GTCTTCATTTTCCTCGCACA R: TGAAACCCACATTCTACTCATG	56.36 56.08	131
<i>stiz300</i>	(CTCTT) ₅	F: GTGTCTTCATTTTCCTCACAAC R: TGACTTTAGTGCTCGATTAGGA	55.85 56.67	154
<i>stiz301</i>	(CTCTT) ₄	F: TCCTCACACAACTTGCTCT R: GTGCTTGATTATGAAACCCACA	56.99 57.22	132
<i>stiz302</i>	(CTCTT) ₄	F: GTCTTCATTTTCCTCACAAC R: CATTCTACTCATGTTGCTGT	52.41 53.32	118
<i>stiz303</i>	(CTCTT) ₄	F: AAGCCTGCTCTTGGTTTCC R: GTGCTTGATTATGAAACCCACA	57.96 57.22	118

<i>stiz304</i>	(CTCTT) ₅	F: CGCACACAAACTTGCTCTTG R: AGGAAACCCACATTCTACTCATG	58.53 58.15	120
<i>stiz305</i>	(CTCTT) ₄	F: GGCATTGTGTCTTCATTTTCCTC R: CGCATTCTACTCATGTTGCCT	58.51 58.72	128
<i>stiz306</i>	(CTCTT) ₇	F: GCTATAAATAGGAGGCATTGTG R: TATGAAACCCGCATTCTACTC	54.75 55.59	150
<i>stiz307</i>	(CTCTT) ₄	F: TCACACAAACTTGCTCTTGGT R: GTGCTCGATTAGGAAACCCAC	58.28 58.99	124
<i>stiz308</i>	(CTCTT) ₄	F: GTGTCTTCATTTTCCTCACA R: CATTCTACTCATGTTGCCTT	53.45 53.28	120
<i>stiz309</i>	(CTCTT) ₄	F: AAAGTTGCTCTTGGTTTCCA R: AGGAAACCCACATTCTACTCA	55.67 56.27	108
<i>stiz310</i>	(TCTCC) ₄	F: CCTCTCCACCTCCACCACAA R: GGAGAGGAGAGACGGCGG	61.13 61.21	105
<i>stiz311</i>	(TGGGGG) ₄	F: TTTTCACTGAGGCTGATTGGT R: ATTCTCCAAAGCATGTTCTGGA	57.77 57.96	100
<i>stiz312</i>	(AACACC) ₄	F: TGGAATATCATAACCTGCACAG R: CATTGCCCTTGGATTAGTGT	55.94 55.69	109
<i>stiz314</i>	(TGGGCG) ₄	F: CGTGGGAAAGGGGAGGGG R: CACCATCATCGCCCACAACA	61.73 60.96	108
<i>stiz315</i>	(CCGGGA) ₄	F: GTCCCGTCCTGATTATTTCCG R: CGGTTTTGAATCGGGACACA	58.52 58.77	107
<i>stiz316</i>	(GGGCGT) ₄	F: CGTGGGAAAGGGGAGGGG R: CACCATCATCGCCCACAACA	61.73 60.96	108
<i>stiz317</i>	(GAAGCC) ₄	F: GCAAGATAGGTACAGTGACAGC R: TTCATCATACCTCCTAGCGGC	58.81 59.38	100
<i>stiz318</i>	(ACACCA) ₄	F: TGGAATATCATAACCTGCACAG R: CATTGCCCTTGGATTAGTGT	55.94 55.69	109
<i>stiz319</i>	(TCTCCT) ₄	F: CGGATGTCCTAGTGTTCGG R: GAGGAGGAGGAGGAGATTGAAT	58.08 58.41	119
<i>stiz320</i>	(CAGGTT) ₄	F: GCAGTCCCCATAAACGGTC R: TGGATTATAAGGAGCCTGAACC	58.22 57.29	124

APPENDIX 2. Voucher and locality information for all individuals of *Stizophyllum* sampled.

Species	Population Code	Locality	Geographic coordinates	Voucher
<i>Stizophyllum riparium</i> (Kunth) Sandwith	CAZ	Brazil, Amazonas, Manaus	3°00'14"N, 59°55'07"W	A. Nogueira 170*
	SWA	Peru, San Martin, Tocache Nuevo	08°11'02"S, 76°30'43"W	J. Schunke-Vigo 12404 ⁵
	SWA	Brazil, Acre, Rio Branco	09°58'29"N, 67°48'36"W	Albuquerque 13980 ³
	SWA	Brazil, Acre, Senador Guilmard	10°03'59"S, 67°37'06"W	M. Beyer 375
	SWA	Brazil, Acre, Senador Guilmard	10°03'59"S, 67°37'06"W	M. Beyer 375
	SWA	Brazil, Acre, Senador Guilmard	10°03'59"S, 67°37'06"W	M. Beyer 375
	SWA	Brazil, Acre, Senador Guilmard	10°03'59"S, 67°37'06"W	M. Beyer 375
	SWA	Brazil, Acre, Senador Guilmard	10°03'59"S, 67°37'06"W	M. Beyer 375
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Epitáciolandia	10°48'36"S, 68°46'09"W	M. Beyer 383
	SWA	Brazil, Acre, Bujari	9°58'29"S, 67°48'36"W	M. Beyer 383

