UNIVERSIDADE DE BRASÍLIA

Vegetative morpho-anatomy of three lianas (Malpighiaceae) used in Ayahuasca analogues

Morfoanatomia vegetativa de três lianas (Malpighiaceae) usadas em análogos da Ayahuasca

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Dissertação apresentada ao Instituto de Ciências Biológicas da Universidade de Brasília, como parte dos requisitos necessários à obtenção do título de Mestre em Botânica.

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Carpe vita!

(Paulo P. Pinheiro)

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TABLE OF CONTENTS P

TABLE INDEX P

FIGURES INDEX P

Extended Abstract in Portuguese

Resumo Expandido em Português

Morfoanatomia vegetativa de três lianas (Malpighiaceae) usadas em análogos da Ayahuasca / Vegetative morpho-anatomy of three lianas (Malpighiaceae) used in Ayahuasca analogues

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INTRODUÇÃO

Ayahuasca é uma bebida psicoativa utilizada por diferentes populações nativas da Bacia Amazônica, cuja ação advém da interação de alcaloides das folhas de *Psicotria viridis* Ruiz & Pav. (Rubiaceae) e de caules da liana *Banisteriopsis caapi* (Spruce ex Griseb.) C.V. Morton (Malpighiaceae) (McKenna, 2004). Alternativamente a esta última, alguns outros membros próximos da família também são utilizados, sobretudo outras *Banisteriopsis* e membros do gênero *Diplopterys* (Garrido & Sabino, 2009).

Não obstante, a classificação e identificação precisas desses e demais parentes de *B. caapi* tem sido desafiadora e controversa, havendo diversas sinonímias dentro do gênero e, ainda, grandes variações morfológicas intraespecíficas (Gates, 1982). Ademais, dados moleculares e de morfologia externa mostraram que o que era considerado como um gênero monofilético por Gates (1982) seriam, na verdade, três clados distintos (Anderson & Davis, 2007).

Nesse contexto, Araújo (2014) defende que características anatômicas podem ser especialmente convenientes para se complementar o embasamento das análises filogenéticas deste gênero, além de auxiliar na circuncisão de suas espécies, precisando-se a identificação. A autora analisa e propõe, para tanto, diversos caracteres da anatomia foliar úteis para esta distinção.

Outro ponto a ser considerado é que a maioria das espécies de *Banisteriopsis* pode apresentar hábito lianescente (Gates, 1982). Como o xilema secundário de lianas apresenta várias especificidades anatômicas, sua análise tem especial papel na identificação de táxons (Angyalossy, Pace, & Lima, 2015; Caballé, 1993).

Por fim, estudos histoquímicos do caule das espécies usadas na Ayahuasca poderiam ser bem-vindos, já que os trabalhos que envolvem a localização de metabólitos de espécies de *Banisteriopsis* e *Diplopterys* foram realizados focando-se majoritariamente as glândulas florais e foliares (*e.g.* Araújo, 2014; Souto, 2012; Nery, 2017).

Neste cenário, os objetivos do trabalho foram 1) caracterizar e comparar, pela primeira vez, a anatomia da madeira de três espécies alternativas utilizadas na bebida: *Banisteriopsis muricata* (Cav.) Cuatrec., *B. laevifolia* (A.Juss.) B. Gates e *Diplopterys pubipetala* (A.Juss) W.R. Anderson & C. Davis; 2) caracterizar e comparar a morfoanatomia da folha destas espécies; 3) comparar a localização histoquímica de alcalóides e outros compostos relevantes na casca e madeira destas espécies e de *B. caapi* (Spruce ex Griseb.) C.V. Morton; 4) fornecer subsídios para identificação das espécies em estado vegetativo.

MATERIAL E MÉTODOS

Após verificar a localização das espécies citadas por Garrido e Sabino (2009) como alternativas a *B. caapi*, três delas foram escolhidas para análise, conforme sua possibilidade de coleta. Além disso, espécies herborizadas foram estudadas para reconhecimento das espécies e suas épocas de floração.

Várias coletas seguiram-se até que as plantas fossem de fato reconhecidas. Uma vez localizados e identificados, três indivíduos de três espécies foram coletados, sendo *B. muricata* em Rio Branco (AC) e *B. laevifolia* e *D. pubipetala* em Brasília (DF), além de um exemplar de *B. caapi*, também advindo de Brasília (DF), este último somente para os testes histoquímicos no caule. Foram retiradas amostras do caule principal a 30 cm do solo e de folhas, sendo estas fixadas em formaldeído, ácido acético e etanol 50 (FAA50).

As técnicas histológicas incluíram cortes da folha e do xilema lianescente à mão livre e em micrótomo de deslize, respectivamente. Ambos foram clarificados com hipoclorito de sódio e corados com azul de Alcian e safranina, após lavagem e desidratação. O acetato de Butila foi utilizado para fixação da coloração. Para os cortes paradérmicos e macerados da madeira, utilizou-se a solução de Franklin (peróxido de hidrogênio e ácido acético 1:1), corados com safranina 1% (Johansen, 1940). Lâminas permanentes foram montadas em verniz vitral incolor, com exceção dos macerados, que foram montados em glicerina. As descrições de madeira seguiram basicamente IAWA Committee (1989) e de padrões de nervação, Ellis *et al.* (2009). Os testes de histoquímica em cortes à mão de madeira e casca fresca seguiram os protocolos propostos por Figueiredo, Barroso & Ascensão (2007).

Obtivemos, com equipamentos Faxitron, imagens de raio-x de folhas e caules para visualização da organização de cristais e padrão de venação. Para *B. muricata*, a diafanização foliar foi realizada no lugar do raio-x, adaptada de Kraus & Arduin (1997) por D. Graciano-Ribeiro e J.G.A. Paiva. As demais imagens foram obtidas com câmeras de celular e um fotomicroscópio Olympus SC30. Os programas Image-Pro Plus 6.0 e Past 3.20 foram usados para medições (30 para características da madeira e 10 para folha) e análises estatísticas, respectivamente.

RESULTADOS E DISCUSSÃO

As três espécies estudadas (*B. laevifolia*, *B. muricata* e *D. pubipetala*, doravante denominados *BL*, *BM* e *DP*, respectivamente) apresentam hábito lianescente quando maduras, tendo um início autossuportante antes de se valer de algum suporte para ascender. Foram identificados tipos de casca diferentes para cada uma, muitas vezes relacionada às suas diferentes variações cambiais: fissurada em *DP* (na qual as fissuras referem-se aos cilindros externos de xilema secundário ou, pelo menos, às cunhas de floema); sulcada em *BL* (em que os sulcos coincidem com arcos de floema) e áspera com lenticelas avermelhadas em *BM* (nesta espécie, os cilindros externos formam uma estrutura um tanto quanto contínua com o principal). De fato, o crescimento secundário anômalo com floema interxilemático tem sido relatado como comum para Malpighiaceae (Metcalfe & Chalk, 1950; Pace, 2016). Esta anomalia pode beneficiar não apenas a condução em lianas, mas proporcionar uma maior flexibilidade e proteção para o tronco, ao intercalar tecidos moles e rígidos (Pace, Lohmann & Angyalossy, 2009; Isnard & Field, 2014; Angyalossy *et al.*, 2015).

Para anatomia da madeira, muitas características qualitativas foram comuns a essas três espécies, como placa de perfuração simples, vasos finos em cadeia radial, pontoações guarnecidas, fibras septadas, abundantes cristais prismáticos no parênquima axial, raios heterocelulares altos e células perfuradas de raio. Tais características estão de acordo com o descrito para a família (Metcalfe & Chalk, 1950; Cabanillas, Pace, & Angyalossy, 2017; Pace *et al.*, 2018). Ainda, todas apresentaram parênquima axial escasso, vasicêntrico e difuso como mais abundantes, assim como achado por Sonsin *et al*. (2016) para *B. caapi*. Assim, estes tipos parenquimáticos parecem variar mais quantitativamente que qualitativamente dentro do gênero, embora outros tipos possam aparecer.