EAM	Brazil, Pará, Parauapebas	06°10'12"S, 50°21'02"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°09'46"S, 50°20'52"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°10'13"S, 50°21'04"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°09'45"S, 50°20'51"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°09'48"S, 50°20'52"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°10'08"S, 50°21'01"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°10'04"S, 50°21'02"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°10'04"S, 50°21'02"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°10'02"S, 50°21'02"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°09'46"S, 50°20'51"W	M. Beyer 295
EAM	Brazil, Pará, Parauapebas	06°09'48"S, 50°20'53"W	M. Beyer 301
EAM	Brazil, Pará, Parauapebas	06°09'36"S, 50°25'21"W	M. Beyer 302
EAM	Brazil, Pará, Parauapebas	06°04'10"S, 50°14'46"W	M. Beyer 303
EAM	Brazil, Pará, Parauapebas	06°03'55"S, 50°12'57"W	M. Beyer 303
EAM	Brazil, Pará, Parauapebas	06°04'14"S, 50°14'44"W	M. Beyer 303
EAM	Brazil, Pará, Novo Repartimento	04°19'57"S, 49°56'57"W	M. Beyer 315
EAM	Brazil, Pará, Altamira	03°12'20"S, 51°14'56"W	M. Beyer 317
EAM	Brazil, Pará, Moju	03°50'25"S, 46°06'40"W	M. Beyer 318
SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353

	SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brasil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brasil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brasil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brasil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brasil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brasil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	-	Brasil, São Paulo, Candido Mota	22°45'27"S, 50°22'06"W	JP. Souza 9703
<i>Stizophyllum perforatum</i>	-	Brasil, Paraná, Londrina	23° 34' 12"S, 50°57'42"W	LHM. Fonseca 105

(Cham.) Miers	-	Brasil Paraná Londrina	23° 34' 12"S, 50°57'42"W	LHM. Fonseca 103
	-	Brasil, Minas Gerais, Belo Horizonte	11° 54'S, 71° 22'W	Lombardi 2431 ¹
	-	Brasil, Rio de Janeiro, São Pedro de Aldeia	22°50'10"S, 42°06'13"W	JA. Kallunki s.n
	-	Brasil, Piaui, Eliseu Martins	08°05'27"S, 43°39'42"W	P. Martins & E. Nunes s.n. ⁴
<i>Stizophyllum</i> <i>inaequilateru</i> <i>m</i> Bureau & K. Schum.	-	Brasil, Acre, Marechal Taumaturgo	08°56'29"S, 72°47'33"W	LG. Lohmann 454
	-	Equador, Amazonia, Orellana	0°40'59" S, 76°24' W	H. Romero-Saltos 2831
	-	Peru, Ucayali, Pedro Abad	09°09'02"S, 75°47'20"W	J. Schunke-Vigo 15997
	-	Frech Guiana, Saul	03°37'22"N, 53°12'34"W	SA. Mori 24242 ²
	-	Peru, San Martin, Tocache	08°11'22"S, 76°30'57"W	J. Schunke-Vigo 14609 ³
	-	Brazil, Amazonas, Letícia	04°12'19"S, 69°55'58"W	A. Gentry 18302

Note: CAZ = Central Amazonia; EAM = East of Amazonia; SAM = South of Amazonia; SWA = South Western Amazonia.

^aMost specimens are deposited at the University of São Paulo Herbarium (SPF), São Paulo, Brazil, except from five samples (¹) that are deposited at the Missouri Botanical Garden Herbarium (MO), St. Louis, USA; one sample (²) that is deposited at the Muséum National d'Histoire Naturelle (P), Paris, France; one sample (³) that is deposited at the New York Botanical Garden Herbarium (NY), New York, USA; one sample (⁴) that is deposited at the Herbarium of the Federal University of Ceará (EAC), Fortaleza, Brazil; and, (⁵) that is deposited at the Herbarium of the Botanical Garden of Rio de Janeiro (RB), Rio de Janeiro, Brazil

APPENDIX 3. Null alleles (Oosterhoute correlation) of the three population of *Stizophyllum riparium*.

Locus	SWA	SAM	EAM
<i>stiz63</i>	0.2001	0.1068	-0.0964
<i>stiz104</i>	-0.0415	-0.055	0.0609
<i>stiz112</i>	-0.1509	-0.1064	-0.4731
<i>stiz123</i>	0.0272	-0.0652	-0.0928
<i>stiz133</i>	0.1314	0.0095	-0.038
<i>stiz135</i>	0.2674	0.2614	0
<i>stiz138</i>	0.1602	-0.0768	-0.0507
<i>stiz154</i>	-0.1285	-0.0701	0.2196
<i>stiz156</i>	0.0723	-0.1464	-0.1027
No. Loci	4	2	1

Note: Bold values correspond to loci with presence of null alleles

Considerações Finais

Essa dissertação apresenta uma filogenia molecular de *Stizophyllum* baseada em uma ampla amostragem de taxa e três marcadores moleculares, um nuclear (*pepC*) e dois plastidiais (*ndhF* e *rpl32-trnL*). Esta filogenia corrobora o monofiletismo de *Stizophyllum* e reconstrói cinco clados principais que são aqui reconhecidos como espécies: (i) *S. coriaceum* Beyer & L.G. Lohmann, restrito ao Estado do Pará, na Amazônia Oriental; (ii) *S. flos-ardeae* (Pitter) Beyer & L.G. Lohmann, distribuído pela América Central; (iii) *S. inaequilaterum* Bureau & K. Schum., distribuído pela Amazônia e América Central; (iv) *S. perforatum* (Cham.) Miers, distribuído pela Mata Atlântica e Áreas Secas do Brasil Central; e, (v) *S. riparium* (Kunth) Sandwith, distribuído por toda Bacia Amazônica. Uma sinopse atualizada é apresentada para o gênero, incluindo uma chave de identificação e breve caracterização de todos os taxa. No entanto, uma revisão taxonômica completa ainda é necessária.

A filogenia de *Stizophyllum* indica que *S. coriaceum* é irmã dos demais *Stizophyllum*, enquanto *S. flos-ardeae* é a segunda linhagem a divergir no grupo. As demais espécies reconhecidas no gênero, i.e., *S. inaequilaterum*, *S. perforatum*, e *S. riparium* fazem parte de uma politomia e o parentesco entre essas elas ainda não está totalmente resolvido, apesar da árvore datada sugerir que *S. inaequilaterum* é irmão de *S. perforatum*. A árvore datada do grupo também indica que *Stizophyllum* divergiu de *Martinella* durante o Eoceno, permanecendo ca. 20 Mya sem diversificar. Diversificação no grupo ocorreu durante o Mioceno, um período com perturbações geológicas intensas.

Por fim, marcadores de microssatélites (do inglês “Simple Sequence Repeats,” SSRs) de núcleo e cloroplasto foram desenvolvidos. Esse trabalho foi importante para demonstrar a característica polimórfica desses marcadores, os quais podem ser de grande utilidade para estudos futuros de filogeografia de *Stizophyllum*. Marcadores de microssatélites destacam-se entre os marcadores moleculares por representarem uma ferramenta poderosa para estimar parâmetros genéticos de populações. Enquanto que os nSSRs que são altamente polimórficos, codominantes e com herança biparental, os cpSSRs são uma molécula não recombinante e com herança uniparental, permitindo rastrear a história das populações através da diversidade de haplótipos. Por cona disso, os marcadores aqui desenvolvidos são complementares para a compreensão da estrutura genética em populações naturais. Estes marcadores servirão como base para estudar a estruturação genética do grupo.

Apêndice

**Using Genomic Data to Develop Chloroplast DNA SSRs for the Neotropical Liana
Stizophyllum riparium (Bignoniaceae, Bignoniaceae)**

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USING GENOMIC DATA TO DEVELOP CHLOROPLAST DNA SSRs FOR THE NEOTROPICAL LIANA *STIZOPHYLLUM RIPARIUM* (BIGNONIEAE, BIGNONIACEAE)¹

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- **Premise of the study:** We developed chloroplast microsatellite markers (cpSSRs) to be used to study the patterns of genetic structure and genetic diversity of populations of *Stizophyllum riparium* (Bignoniaceae, Bignoniaceae).
- **Methods and Results:** We used genomic data obtained through an Illumina HiSeq sequencing platform to develop a set of cpSSRs for *S. riparium*. A total of 36 primer pairs were developed, of which 28 displayed polymorphisms across 59 individuals from three populations. Two to 12 alleles were recorded, and the unbiased haploid diversity per locus ranged from 0.037 to 0.905. All 28 cpSSRs presented transferability to two closely related species, *S. inaequilaterum* and *S. perforatum*.
- **Conclusions:** We report a set of 28 cpSSRs for *S. riparium*. All markers were shown to be variable in *S. riparium*, indicating that these markers will be valuable for population genetic studies across *S. riparium* and congeneric species.

Key words: Bignoniaceae; chloroplast microsatellites; cross-amplification; Neotropical flora; *Stizophyllum inaequilaterum*; *Stizophyllum perforatum*.

Stizophyllum Miers (Bignoniaceae) is a small genus of Bignoniaceae, the largest tribe in the Bignoniaceae (Lohmann and Taylor, 2014). The genus is clearly monophyletic (Lohmann, 2006) and includes three species, i.e., *S. inaequilaterum* Bureau & K. Schum., *S. perforatum* (Cham.) Miers, and *S. riparium* (Kunth) Sandwith (Lohmann and Taylor, 2014). All species of *Stizophyllum* are lianas with trumpet-shaped flowers that are pollinated by medium-sized bees (Gentry, 1974). Their winged seeds are dispersed by wind (Gentry, 1974). Members of *Stizophyllum* are easily recognized by their hollow stems, pellucid-punctate leaflets, urceolate calyces, and linear fruits (Lohmann and Taylor, 2014). The genus as a whole is broadly distributed, occurring from southern Mexico to the Atlantic Forest in southern Brazil (Lohmann and Taylor, 2014). Although the generic circumscription is clear, the three species of *Stizophyllum* currently recognized are morphologically similar and can occur sympatrically in Amazonia.