Outras características do xilema secundário presentes nas três espécies estudadas foram parênquima axial não-lignificado, variações cambiais, dimorfismo de vasos e raios altos, estas relacionadas ao que Angyalossy *et al.* (2015) cunhou como “Síndrome Vascular Lianescente”. Acredita-se que lianas são derivadas dentro de Malpighiaceae

(Davis & Anderson, 2010; Pace, 2016). Isso pode explicar por que essas últimas características não são comumente relatadas para toda a família.

Placas de perfuração simples são entendidas como uma adaptação à maior condutividade em climas mais secos e mais quentes, oferecendo menos resistência ao fluxo de água (Baas & Schweingruber, 1987; Christman & Sperry, 2010) Isto pode ser bastante importante em cipós, já que a água tem que atingir grandes distâncias através de seus longos caules (Isnard & Field, 2014).

Vasos múltiplos podem espalhar embolias quando estas ocorrem (Knipfer *et al*., 2015; Fukuda *et al.*, 2015). Isso poderia ser um problema para as espécies aqui estudadas, uma vez que pelo menos 60% de seus vasos são múltiplos, chegando a atingir, em um único agrupamento, mais de 20 vasos, mas sempre de pequeno diâmetro. Assim, pode haver uma compensação, já que a suscetibilidade de vasos múltiplos à cavitação é significativamente reduzida se estes são estreitos (Fukuda *et al.*, 2015). O dimorfismo de vasos, portanto, também é de grande importância e prevalência nos cipós, já que os vasos estreitos garantem a condução da água quando os vasos mais amplos sofrem embolia (Carlquist, 1985; Ewers, 1985). Ainda assim, estes últimos são de grande utilidade, já que o diâmetro maior dos vasos permite menor resistência à água e aumenta exponencialmente o fluxo interno, podendo compensar a área transversal relativamente pequena das lianas (Carlquist, 1985).

Pontoações areoladas também ajudam a restringir bolhas de ar durante a passagem de água (Carlquist, 1988; Baas & Wheeler, 1991; Jansen, Smets, & Baas, 1998; Choat, Cobb & Jansen, 2008). Guarnições também podem ajudar a evitar a embolia por aumentar a quantidade de água próxima à parede do vaso, além de permitir que esta suporte tensões mais altas (Carlquist, 1982; Jansen *et al*., 1998). Assim, a presença de Pontoações areoladas e guarnecidas nas espécies estudadas pode compensar a vulnerabilidade à embolia à qual os seus amplos elementos de vasos estão sujeitos.

Ainda, tem sido sugerido que células perfuradas de raio são importantes para manter o transporte de água eficientemente em diferentes períodos do ano (Machado & Angyalossy-Alfonso, 1995). De fato, tais células foram encontradas neste trabalho, conectando vasos estreitos.

Foi possível observar em todas as espécies um alinhamento horizontal dos elementos de vaso conforme visto na secção radial, aqui denominado "estratificação radial de elementos de vasos". Esta característica parece não ter sido descrita em trabalhos anteriores, embora possa ser encontrada em diferentes graus em muitas espécies que possuem padrão radial, tanto dentro de Malpighiaceae quanto em outras famílias (ver Sonsin *et al*., 2014). Este traço pode ser relevante em descrições, sugerindo-se estudos ontogenéticos aprofundados para sua melhor compreensão.

Fibras gelatinosas também foram encontradas em certa abundância em todos os espécimes observados. Isto pode ter uma relação com o seu hábito, pois elas permitem ondulações, curvas e entrelaçamento dos caules de lianas, aumentando também sua flexibilidade (Bowling & Vaughn, 2009; Fisher & Blanco, 2014). *BL* parece ter desenvolvido uma organização ainda mais especializada de fibras gelatinosas, na qual cada linha de parênquima axial (lignificada ou não) é comumente seguida por uma área mais externa de fibras gelatinosas, que por sua vez é adjacente a uma área sem fibras gelatinosas imediatamente antes da linha seguinte. Nós especulamos que esse arranjo concede maiores graus de flexibilidade e resistência, tão indispensáveis para plantas deste hábito.

Quantidades relativamente altas de tecidos parenquimatosos nas espécies estudadas também podem refletir essa busca por flexibilidade. Isso explicaria, por exemplo, por que todos os nove espécimes têm raios com mais de 1 mm de altura, o que não é esperado para a família (ver Metcalfe & Chalk, 1950; Amorim *et al*., 2017; Pace *et al*., 2018). O parênquima não-lignificado encontrado também é importante para esse objetivo, além de manter sua capacidade meristemática e, assim, permitir que o xilema secundário sofra mudanças estruturais e repare lesões (Angyalossy *et al*., 2015; Carlquist, 1985; Ewers & Fisher, 1991). Além disso, linhas de parênquima não-lignificado nas espécies estudadas parecem adquirir atividade cambial para formar floema secundário e talvez até mesmo xilema secundário, similarmente ao câmbio interxilemático proposto por Pace *et al*. (2018), mas análises ontogenéticas devem ser realizadas para elucidação completa deste fenômeno.

Mesmo havendo todos esses atributos em comum, a análise de componentes principais (PCA) para dados quantitativos dividiu bem as três espécies dentro de fatores que explicam 75% da variância. O primeiro eixo foi influenciado substancialmente por menores medições para comprimento de vasos (tanto largos quanto finos) e lume de vasos finos, explicando 47% da variância e separando *BL* das demais. O segundo fator, responsável por 28% da variância, teve como as mais influentes a largura de raio e a densidade de vasos solitários, menores em *DP*. Ainda, a análise de variância corroborou o PCA e separou as espécies para todas as características quantitativas. A análise de agrupamento corroborou a divisão dos clados proposta por Anderson & Davis (2007) pois consistentemente agrupou os indivíduos dentro de suas espécies, com *DP* como um grupo externo.

Para esta última espécie, uma importante característica distintiva foi a presença de cristais prismáticos nas células dos raios, geralmente aos pares em células eretas. Embora essas inclusões não sejam incomuns em raios de espécies de Malpighiaceae (ver Andrade, 1997), eles se dispõem em um arranjo ímpar em *DP*, alinhando-se tangencialmente, por vezes seguindo anéis de crescimento. Os mecanismos exatos de desenvolvimento desta característica ainda devem ser explorados em um estudo separado.

Além disso, esta espécie foi separada por sua composição de raios, sendo a maioria com células procumbentes com mais de 4 fileiras de células marginais eretas e/ou quadradas, e paredes de fibra mais espessas. Por sua vez, ambas as espécies de *Banisteriopsis* apresentaram traqueídes, tiloses e linhas irregulares de parênquima axial, características não encontradas em *DP*.

Ainda, *BL* possui raios aproximadamente duas vezes mais altos que as demais espécies, pontoações menores e menor densidade de vasos, além do já mencionado anteriormente. Quanto a *BM*, foram encontrados raios de tamanho distinto, sendo os mais largos comumente com mais de 4 células de largura. Adicionalmente, a análise de variância isolou esta espécie por seus maiores diâmetros de vasos (superiores a 200 µm) e fibras mais longas. Possivelmente, algumas das características distintivas desta espécie estão relacionadas a uma melhor adaptação para o hábito lianescente, já que esta possuía indivíduos mais desenvolvidos. Raios maiores, por exemplo, permitem torções e ajustes sob diferentes taxas de crescimento em lianas, e vasos mais largos também podem estar relacionados ao hábito, pois são muito característicos de lianas (Carlquist, 1985; Angyalossy *et al.*, 2015). Anel semi-poroso foi também encontrado apenas em *BM*, tendo sido previamente relatado em *B. oxyclada* e em várias lianas da família Bignoniaceae (Andrade, 1997; Lima, Pace, & Angyalossy, 2010).

Além disso, pontoações são maiores em *BM*, havendo possível relação com o ambiente em que foi coletada (na Floresta Amazônica, em contraste com o Cerrado, onde foram coletados *DP* e *BL*). Carvalho *et al.* (2015) mostraram que cipós de ambientes mais secos tendem a ter pontoações menores do que aqueles de floresta úmida para evitar a cavitação, o que pode se aplicar às espécies aqui estudadas.