Nuclear DNA polymorphisms based on microsatellites (simple sequence repeats [SSRs]) are powerful sources for population

genetic studies (Kalia et al., 2011). These molecular markers, present also in the genomes of organelles (e.g., chloroplast), allow us to access genetic information that facilitates genotype identification, mainly due to their multiallelic nature (Masi et al., 2003). The chloroplast microsatellite and nuclear microsatellite markers (cpSSR and nSSR, respectively) have been widely used to study phylogenetic and genetic diversity in plants (Kalia et al., 2011). Unlike nSSRs, which are highly polymorphic, codominant, and biparentally inherited, cpSSRs are a nonrecombinant molecule and uniparentally inherited, allowing us to trace the history of populations through their haplotype diversity (Ebert and Peakall, 2009). Despite these differences, the two markers are complements for understanding the genetic structure in natural populations (Ebert and Peakall, 2009; Kalia et al., 2011).

Furthermore, the advent of high-throughput sequencing technologies (Metzker, 2010) has allowed the development of SSR markers for multiple taxonomic groups (Zalapa et al., 2012; Francisco et al., 2016). Here, we used chloroplast genome sequence data obtained by de novo and reference-guided assembly to develop and characterize chloroplast microsatellite markers for *S. riparium*. Cross-amplification of the cpSSR markers developed for *S. riparium* was tested in all congeneric species to evaluate the utility of those markers for population genetic studies in *Stizophyllum* as a whole.

METHODS AND RESULTS

We first obtained the chloroplast genome sequence of *S. riparium* using an Illumina platform. For that, we extracted the genomic DNA from fresh leaf material dried in silica gel from a single individual of *S. riparium* (voucher: *Nogueira*

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TABLE 1. Characteristics of 28 intergenic chloroplast microsatellite markers developed for *Stizophyllum riparium*.