As análises histoquímicas revelaram a presença de importantes compostos de potencial uso farmacológico: alcalóides, saponinas, óleos essenciais, lipídios, pectinas, taninos e compostos fenólicos em geral, principalmente em tecidos parenquimatosos da casca e, em segundo lugar, da madeira, com exceção dos lipídios em *DP*, encontrados dentro de vasos e em células companheiras do floema secundário, e pela presença de ácidos resínicos e óleos essenciais no conteúdo de vasos de *B. caapi*.

As espécies aqui estudadas parecem ser alternativas confiáveis para *B. caapi* na Ayahuasca devido à presença de alcalóides no caule, mas estudos adicionais são necessários para garantir sua segurança de uso. Além disso, novas técnicas de extração que utilizam apenas pedaços de casca em preparações de Ayahuasca poderiam ser uma opção, possivelmente melhorando o manejo florestal relacionado à produção desta bebida.

Em relação às folhas, todas são simples, opostas, pecioladas, com lâmina simétrica, margem inteira e venação pinada. Os caracteres taxonômicos mais relevantes para distinção pela morfologia foliar se referem ao comprimento e forma do pecíolo (muito curtos em *BL*); forma de lâmina (com base cordiforme em *BL* e ápice acuminado em *DP*); posição das glândulas (basilaminares nas espécies de *Banisteriopsis* e marginais em *Diplopterys*); tricomas (maiores, mais densos e com longos pedúnculos centralizados em *BL*); ângulo das principais veias secundárias até a variação mediana (descescente em *DP*, crescente em *BL* e irregular em *BM*); e aréolas (menos desenvolvidas em *BM*).

A variação e relevância taxonômica de tricomas e glândulas já eram esperadas para Malpighiaceae, sendo estas últimas denominadas nectários extraflorais por funcionam como atrativos a formigas que, de fato, foram observadas nas folhas das espécies deste trabalho (Metcalfe & Chalk, 1950; Gates,1982; Elias, 1983; Cronquist, 1988). Enquanto a forma da glândula de *DP* e *BL* confirmaram os descritos em trabalhos anteriores (ex. Araújo, 2014; Possobom, 2008), parece não haver concordância em relação a *BM*, já que Araújo (2014) afirma que são sésseis como encontrados no presente trabalho, enquanto Nery e coautores (2017) as descrevem como pedunculadas.

O padrão de venação pareceu bastante estável, sem grandes variações entre as espécies. De fato, venação broquidódroma é comum em vários gêneros de Malpighiacae, particularmente frequentes em espécies do Cerrado (Mamede, 1993).

Em relação à anatomia foliar, características distintivas foram relacionadas à espessura da parede da epiderme e cutícula (maiores em *DP* em menores em *BM*); forma de células adaxiais (com paredes retas em *DP*); camadas de epiderme (biestratificada em algumas regiões em *BL*); posição dos estômatos (abaixo das demais células epidérmicas em *BM*), sua densidade (menor em *BM*) e cristas (presentes em *DP* e *BL*); alturas e proporções do parênquima paliçádico e esponjoso (parênquima paliçádico constituindo metade do mesofilo em *BL* e um terço nas demais e tendo *DP* mais camadas de lacunoso, *Bm* com a menor altura de mesofilo); inclusões minerais (com diferentes localizações de drusas e cristais prismáticos em cada espécie); extensão da bainha colenquimática de feixes secundários (ausente em *DP*); nervura mediana (plano-convexa em *BM* e biconvexa nas demais); e características do pecíolo.

Este último caráter foi bastante distintivo entre as espécies, conforme também encontrado por Araújo (2014). O sistema vascular do pecíolo permanece em feixes separados somente em *BM* e se apresenta lobado em *DP*, o que podem ser características diagnósticas. Bainhas esclerenquimáticas não sem encontradas apenas em *DP*, embora estas possam estar não-lignificadas em *BM*. O comprimento do pecíolo também varia entre estas espécies, como afirma Gates (1982). Embora muitos autores defendam que o número de feixes acessórios no pecíolo pode ser de valor taxonômico, seu número exato pode não ser bastante confiável, pois, como corroborado neste trabalho, pode variar dentro das espécies de *Banisteriopsis* e *Diplopterys* (Araújo, 2014).

A diferenças de espessura de parede e cutícula deve estar relacionada ao ambiente de coleta, já que *BM* veio de uma floresta úmida e, assim, não sofreu a mesma pressão adaptativa para evitar a perda de água, uma vez que a espessura da parede celular epidérmica e a cutícula são inversamente proporcionais à perda de água epidérmica (Ristic & Jenks, 2002). A diferença na pubescência também pode estar relacionada a isso, já que *BM* tem uma pubescência esparsa enquanto *BL* exibe até mesmo um padrão discolor associado aos abundantes tricomas em sua superfície abaxial. Essas estruturas são importantes e mais prevalentes em ambientes mais secos, pois reduzem o movimento do ar na superfície foliar e refletem a radiação, diminuindo as taxas de transpiração (Ehleringer & Mooney, 1978; Fahn & Cutler, 1992). As cristas formadas pela cutícula nas células guarda (encontrados em *DP* e *BL*) também podem atuar nesse sentido. Elas auxiliam o fechamento do ostíolo, como uma vedação adicional que pode até reter água, a fim de evitar a perda de água neste ambiente frequentemente seco (Mauseth, 1988; Fricker & Willmer, 1996).

Epiderme de duas camadas em algumas regiões foi registrada para *Tetrapteris, Janusia* e outras espécies de *Banisteriopsis* (inclusive *B. laevifolia*), algumas vezes relacionadas à secreção mucilaginosa (Andrade, 1997; Araújo, 2014; Metcalfe & Chalk, 1950).

Várias características que encontramos seguiram descrições para Malpighiaceae, como a lâmina dorsiventral, drusas e cristais prismáticos no mesofilo, estômatos

paracíticos na superfície inferior, sistema vascular em forma de arco com feixes acessórios no peciolo e idioblastos (Watson & Dallwitz; Metcalfe & Chalk, 1950). Tais características parecem coincidir com o que foi encontrado para a maioria dos etnótipos de *B. caapi* (Mystery, Ourinho e Tucunacá) (Araújo *et al*., 2016).

Portanto, é possível separar *B. laevifolia*, *B. muricata* e *D. pubipetala* tanto pela morfo-anatomia da madeira quanto da folha, e suas descrições podem auxiliar na identificação em estado vegetativo. Além disso, alcaloides e outros compostos com potencial farmacêutico foram encontrados majoritariamente em sua casca, sugerindo estudos mais aprofundados para suas exatas identificações.

Palavras-chave: anatomia, *Banisteriopsis*, histoquímica, folha, madeira.

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Vegetative Morpho-Anatomy of Three Lianas (Malpighiaceae) Used as Ayahuasca Analogs

1. INTRODUCTION

1.1. Ayahuasca

For the past two decades, Ayahuasca has attracted much attention from both the population in general and the scientific community (Teixeira *et al.*, 2008; Frecska, Bokor, & Winkelman, 2016). It is an intriguing psychoactive beverage that has been used for millennia originally by indigenous populations of the Amazon Basin, also known as *daime*, *hoasca*, *caapi*, *natema*, *yagé*/*yajé/iajé* and *pindé*, depending on whether you are in Brazil, Ecuador, Colombia or Peru (McKenna, 2004; Pereda-Miranda, Taketa, & Villatoro-Vera, 2007; Teixeira *et al.*, 2008; Meneguetti & Meneguetti, 2014; Frecska *et al.*, 2016). As these same authors state, it is often used as a sacrament to reveal the true reality, elevate the state of consciousness and reach spiritual evolution and equilibrium. It has legal support in Brazil and does not evoke chemical dependence on its users (Mabit, 2002).

The interest in this brew is not unusual. It has been proving to be promising in a great variety of treatments (Meneguetti & Meneguetti, 2014). Ayahuasca has shown significant effects on attenuating feelings of hopelessness and panic, as well as antidepressant and anxiolytic efficiency (Santos *et al.*, 2007; Osório *et al.*, 2015; Teles, 2016). Improvements have also been reported for autism, schizophrenia, Parkinson's disease, attention deficit hyperactivity disorder and senile dementia, and there are even rehabilitation clinics that use the beverage for addiction treatment (Serrano-Dueñas, Cardozo-Pelaez, & Sánchez-Ramos, 2001; McKenna, 2004). Even for cancer treatment, benefits can be recognized in many spheres for its users, ranging from the social context to neuropsychological and physio-immunological improvements (Meneguetti & Meneguetti, 2014). A better understanding of the chemical nature of its active constituents can collaborate with advances in contemporary neuropharmacology, neurophysiology and psychiatry (McKenna, 2004).

In spite of all these benefits, little focus has been given to the risks of Ayahuasca's ingestion (Ray & Lassiter, 2016). Although most of the literature defends that it is harmless to human health, some side effects have also been reported, ranging from exhaustion and insomnia to psychosis and possibly death (Grob *et al.*, 1996; Callaway *et al.*, 1999; Halpern *et al.*, 2008; Santos, 2013; Meneguetti & Meneguetti, 2014). In contrast, neurotoxicity studies have endorsed the safety of its use – especially in a religious context –, indicating that the lethal oral dose would be very much higher than that usually consumed.

Concerning its confection, Ayahuasca (*aya*=spirit, ancestor; *waska*=vine) is prepared by boiling or steeping the bark and stems of, customarily, *Banisteriopsis caapi* (Spruce *ex* Griseb.) C.V. Morton (Malpighiaceae) in a concoction with other hallucinogenic plants, usually the leaves of the Rubiaceae species *Psychotria viridis* Ruiz & Pav. (McKenna, 2004; Lunna, 2011). A synergistic interaction between alkaloids in both plants brings about the psychoactive effect: *B. caapi* contains monoamine oxidase (MAO) inhibitors and h-carboline alkaloids, while *P. viridis* or related species contains N,N-dimethyltryptamine (DMT) in its leaves, which becomes active when combined with peripheral MAO inhibitors, altering the serotonergic system (McKenna, 2004; Santos *et al.*, 2007).

Moreover, adepts of the sects discern various types of vines used for the drink, based on different bark and trunk morphologies and their effects (Lunna, 2011). In fact, many other *Banisteriopsis* species are used, such as *B. laevifolia* (A.Juss.) B.Gates, *B. martiniana* (A.Juss.) Cuatrec., *B. muricata* (Cav.) Cuatrec., *B. oxyclada* (A.Juss.) B.Gates, *B. padifolia* (Poepp. ex Nied.) B.Gates, as well as former *Banisteriopsis* species that are now classified under other genera, which are *Diplopterys caduciflora* (Nied.) W.R.Anderson & C.Davis, *D. cristata* (Griseb.) W.R.Anderson & C.Davis, *D. heterostyla* (A.Juss.) W.R.Anderson & C.Davis, *D. leiocarpa* (A.Juss.) W.R.Anderson & C.Davis, *D. longialata* (Nied.) W.R.Anderson & C.Davis, *D. lucida* (Rich.) W.R.Anderson & C.Davis, *D. nigrescens* (A.Juss.) W.R.Anderson & C.Davis, *D. nutans* (Nied.) W.R.Anderson & C.Davis, *D. peruviana* (Nied.) W.R.Anderson & C.Davis, *D. pubipetala* (A.Juss.) W.R.Anderson & C.Davis, and *Bronwenia cornifolia* (Kunth) W.R.Anderson & C.Davis (Pereda-Miranda *et al.*, 2007 *apud* Garrido & Sabino, 2009). Of those, only *Banisteriopsis padifolia*, *D. heterostyla*, *D. longialata*, *D. nutans* and *D. peruviana* have no collection register in Brazil (Flora do Brasil, 2020 under construction, The Plant List, 2013).

The term “Ayahuasca analogues” has been used as nontraditional combinations of plants with the same active principle of the two beforementioned (Ott, 1994; Schultes, Hofmann, & Rätsch, 2001; Labate & Araújo, 2002). Schultes *et al.* (2001) agree that many *Banisteriopsis* species are especially appropriate for use in these analogues, because they contain large amounts of MAO inhibitors such as harmine.

Specifically for *B. muricata*, Davis & Yost (1983) report that Waorani Indians from Ecuador scrape the bark of these wild plants and boil a brew (*mii*) that is drunk in order to call malevolent spirits to curse enemies. They add that Peruvian Witotos of the Ampiyacu river call it *sacha Ayahuasca* ("wild Ayahuasca"), claiming it can be used in the same way as the one from *B. caapi*, even though it induces weaker effects. Ghosal and Mazumder (1971) have shown that this species presents many alkaloids, which would be responsible for its hallucinogenic effects.

Regarding *B. laevifolia*, it has been described that its roots, leaves and flowers have medical properties, ranging from anti-inflammatory effect to renal treatments (Rodrigues & Carvalho, 2001). As to *D. pubipetala*, phytochemical tests identified in its bark important secondary metabolites that could be useful for pharmaceutical industry, such as saponins, tannins, flavonoids, terpenoids and steroids (Santos *et al.*, 2015).

It is relevant to note that Ayahuasca plays a vital role in Amazonian tribal cultures, which embrace it as an intrinsic part of their world view despite having different origins and languages (Williams, 2015). Nonetheless, they are not the only ones which consume Ayahuasca nowadays, mostly due to advanced communications technology and tourism (Tupper, 2008; Ray & Lassiter, 2016). This globalization along with fast acculturation and westernization can threaten both native plant knowledge and environmental conservation (Schultes, 1994).

In this matter, even though some Ayahuasca territorial expansion is coming along with maintenance of forests area, growing interest not only in Brazil but all around the world may still promote indiscriminate exploitation, which is already concerning environmental authorities (Teixeira *et al.*, 2008; Thevenin, 2017). In Acre state, for example, Portaria nº4-2001 of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis of Acre state-IBAMA/AC regulates that transport of both *B. caapi* and *P. viridis* is restricted to registered religious entities which undertake to collect them under specific conservational conditions.

Therefore, it is imperative that we make all the effort necessary so that this cultural patrimony is preserved. Ethnobotanical information can only survive with urgent investigation on related plants and iterant field works (Schultes, 1988). Findings from studies on species related to Ayahuasca can be relevant not only to aid the conservation of these plants but also to strengthen essential cultural legacies, being even more significant under the controversial political tendencies on environmental conservation and first peoples' issues that Brazil is currently facing.

1.2. *Banisteriopsis* C.B.Rob. ex Small (Malpighiaceae)

Malpighiaceae comprises 81 genera and ca. 1300 species of trees, shrubs and vines, distributed in tropical and subtropical forests and savannas; of these, 45 genera and 574 species occur in Brazil, which has in Cerrado the presumable diversity center of the family (Davis & Anderson, 2010; The Plant List, 2013; Francener, de Almeida, & Sebastiani, 2018; Flora do Brasil, 2020 under construction). This family is recognized by their simple opposite leaves, stipules, pedicellate bisexual flowers with five sepals and five clawed petals, ten stamens and a three-parted pistil (Cuatrecasas, 1958; Gates, 1982). Several species of this family are important as food for humans and animals, wood for civil construction, luxury joinery, ornamental trees and medicines (Carvalho, 2007).

As one of the largest genera in the family, with 61 accepted names on The Plant List (2013), *Banisteriopsis* C.B.Rob. ex Small is represented by various woody plants widespread throughout the Neotropics (Gates, 1982). They can be found in tropical forests, mountains, savannas and semiarid regions, being particularly abundant in Brazil, with 47 accepted species, 34 of them endemic (Cuatrecasas, 1958; Flora do Brasil, 2020 under construction). At least half of the *Banisteriopsis* species are vines, which are probably derived within the family from multiples origins; the genus also comprises subshrubs, shrubs and small trees (Gates, 1982; Davis & Anderson, 2010; Pace, 2015). Still, several shrubby species are facultative vines, when the ends of the branches twine around an available support (Gates, 1982).