Locus	Primer sequences (5'–3')	Repeat motif	Allele size range (bp)	T _a (°C)	Fluorescent dye ^a	Position	GenBank accession no.
Stiz2	F: CCTTGTGGTTAGTTGAGTCC R: GGGTAGCGACTTGTATATACT	(A) ₆ (G) ₁₀	211–216	49.9	JOE	<i>trnK-UUU/rps16</i>	KP863512
Stiz3	F: AGGAAGTTTCTCCTCGTAC R: CTTGAGTTATGAGTACGAATGG	(T) ₁₀	170–176	54.0	JOE	<i>rps16/rps16</i>	KP863513
Stiz4	F: ATCTAATGATCCGGGGCG R: TTCCCTTCCGTTGATGACT	(A) ₁₀	160–169	55.8	6-FAM	<i>psbI/trns-GCU</i>	KP863514
Stiz5	F: CCCTTCCCGAACCAACATG R: TTGTGAGAAGAGTCCCTCCAAGT	(A) ₁₁	176–181	57.4	JOE	<i>atpF/atpF</i>	KP863515
Stiz6	F: GCCACACATACATTGCTTTGTC R: TCCCTGTCTGTTCCCTTGGA	(A) ₁₂	143–150	57.4	JOE	<i>atpH/atpI</i>	KP863516
Stiz7	F: TCCTAGTATGCTGGCCAACA R: AGAAGAGAAGGTTCCATCGGA	(A) ₁₀	164–171	57.4	JOE	<i>rps2/rpsC2</i>	KP863517
Stiz10	F: CCAACCTAAAATTCTTCCGA R: CTTATAGAAAGTGGGTGTC	(T) ₁₀	165–173	57.4	6-FAM	<i>petN/psbM</i>	KP863519
Stiz11	F: CAGATTGCTTTCCACTTCGA R: TCGAAGAAATCCCCAACCCCT	(A) ₁₀	190–199	54.0	JOE	<i>psbM/trnD-GUC</i>	KP863520
Stiz12	F: GGATTTCCTCGATGGGCCCT R: GCCAGTCCCGACAGATCC	(A) ₁₁	167–180	56.9	6-FAM	<i>psbM/trnD-GUC</i>	KP863521
Stiz13	F: CCTGTCTTTCCATGACCCC R: AAAGAAAGGGGAATGGCTCG	(T) ₁₀	201–210	54.0	JOE	<i>psbC/trnS-UGA</i>	KP863522
Stiz14	F: GCATAGCTAGCAATCCATTCT R: GGAACAATTTGAATAGAATGCC	(A) ₁₃	136–149	54.0	JOE	<i>psaA/ycf3</i>	KP863523
Stiz15	F: CATTCCTCGGCTTTCATTCTGT R: TCTTCTGCCATTTCTCCCA	(A) ₁₀	90–96	51.7	6-FAM	<i>trnT-UGU/trnL-UAA</i>	KP863525
Stiz18	F: CATTCGTCTTCAGCCTCA R: TTGAGTTGCATGGATTGGGA	(T) ₁₀	179–184	54.7	JOE	<i>ycf4/cemA</i>	KP863527
Stiz20	F: TTCTAGGAGGCTGTCTTCC R: TGTCCTACTTTACTGTACGT	(T) ₁₂	183–191	56.9	6-FAM	<i>petA/psbJ</i>	KP863528
Stiz21	F: TACTGCGTCAATTGCCAATT R: TCACCACGCTGTATTGTAA	(T) ₁₀	186–197	48.6	6-FAM	<i>petL/petG</i>	KP863529
Stiz22	F: ACCCACCTATACAGTAACGGT R: TCTCCATTGGTAGCAATAGGT	(T) ₁₀	161–169	56.9	JOE	<i>rpl20/rpl1s</i>	KP863531
Stiz23	F: GATTCTCTATCTGCTGTGTG R: TCTTGAATGGAAGTAAGGG	(A) ₁₀	153–162	56.9	6-FAM	<i>ClpP/psi-psbT</i>	KP863532
Stiz24	F: AGTCGAGTATCTGAACACGA R: AGAACCATTGGATCATCACGT	(A) ₁₁	179–183	61.4	6-FAM	<i>psbH/petB</i>	KP863534
Stiz25	F: TGGAGGAGAAACGATATTAG R: CTCTCGAGGTATAATGACA	(T) ₁₁	168–175	61.4	JOE	<i>infA/rps8</i>	KP863535
Stiz26	F: TCTAGCCATATCAGCATTTCTGT R: GTGTCTCAGGCATATACCT	(T) ₁₀	168–170	61.4	6-FAM	<i>rps8/rpl14</i>	KP863536
Stiz27	F: TCTTCTCATCCAGTCTCTCG R: CGAATAAGCGCTACGACTGA	(T) ₁₀	243–248	53.2	JOE	<i>rpl16/rps3</i>	KP863537
Stiz28	F: GTCCTTTATCCAAGTTTACC R: TCTTACTGATTCACTAGTCG	(A) ₁₀	168–169	55.8	JOE	<i>rpl16/rps3</i>	KP863538
Stiz33	F: TGGATCTCTCAGTCTAAGCAGG R: CCACAAGCCGAGGAGATCTT	(T) ₁₃	230–238	49.9	6-FAM	<i>ycf1/ycf1</i>	KP863539
Stiz34	F: GACACGCAGGATAATTCATA R: TCCAGAAATGAATACAGAAC	(T) ₁₀	182–206	56.9	JOE	<i>ycf1/ycf1</i>	KP863540
Stiz36	F: ACATCCCTATTCTCTCCATTG R: TCTTAGCGAGTAGTCCGA	(TA) ₆	168–170	56.9	6-FAM	<i>trnP-UGG/psaJ</i>	KP863530
Stiz39	F: CTCTAACCTCTGAGCTAAGC R: CGAAATTTCTATATCGCTGCA	(ATA) ₄	175–176	58.0	JOE	<i>trnT-UGU/trnL-UAA</i>	KP863524
Stiz40	F: GATCCAAGAAATTACAGGAC R: TGAGCTATCCTGACCATTT	(TAA) ₄	130–132	53.2	JOE	<i>trnL-UAA/trnF-GAA</i>	KP863526
Stiz45	F: GAAATCCCATATGACCCA R: CCCCCAAGAAAGAGAAAG	(TCTCTT) ₇	156–171	53.2	JOE	<i>ClpP/psi-psbT</i>	KP863533

Note: T_a = annealing temperature.

^aFluorescent label used for the forward primer sequence.

170; Appendix 1) collected in Manaus (Amazonas State, Brazil) using the Invisorb Spin Plant Mini Kit (Invitex, Berlin, Germany) and following the manufacturer's instructions. Approximately 5 µg of total DNA was fragmented using a Covaris S-Series Focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA) and a short-insert (300 bp) library was constructed with NEBNext DNA Library Prep Master Mix Set and NEBNext Multiplex oligos for Illumina (New England BioLabs, Ipswich, Massachusetts, USA), following the manufacturer's protocol. The library concentration was diluted to 10 mM and sequenced (single end) on an

Illumina HiSeq 2000 system (Illumina, San Diego, California, USA) at the University of São Paulo (Escola Superior de Agricultura Luiz de Queiroz da Universidade de São Paulo [ESALQ]) in Piracicaba, Brazil. A Perl script was used to filter for quality using a Phred score of 20 or more for the cleaned reads, with the exclusion of reads with more than three uncalled bases, or shorter than 40 bp. We used a combination of reference-guided and de novo assembly to construct the chloroplast genome of *S. riparium* following Nazareno et al. (2015). The chloroplast genome for *S. riparium* was annotated using DOGMA (Dual Organellar

TABLE 2. Characteristics of 28 polymorphic chloroplast microsatellite markers in three populations of *Stizophyllum riparium*.^a