Many species of the genus have synonyms, and, still, the same species can show great morphological variations (Gates, 1982). Hence, accurate identification of these plants has been somewhat challenging, since the very name and understanding of the genus have suffered changes over the past. Additionally, their identities are not entirely known, mostly because collections are usually of vegetative samples and taxonomic understanding of the group is still under development (Wang *et al.*, 2010).

In fact, what used to be believed as a monophyletic group with three subgenera by Gates (1982) is now considered three distinct clades supported by molecular data, within the stigmaphylloids clade (Anderson & Davis, 2007) (Fig. 1). While most *Banisteriopsis* of the homonym subgenus remained as accepted members of the present genus, the subgenus Hemiramma (Griseb.) B. Gates is now recognized as the genus *Bronwenia* W.R.Anderson & C.C.Davis. Members of the subgenus Pleiopterys (Nied.) B. Gates, in turn, were included in an expanded *Diplopterys* A.Juss. genus, whose representatives had extremely reduced dorsal wings on its fruits but now comprehend three wing-fruited plants as well (Anderson & Davis, 2006, 2007; Davis & Anderson, 2010).

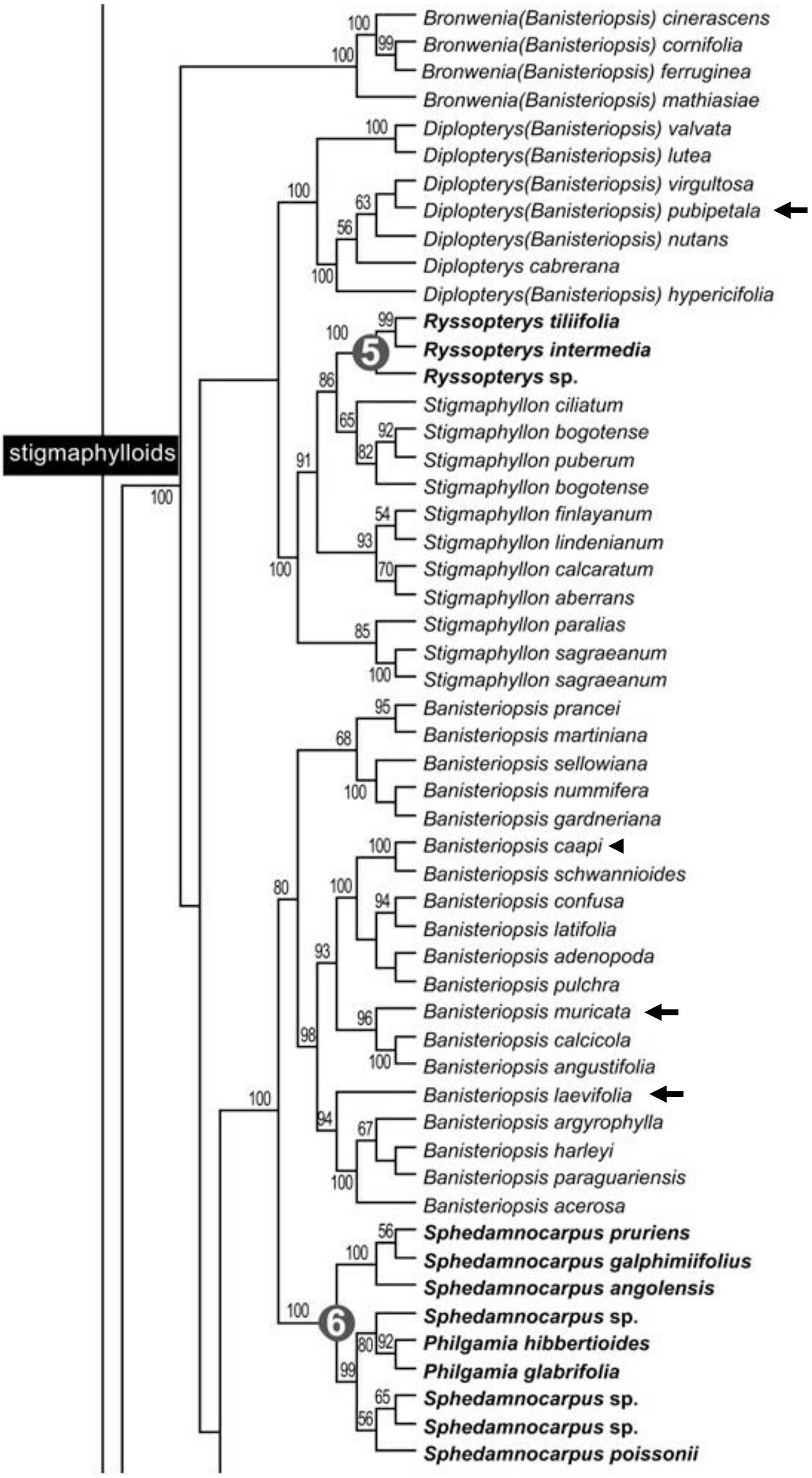

Figure 1. Stigmaphylloids clade. Excerpt adapted from Davis and Anderson (2010) topology based on genes and morphology data set, with previous generic names in parentheses. Arrows=studied species; arrowhead=*B. caapi*.

1.3. Morpho-anatomical studies

Lianas are woody plants that germinate in the soil and become dependent on some external mechanical support to ascend (Gerwing *et al.*, 2006). They are particularly abundant in the tropics, being important for many aspects of forest ecology, from providing a food source and structural pathways for animals to diversity maintenance and regeneration (Schnitzer & Bongers, 2002; Yanoviak & Schnitzer, 2013).

In their search for sunlight, lianas' long stems have special characteristics for guaranteeing efficient hydraulic conduction (Angyalossy, Pace, & Lima, 2015). Thus, evolutionary adjustments are expected to be found in xylem tissue, since it is responsible for both mechanical support and water transport in vascular plants (Ewers & Fisher, 1991). Their internal anatomy reflects the capacity to twist and fold, without interference in their conduction ability (Figueiredo, 2011).

Carlquist (1985) has listed a number of general characteristics found on liana stems, as follows: both phloem and xylem constitute a relatively greater area of conduction when compared with other types of habits; the vessels are typically wide, visible to the naked eye; the presence of vessel dimorphism, in which these wide vessels are combined with narrow ones, is quite frequent; cambial variants are also common and may reflect an evolutionary peculiarity within the group. The author also mentions that these plants have broad rays and abundant axial parenchyma, both not uncommonly non-lignified, to increase the mechanical flexibility, regeneration ability and storage capacity. In addition, xylem fibers usually have a reduced volume, and all these characteristics together characterize the so called lianescent vascular syndrome (Angyalossy *et al.*, 2015).

The anatomical complexity of lianas is poorly explored when compared with the number of studies on the tree and herbaceous plants (Angyalossy *et al.*, 2015). Nevertheless, wood anatomy analysis represents a great tool to aid the identification of families and species, as well as understanding their ecology and development (Figueiredo, 2011).

Brazilian lianas led the first anatomical studies of Malpighiaceae species, on which researchers have addressed cambial variations and other stem's features (Guimarães *et al.*, 2016) while Metcalfe & Chalk (1979) and Solereder (1908) included information on the leaf anatomy. Later, some anatomical works aimed at further taxonomic studies in the family, but still many focused solely on secretory structures in flowers and leaves (Guimarães *et al.*, 2016).

For *Banisteriopsis* leaf morpho-anatomy, some useful traits for species distinction were proposed: petiole shape, vascular pattern and presence of accessory bundles on the petiole, shape of the main vein, presence of sheath extensions, type of mesophyll, presence of leaf glands and their shape (Araújo, 2014). Their wood anatomy, on the other hand, has not been much explored. Some exceptions are the work on *B. oxyclada* (A.Juss.) B.Gates by Andrade (1997), Sonsin-Oliveira *et al.* (2016) on *B. caapi* (Spruce ex Griseb.) C.V. Morton and the recent paper by Pace *et al.* (2018), which studies *B. gardneriana* (A.Juss.) W.R.Anderson & B.Gates and *B. nummifera* (A.Juss.) B. Gates.