Locus	CAM (n = 19)		SAM (n = 22)		EAM (n = 18)		All (n = 59)	
	A	h	A	h	A	h	A	h
Stiz2	3	0.368	4	0.614	4	0.654	6	0.660
Stiz3	4	0.551	2	0.524	4	0.575	6	0.671
Stiz4	5	0.719	3	0.626	3	0.451	9	0.773
Stiz5	3	0.550	2	0.095	2	0.125	4	0.533
Stiz6	3	0.433	4	0.726	2	0.209	6	0.668
Stiz7	4	0.618	3	0.511	2	0.529	6	0.719
Stiz10	5	0.693	2	0.505	2	0.118	6	0.633
Stiz11	2	0.529	3	0.426	4	0.802	5	0.653
Stiz12	5	0.683	4	0.671	4	0.712	6	0.760
Stiz13	4	0.419	6	0.766	3	0.385	7	0.584
Stiz14	7	0.850	8	0.884	4	0.600	10	0.893
Stiz15	4	0.575	2	0.467	4	0.582	6	0.627
Stiz18	3	0.608	4	0.633	3	0.228	6	0.588
Stiz20	3	0.342	8	0.833	2	0.294	9	0.780
Stiz21	7	0.596	5	0.745	3	0.601	8	0.717
Stiz22	1	0.000	2	0.416	4	0.467	5	0.703
Stiz23	4	0.642	4	0.676	3	0.582	6	0.698
Stiz24	2	0.441	3	0.706	2	0.111	4	0.665
Stiz25	2	0.525	3	0.552	2	0.523	4	0.732
Stiz26	3	0.632	2	0.395	1	0.000	3	0.420
Stiz27	4	0.608	3	0.386	3	0.600	4	0.625
Stiz28	2	0.125	2	0.233	1	0.000	2	0.458
Stiz33	4	0.419	5	0.737	2	0.309	9	0.796
Stiz34	2	0.485	3	0.338	2	0.476	5	0.725
Stiz36	2	0.264	3	0.451	2	0.111	3	0.516
Stiz39	3	0.542	2	0.521	1	0.000	3	0.493
Stiz40	1	0.000	2	0.100	1	0.000	2	0.037
Stiz45	5	0.788	9	0.900	3	0.621	12	0.905
Mean	3.4	0.500	3.6	0.551	2.6	0.381	5.7	0.644

Note: A = number of alleles; h = unbiased haploid diversity.

^aVoucher and locality information are provided in Appendix 1.

GenoMe Annotator; Wyman et al., 2004; <http://dogma.cccb.utexas.edu/>). Start and stop codons were inspected and adjusted manually.

The Imperfect Microsatellite Extractor (IMEx) interface (Mudunuri and Nagarajaram, 2007) was used to detect perfect microsatellites, with a threshold of 10 repeat units for mononucleotide and six repeats for di-, tri-, tetra-, penta-, and hexanucleotides. The annotated chloroplast genome was used to select markers located in noncoding regions exclusively. Primer pairs were designed from microsatellite sequence sites using Primer3web 4.0 (Rozen and Skaletsky, 1999), with the following parameters: primer size of 18–23 bp, temperature of 50–62°C with maximum difference between forward and reverse primers of 1°C, and GC content of 40–80%.

In total, 36 primer pairs were designed. The PCR amplifications were performed in a final volume of 10 µL and contained 15 ng of genomic DNA, 0.5 µL (10 mM) of each primer with forward primers labeled with 6-FAM or JOE fluorescent dyes (Macrogen, Seoul, South Korea; Table 1), 0.6 µL (25 mM) MgCl₂ (Promega Corporation, Madison, Wisconsin, USA), and 5 µL 1× of KAPA2G Fast ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA). The cycling conditions were as follows: an initial denaturation step of 3 min at 94°C; followed by 30 cycles of 30 s at 94°C for denaturation, 30 s at the specific annealing temperature for each primer pair (Table 1), and 72°C for 60 s; and a final extension of 5 min at 72°C. To test the utility of the individual primers, PCR products were detected using a 1.0% agarose gel electrophoresis with a 100-bp range DNA ladder (Promega Corporation).

Of the 36 primer pairs tested, 28 successfully amplified (Table 1) and were checked for polymorphism in 59 individuals from three *S. riparium* populations (ranging from 18 to 22 individuals; Appendix 1). All samples tested were also extracted using fresh leaf material dried in silica gel or from herbarium specimens using the Invisorb Spin Plant Mini Kit, following the manufacturer's instructions. The amplicons with fluorescent labels were resolved to genotype on an ABI 3500 XL automated DNA sequencer with GeneScan 500 ROX Size Standard (Applied Biosystems, Foster City, California, USA). The microsatellite marker profiles were analyzed using GeneMarker (Holland and Parson, 2011). For each cpSSR, the number of alleles (A) and unbiased haploid diversity index (h) were obtained using GenAlEx 6.41 (Peakall and Smouse, 2006). In addition, we calculated

TABLE 3. Transferability of 28 chloroplast microsatellite markers developed for *Stizophyllum riparium* across two related *Stizophyllum* species.^a