Because *Banisteriopsis* have a great number of species that are hard to identify if not flowering, anatomical characteristics should be specially assessed for distinguishing species within this Malpighiaceae genus (Araújo *et al.*, 2010). Gates (1982) stated that *Banisteriopsis* and *Diplopterys* are hard to discriminate even in flower. Wood anatomy data can assist in solving taxonomical and evolutionary puzzles, as well as correctly identifying and separating species (Soffiatti & Angyalossy-Alfonso, 1999). Given that all species from these clades are woody plants, the analysis of their secondary xylem can be very useful. Furthermore, lianas show a variety of special anatomical traits that reflect this habit on their stems, often with different types of cambial variants that are often taxon-specific (Angyalossy *et al.*, 2015). Anatomical analysis of vine wood could efficiently assist in taxa identification (Caballé, 1993); thus, the analysis of their secondary xylem could represent a key instrument for distinguishing *Banisteriopsis* and *Diplopterys* species.

Moreover, leaf anatomy can provide further information for this elucidation. They serve as additional data along with general morphological features for solving taxonomic problems and distinguishing species (Metcalfe & Chalk, 1983; Araújo *et al.*, 2010). Nevertheless, anatomical studies applied to the taxonomy of Malpighiaceae family are still very scarce (Araújo *et al.*, 2010). In short, all anatomical data provided by this paper could further support systematic studies for the genus.

1.4. Histochemistry

Although much effort has been spent on the histochemical characterization of flower and leaf glands, seeds and fruits in many Malpighiaceae – including *Banisteriopsis* and *Diplopterys* (*e.g.* Souto & Oliveira, 2012; Araújo, 2014; Nery, Vieira, & Ventrella, 2017), stem histochemistry has not been extensively analyzed. Guilhon-Simplicio *et al.* (2013) addressed *Byrsonima japurensis* A. Juss. stem bark chemical composition and found a variety of substances with pharmacological potential. Pace *et al.* (2018) tested some *Banisteriopsis* for phenolic compounds, which were found mostly in cells of the cortex and phelloderm. Only chemical works on whole aerial organs of *Banisteriopsis caapi* have been performed, revealing that most bioactive markers were in dried bark of matured stem and branches, but not specifying in which exact cells (Wang *et al.*, 2010).

2. OBJECTIVES

Within the projects "Taxonomia e fitoquímica de *Banisteriopsis caapi* (Spruce *ex* Griseb.) C.V. Morton (Malpighiaceae) componente do chá Ayahuasca" (number 0193001773/2017-FAPDF) and "Valorização das plantas medicinais e de uso religioso do Cerrado através de estudos profundos da taxonomia, morfologia, anatomia, genética e status de conservação" (number 0193000881/2015-FAPDF), this work contributes to a broader botanical understanding of this complex and fascinating brew. This is the first study that aggregates information on leaf and wood anatomy as well as histochemical characterization for important alternative species used for Ayahuasca, enhancing the current knowledge about *Banisteriopsis* and *Diplopterys* genera and lianas in general.

In this context, the present study aims to reach the following objectives:

1. Characterize and compare, for the first time, the wood anatomy of:
    - *a.* *Diplopterys pubipetala* (A.Juss.) W.R.Anderson & C.Davis (=*Banisteriopsis pubipetala* (A.Juss.) Cuatrec.);
    - *b.* *Banisteriopsis laevifolia* (A.Juss.) B.Gates;
    - *c.* *Banisteriopsis muricata* (Cav.) Cuatrec.

2. Characterize and compare the leaf morpho-anatomy of the same species;

3. Compare histochemical localization of alkaloids and other compounds in the bark and wood of these species and of *B. caapi* (Spruce *ex* Griseb.) C.V. Morton;

4. Support further identification of species in vegetative state with morpho-anatomical descriptions.

3. MATERIAL AND METHODS

3.1. Herbarium survey

Specimens of the alternative Malpighiaceae species used for Ayahuasca as stated by Pereda-Miranda *et al.* (2007) *apud* Garrido & Sabino (2009) were localized on SpeciesLink website. Three of them were chosen to be studied, based on their distribution and possibility for collection. Vouchers of the University of Brasilia Herbarium (UB) and images provided by the same website were analyzed in order to support the species recognition, as well as to obtain information on their flowering seasons.

3.2. Botanical material

Populations of the species were plotted on Google Maps to guide the field expeditions, which followed the usual techniques in taxonomy. However, the species were hard to find and identify, due to inaccurate coordinates records, areas of difficult access, absence of flowers and intrinsic difficulties of the genera division. Therefore, at least 12 different *Banisteriopsis* species and four from other genera were collected in 11 field trips in Distrito Federal and one in Acre. Eventually, identification was undertaken with the aid of further herbarium comparisons, literature guidance and confirmation by Malpighiaceae specialists Dra. Renata Sebastiani and Dra. Maria Candida H. Mamede.

Thereafter, samples from three specimens of each confirmed species as well as from one *B. caapi* were collected, as follows in Table 1. While *B. muricata* required a trip to Rio Branco (AC), *D. pubipetala*, *B. laevifolia* (Fig. 2) and *B. caapi* (tucunacá type) were collected in Brasília (DF).

Hacksaw, machete and pruning shears were used to cut the lianas' stems for herborization. They were measured and collected at 30 cm distance from the base in the ground, as it was not always possible to reach 1.3 m as proposed by Gerwing *et al.* (2006), except *B. caapi*, from which we collected only the branch, in order to avoid a destructive collection to a cultivated plant. Some small proof samples were kept fresh for histochemical analysis; the remaining material was stocked in ethanol 70% for microscopic analysis. Some previously collected samples of *B. caapi* by J. Sonsin-Oliveira, C. W. Fagg, N. Nagamine-Pinheiro, J. A. Pereira, R. C. Oliveira (unpubl. data.) were also analyzed for histochemical composition.

Vouchers were deposited at University of Brasilia Herbarium (UB), and wood at the wood collection (UBw) after been frozen for a week. Six leaves from each specimen except from *B. caapi* were collected and fixed in formaldehyde, acetic acid and 50% ethanol (FAA50), as described by Johansen (1940), being taken from the fourth node (or subsequent, if not possible) after the apical meristem of a branch.

Table 1. Studied species. nº=collected individuals for each species.

Species	nº	Coordinates	Collectors	UB
Diplopterys pubipetala (A.Juss.) W.R.Anderson & C.Davis	1	15°30'33.8"S 47°57'31.8"W	Fagg, C.W.; Sonsin-Oliveira, J.; Nagamine-Pinheiro; N.	CWF 2479
	2	15°30'33.8"S 47°57'31.8"W	Fagg, C.W.; Sonsin-Oliveira, J.; Nagamine-Pinheiro; N.	CWF 2467
	3	15°30'32.1"S 47°57'14.9"W	Fagg, C.W.; Sonsin-Oliveira, J.; Nagamine-Pinheiro; N.	CWF 2469
Banisteriopsis laevifolia (A.Juss.) B.Gates	1	15°31'44.8"S 47°57'06.8"W	Fagg, C.W.; Sonsin-Oliveira, J.; Nagamine-Pinheiro; N.	CWF 2471
	2	15°31'45.5"S 47°57'06.4"W	Fagg, C.W.; Sonsin-Oliveira, J.; Nagamine-Pinheiro; N.	CWF 2482
	3	15°31'46.2"S 47°57'06.0"W	Fagg, C.W.; Sonsin-Oliveira, J.; Nagamine-Pinheiro; N.	CWF 2483
Banisteriopsis muricata (Cav.) Cuatrec	1	10°03'15.0"S 68°00'20.0"W	Oliveira, R.C.	RCO 3392
	2	10°03'15.0"S 68°00'20.0"W	Oliveira, R.C.	RCO 3392
	3	10°03'15.0"S 68°00'20.0"W	Oliveira, R.C.	RCO 3392
Banisteriopsis caapi (Spruce ex Griseb.)	1	15°51'57.0"S 47°47'20.0"W	Sonsin-Oliveira, J.; Nagamine-Pinheiro, N.	JSO 414w

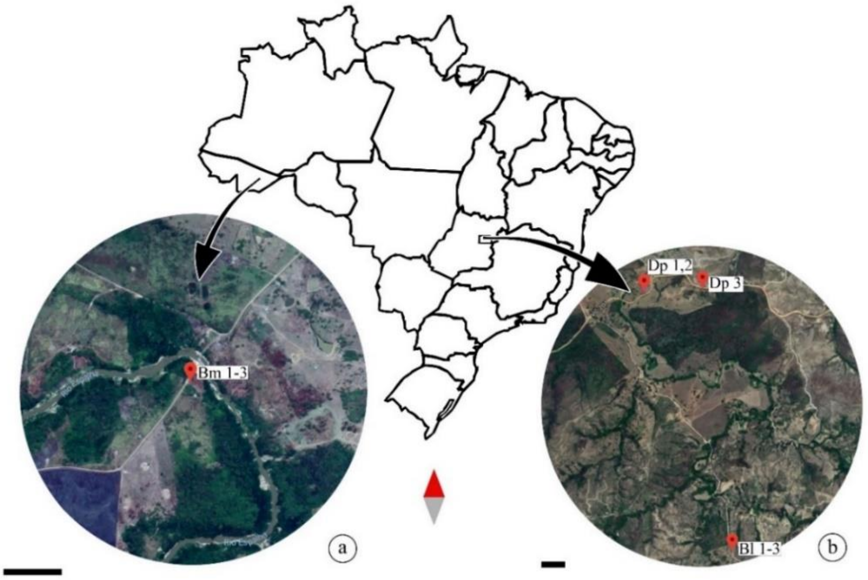

Figure 2. Collection sites. a. Bm=*B. muricata* - Rio Branco (AC); b. Dp=*D. pubipetala*; Bl=*B. laevifolia* - Brasília (DF). Image: @2019 DigitalGlobe, data from map @2019Google.

3.3. Histological techniques

Wood

Wood samples were sectioned 15 to 20 µm thick on their transversal and longitudinal (tangential and radial) planes, by a slide microtome. These samples were taken from the lianescent xylem of the specimens. The sections were clarified with 50% sodium hypochlorite, washed with distilled water and dehydrated in ethanol 30 and 50, respectively, then stained with ethanolic Alcian blue and safranin 50% (1:4) (Johansen, 1940). Increasing ethanolic series (50-70-90-95-100) were followed by butyl acetate, and slides were assembled in colorless glass varnish (Paiva *et al.*, 2006).

Furthermore, the cells were macerated using the method proposed by Franklin (1945) and modified by Kraus & Arduin (1997), and then stained with alcoholic safranin by itself or together with Alcian blue, since phloem fragments could be found in some samples. Semi-permanent slides were mounted in diluted glycerin (1:1).

Microscopy analysis were based on “The International Association of Wood Anatomists (IAWA) Hardwood List” (IAWA Commitee, 1989) together with other studies on liana descriptions: Angyalossy *et al.* (2015), Figueiredo (2011), Pace *et al.* (2018).

For quantitative analysis, 30 measurements of each anatomical feature were taken, using Image-Pro Plus 6.0 software. The principal components analysis (PCA) was performed to assess the more variant factors, as well as an analysis of variance (ANOVA) of normalized data, followed by Tukey’s test at 5% probability. For further similarity analysis, the individuals were clustered with Euclidean similarity index (Sneath & Sokal, 1963) and Unweighted Pair Group Method using Arithmetic averages (UPGMA), considering both quantitative and qualitative data. All statistical analyses were performed using Past 3.20 program.

Leaves

The collected material was fixed in formaldehyde, acetic acid and 50% ethanol (FAA50) for around 48 h, then transferred to 70% ethanol, according to the classic procedure performed in plant anatomy (Johansen, 1940).

Free-hand cross-sections were obtained from samples of the middle third of the leaf blade and from the petiole at three different lengths (base, middle, apex), with the aid of razor blades. After clarification in 20% sodium hypochlorite, rinsing and dehydratation (ethanolic gradient 10-20-30-40-50%), the material was stained with, again, ethanolic Alcian blue and safranin 50% (1:4) (Johansen, 1940). Dehydration in ethanolic series (50-60-70-80-90-95-100) was followed by increasing gradients of butyl acetate. Permanent histological slides were finally assembled using a colorless glass varnish (Paiva *et al.*, 2006).

In addition, leaf samples were heated (60ºC) in Franklin's solution (hydrogen peroxide and acetic acid 1:1) for epidermal dissociation (Johansen, 1940). After washing in distilled water, a brush was used to remove any cell fragment of the mesophyll. Where possible, we also put the venation network of the sample aside with tweezers. The paradermal preparations and veins were stained with 1% aqueous safranin (Sass, 1958) and the slide mounting followed as previously described by Paiva *et al.* (2006).

For leaf architecture study of *B. muricata*, which was undertaken after imaging of *D. pubipetala*'s and *B. laevifolia*'s venation pattern, we followed clarification and staining diaphanization method from Strittmater, adapted by D. Graciano-Ribeiro and J.G.A. Paiva (Kraus & Arduin, 1997). We first rehydrated dried leaves in distilled water, then brushed them with coconut soap, rinsed and let them rest for about 10 minutes in distilled water. They were left overnight in 20% sodium hydroxide, which was periodically exchanged. After washing them in running water, we left the material in 20% sodium hypochlorite until clarification, then washed them again but with distilled water (at least 3 times, for 5 minutes). After staining with 1% aqueous safranin, we followed for dehydration in ethanolic series for 1 hour in each alcohol, to finally put them in increasing concentrations of butyl acetate. Colorless glass varnish was used again to mount them between 0.5 mm glasses (Paiva *et al.*, 2006).

At least 10 measurements were undertaken whenever possible, joining information from the three specimens of each species. Hair stalk could not be assessed in *D. pubipetala* and *B. muricata*, as they were very small. ANOVA tests and cluster analysis were performed with this data in the same way as for wood, as well as t-test was to verify the difference between hair arms. The same programs as abovementioned were used.

3.4. Histochemical tests

Some histochemical analysis was performed to test the major chemical classes of metabolites and cell wall nature in these plants, especially for alkaloids - since those are more important regarding hallucinogenic effects - and other potential phytotherapic compounds. We followed Figueiredo; Barroso; & Ascensão (2007) for protocols. Fresh stem wood and bark from the tree species and *B. caapi* were sectioned with razor blades and submitted to the following tests: Wagner and Dittmar reagents for alkaloid detection (Furr & Mahlberg, 1981; Svendsen & Verpoorte, 1983); vanillin-hydrochloric acid for tannins (Gardner, 1975), ferric chloride for phenolic compounds (Johansen, 1940); Sudan Black and Red Sudan IV for lipids (Pearse, 1980); NADI reagent for detection of essential oil (David & Carde, 1964) and ruthenium red for pectin (Gregory & Baas, 1989).We also verified the presence of saponins in bark and wood samples (Zenid & Ceccantini, 2007).

3.5. X-ray

In order to obtain images of venation patterns on leaves of *D. pubipetala* and *B. laevifolia*, they were X-rayed in an Faxitron MX-20 digital radiography system with a DC-12 camera (3 seconds exposure, 25 kV accelerating voltage, 0.4 heating current of the cathode; Fig. 3a, 3b), at *Laboratório de Anatomia, Identificação e Densitometria de Raios X em Madeira* (ESALQ/USP, Piracicaba-SP). When necessary for detailed pictures, trichomes were removed using a razor blade.

Wood samples (2.0 mm thick) were cut with IsoMet 5000 linear precision saw (Fig. 3c) and stored in a conditioning chamber to be dried. Afterwards, they were also X-rayed in their transverse section, allowing the visualization of crystals patterns. All images were captured by the software Faxitron Bioptics LLC – Vision Version 2.4.1U.