Locus	Repeat motif	<i>S. perforatum</i>	<i>S. inaequilaterum</i>
Stiz2	(A) ₆ (G) ₁₀	+	+
Stiz3	(T) ₁₀	+	+
Stiz4	(A) ₁₀	+	+
Stiz5	(A) ₁₁	+	+
Stiz6	(A) ₁₂	+	+
Stiz7	(A) ₁₀	+	+
Stiz10	(T) ₁₀	+	+
Stiz11	(A) ₁₀	+	+
Stiz12	(A) ₁₁	+	+
Stiz13	(T) ₁₀	+	+
Stiz14	(A) ₁₃	+	+
Stiz15	(A) ₁₀	+	+
Stiz18	(T) ₁₀	+	+
Stiz20	(T) ₁₂	+	+
Stiz21	(T) ₁₀	+	+
Stiz22	(T) ₁₀	+	+
Stiz23	(A) ₁₀	+	+
Stiz24	(A) ₁₁	+	+
Stiz25	(T) ₁₁	+	+
Stiz26	(T) ₁₀	+	+
Stiz28	(T) ₁₀	+	+
Stiz27	(A) ₁₀	+	+
Stiz33	(T) ₁₃	+	+
Stiz34	(T) ₁₀	+	+
Stiz36	(TA) ₆	+	+
Stiz39	(ATA) ₄	+	+
Stiz40	(TAA) ₁	+	+
Stiz45	(TCTCTT) ₇	+	+

^aSuccessful amplification (+) evidenced by the occurrence of a distinct single band on the sequencing gel.

chloroplast haplotype variation within populations by estimating the total number of haplotypes and the unbiased haplotype diversity as $H_e = [n/(n-1)](1 - \sum p_i^2)$, where n is the number of individuals analyzed in each population and p is the frequency of the i^{th} haplotype in the respective population (Nei, 1978). Cross-amplification of polymorphic cpSSRs was tested in six individuals of *S. inaequilaterum* and *S. perforatum* using the same PCR conditions described above.

A partial chloroplast genome (128,239 bp) was obtained and used to develop a set of 28 polymorphic cpSSRs (Table 2). The number of alleles ranged from two to 12, and unbiased haplotype diversity varied from 0.037 (Stiz40) to 0.905 (Stiz45) (Table 2). When alleles at each of the 28 loci were jointly analyzed, we observed 18, 19, and 22 haplotypes for EAM (i.e., east of Amazonia), CAM (i.e., Central America), and SAM (i.e., south of Amazonia) populations, respectively. Furthermore, the maximum unbiased haplotype diversity ($H_e = 1.00$) was observed for all *S. riparium* populations. All polymorphic cpSSR markers successfully amplified for *S. inaequilaterum* and *S. perforatum* (Table 3).

CONCLUSIONS

We developed and characterized 28 chloroplast microsatellite markers for *S. riparium*. Due to the high rate of cross-amplification (100%), these chloroplast microsatellite markers will be useful for genetic studies involving *Stizophyllum* as a whole.

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APPENDIX 1. Voucher and locality information for all individuals of *Stizophyllum* sampled.

Species	Population code	Locality	Geographic coordinates	Voucher no. ^a
<i>Stizophyllum riparium</i> (Kunth) Sandwith	CAZ	Brazil, Amazonas, Manaus	3°00'14"N, 59°55'07"W	A. Nogueira 170*
	CAM	Mexico, Campache, Calakmul	18°48'39"N, 89°18'28"W	D. Álvarez 5027 ¹
	CAM	Mexico, Quintana Roo, Playa del Carmen	20°36'58"N, 87°03'58"W	E. Cabrera 6450 ¹
	CAM	Mexico, Yucatan, Buctotz	21°12'60"N, 88°45'60"W	E. Cabrera 13716 ¹
	CAM	Mexico, Quintana Roo, Adolfo de la Huerta	19°34'45"N, 89°03'31"W	D. Álvarez 9469 ¹
	CAM	Mexico, Campache, Calakmul	18°32'27"N, 89°54'53"W	D. Álvarez 5168 ¹
	CAM	Belize, Toledo, Maya Mts.	16°24'25"N, 89°06'07"W	B. K. Holst 4319 ¹
	CAM	Belize, Cayo	17°21'05"N, 88°55'12"W	D. E. Atha 1040 ¹
	CAM	Mexico, Chipas, Ocosingo	16°54'18"N, 92°02'17"W	E. Martinez 12499 ¹
	CAM	Mexico, Chipas, Ocosingo	17°01'39"N, 91°16'31"W	J. P. Abascal 79 ¹
	CAM	Mexico, Chipas, Ocosingo	17°01'30"N, 91°18'22"W	G. Aguilar 1688 ¹
	CAM	Mexico, Veracruz, Los Tuxtlas	18°34'60"N, 95°05'60"W	C. S. Sinaca 1561 ¹
	CAM	Mexico, Veracruz, San Andres Tuxtla	18°26'60"N, 95°12'58"W	C. S. Sinaca 339 ¹
	CAM	Guatemala, Peten, San Jose	17°01'39"N, 89°52'60"W	B. Wallnöfer 5907 ¹
	CAM	Costa Rica, Puntarenas, Peninsula de Osla	8°24'01"N, 83°17'30"W	R. Aguilar 1960 ¹
	CAM	Costa Rica, Guanacaste, Nandayure	9°50'57"N, 85°20'26"W	A. Estrada 87 ¹
	CAM	Costa Rica, Puntareans, Palmar Norte	8°58'60"N, 83°27'60"W	A. H. Gentry 78803 ¹
	CAM	Costa Rica, San José, Tircoles	9°47'29"N, 84°31'37"W	J. F. Morales 4012 ¹
	CAM	Costa Rica, Puntarenas, Golfito	8°33'03"N, 83°20'60"W	A. Azofeifa 719 ¹
	CAM	Costa Rica, Puntarenas, Bueno Aires	9°02'04"N, 83°25'49"W	L. González 897 ¹
	EAM	Brazil, Pará, Parauapebas	06°10'12"S, 50°21'02"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°09'46"S, 50°20'52"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°10'13"S, 50°21'04"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°09'45"S, 50°20'51"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°09'48"S, 50°20'52"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°10'08"S, 50°21'01"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°10'04"S, 50°21'02"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°10'04"S, 50°21'02"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°10'02"S, 50°21'02"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°09'46"S, 50°20'51"W	M. Beyer 295
	EAM	Brazil, Pará, Parauapebas	06°09'48"S, 50°20'53"W	M. Beyer 301
	EAM	Brazil, Pará, Parauapebas	06°09'36"S, 50°25'21"W	M. Beyer 302
	EAM	Brazil, Pará, Parauapebas	06°04'10"S, 50°14'46"W	M. Beyer 303
	EAM	Brazil, Pará, Parauapebas	06°03'55"S, 50°12'57"W	M. Beyer 303
	EAM	Brazil, Pará, Parauapebas	06°04'14"S, 50°14'44"W	M. Beyer 303
	EAM	Brazil, Pará, Novo Repartimento	04°19'57"S, 49°56'57"W	M. Beyer 315
	EAM	Brazil, Pará, Altamira	03°12'20"S, 51°14'56"W	M. Beyer 317
	EAM	Brazil, Pará, Moju	03°50'25"S, 46°06'40"W	M. Beyer 318
	SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353

APPENDIX 1. Continued.

Species	Population code	Locality	Geographic coordinates	Voucher no. ^a
	SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brazil, Mato Grosso, Paranaíta	9°35'28"S, 56°30'49"W	M. Beyer 353
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°23'13"S, 56°30'07"W	M. Beyer 354
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
	SAM	Brazil, Mato Grosso, Paranaíta	9°31'48"S, 9°31'48"W	M. Beyer 356
<i>Stizophyllum perforatum</i> (Cham.) Miers	—	Brazil, São Paulo, Candido Mota	22°45'27"S, 50°22'06"W	J. P. Souza 9703
	—	Brazil, Paraná, Londrina	23°34'12"S, 50°57'42"W	L. H. M. Fonseca 105
	—	Brazil, Paraná, Londrina	23°34'12"S, 50°57'42"W	L. H. M. Fonseca 103
	—	Brazil, Minas Gerais, Belo Horizonte	11°54'S, 71°22'W	J. Lombardi 2431 ¹
	—	Brazil, Rio de Janeiro, São Pedro de Aldeia	22°50'10"S, 42°06'13"W	J. A. Kallunki s.n.
	—	Brazil, Piauí, Eliseu Martins	08°05'27"S, 43°39'42"W	P. Martins & E. Nunes s.n. ⁴
<i>Stizophyllum inaequilaterum</i> Bureau & K. Schum.	—	Brazil, Acre, Marechal Taumaturgo	08°56'29"S, 72°47'33"W	L. G. Lohmann 454
	—	Ecuador, Amazonia, Orellana	0°40'59"S, 76°24'W	H. Romero-Saltos 2831
	—	Peru, Ucayali, Pedro Abad	09°09'02"S, 75°47'20"W	J. Schunke-Vigo 15997
	—	French Guiana, Saul	03°37'22"N, 53°12'34"W	S. A. Mori 24242 ²
	—	Peru, San Martín, Tocache	08°11'22"S, 76°30'57"W	J. Schunke-Vigo 14609 ³
	—	Brazil, Amazonas, Letícia	04°12'19"S, 69°55'58"W	A. H. Gentry 18302

Note: CAM = Central America; CAZ = Central Amazonia; EAM = east of Amazonia; SAM = south of Amazonia.

^a Most specimens are deposited at the Herbarium of the University of São Paulo (SPF), São Paulo, Brazil, except for 20 samples (¹) that are deposited at the Missouri Botanical Garden Herbarium (MO), St. Louis, Missouri, USA; one sample (²) that is deposited at the Herbarium of the Muséum National d'Histoire Naturelle (P), Paris, France; one sample (³) that is deposited at the New York Botanical Garden Herbarium (NY), Bronx, New York, USA; and one sample (⁴) that is deposited at the Herbarium of the Federal University of Ceará (EAC), Fortaleza, Ceará, Brazil.

* Sample used for DNA extraction.