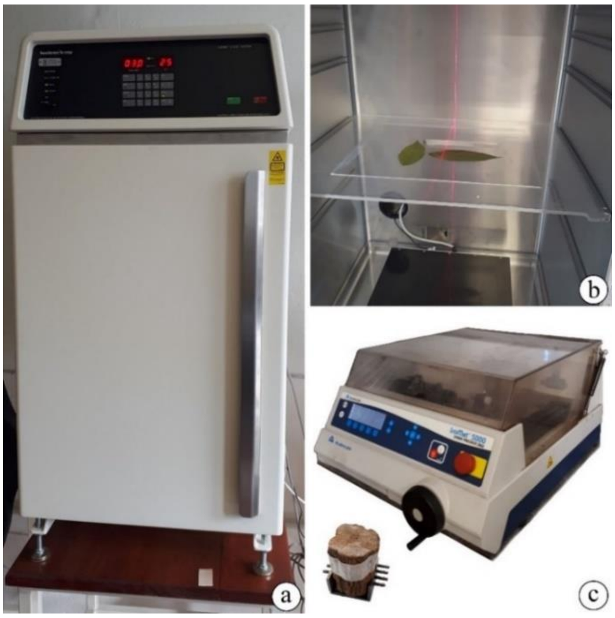

Figure 3. Equipment used for X-ray techniques. a-b. Faxitron MX-20 digital radiography system; c. IsoMet 5000 linear precision saw and wood sample.

3.6. Morphological descriptions

In order to aid further identifications, we observed general macroscopic vegetative characteristics of the three species. Descriptions of bark external morphology followed Ribeiro *et al.* (2002). For leaf morphology and venation analysis, we used chiefly Ellis *et al.* (2009) as a guide, in addition to Hickey (1973) and Leaf Architecture Working Group (1999), this last chosen to guide base angle descriptions, as different authors vary in methods for assessing this characteristic.

3.7. Imaging

Apart from x-Ray images, macroscopic photos were taken by regular cellphone cameras. Microscopical results were registered using an Olympus SC30 photomicroscope coupled with digital image capture system with analySIS getIT software. Additionally, we used Microsoft Power Point and Paint 3D to make sketches of transverse sections of the petiole. Images were, finally, digitally treated on Photoshop 5.0.

4. RESULTS

4.1. Macroscopic vegetative characteristics

A compilation of general macroscopic vegetative characteristics of *B. laevifolia*, *B. muricata* and *D. pubipetala* are shown in Fig. 4-6. They are all scandent when mature, self-supporting at the beginning and then relying on other plants or objects to ascend.

Each species may show a different type of bark surface, often relating to its cambial variant. It is considered cleft for *D. pubipetala*, on which hollows refers to external cylinders of secondary xylem or at least phloem wedges in different degrees (Fig 4b; see anomalous growth in 4e-f). *B. laevifolia* has a furrowed bark, with shallower furrows coinciding with phloem arcs (Fig. 5b, see anomalous growth in 5e-f). Lastly, a rough bark with reddish lenticels is described for *B. muricata* (Fig. 6b, detail), being less ridged as their external cylinders are quite continuous with the main one (Fig. 6b, see anomalous growth in 6d-e).

Leaves for all studied species were simple, opposite, petiolate with marginal attachment; lamina symmetrical, with entire margin and pinnate venation. (Fig. 4c, 5c, 6c). We can acknowledge some distinctive qualities, such as leaf shape and glands position (Fig. 4d, 5d, 6c-detail), but these and other traits are further explored in leaf morpho-anatomy section.

Finally, macroscopic transverse sections can help in distinguish the species, since the already mentioned cambial variants of diverse aspects (Fig. 4e-f, 5e-f, 6d-e). It is relevant to mention that semi-ring porosity may be found at some stages for *B. muricata* (Fig. 6f).

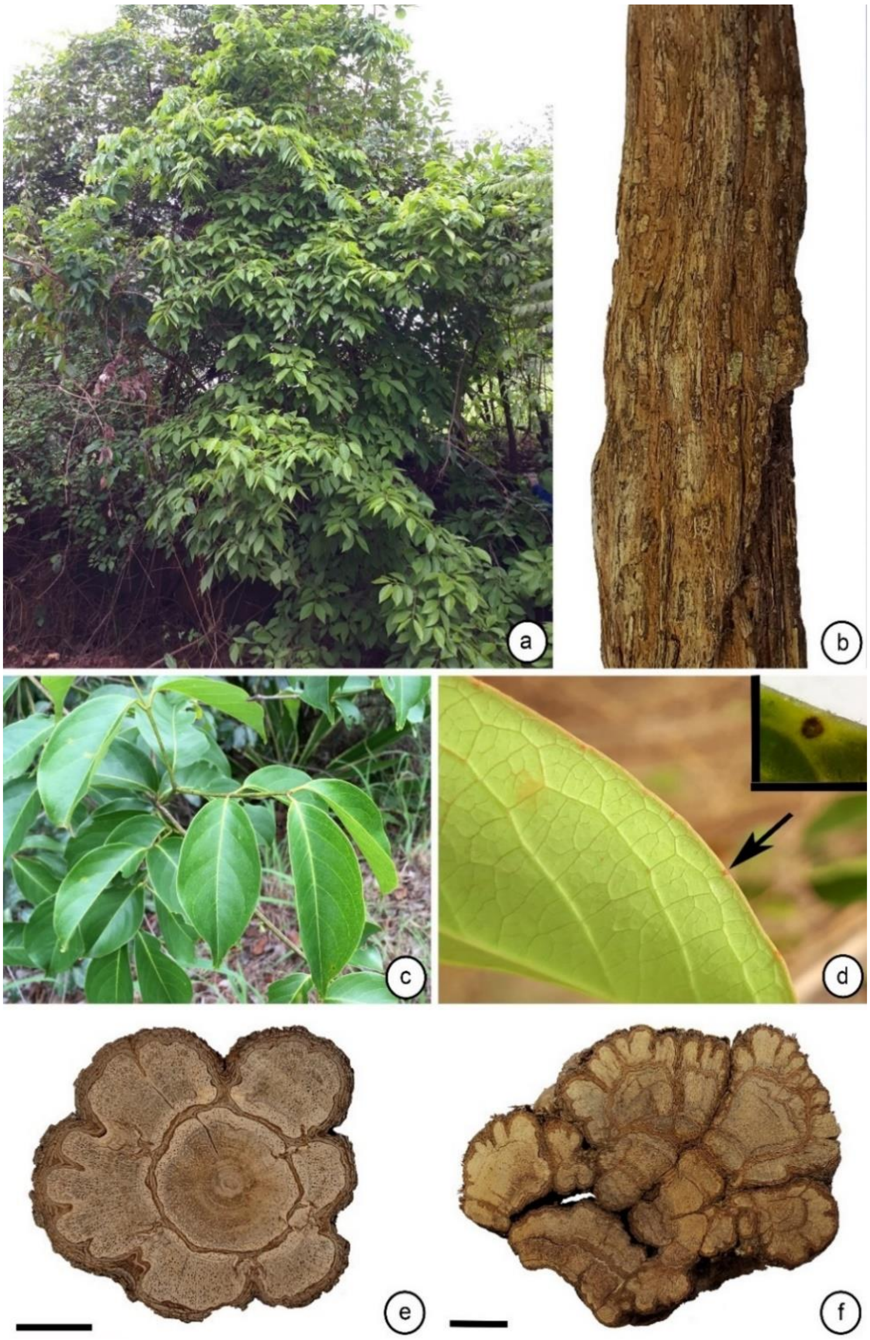

Figure 4. *D. pubipetala* macroscopic vegetative characteristics. a. Plant habit. b. Cleft bark surface. c. Branch. d. Detail of the minute marginal leaf glands. e-f. Macroscopic transverse sections with different stages of cambial variants. Scale bars: d=0,5 cm; e-f=1 cm.

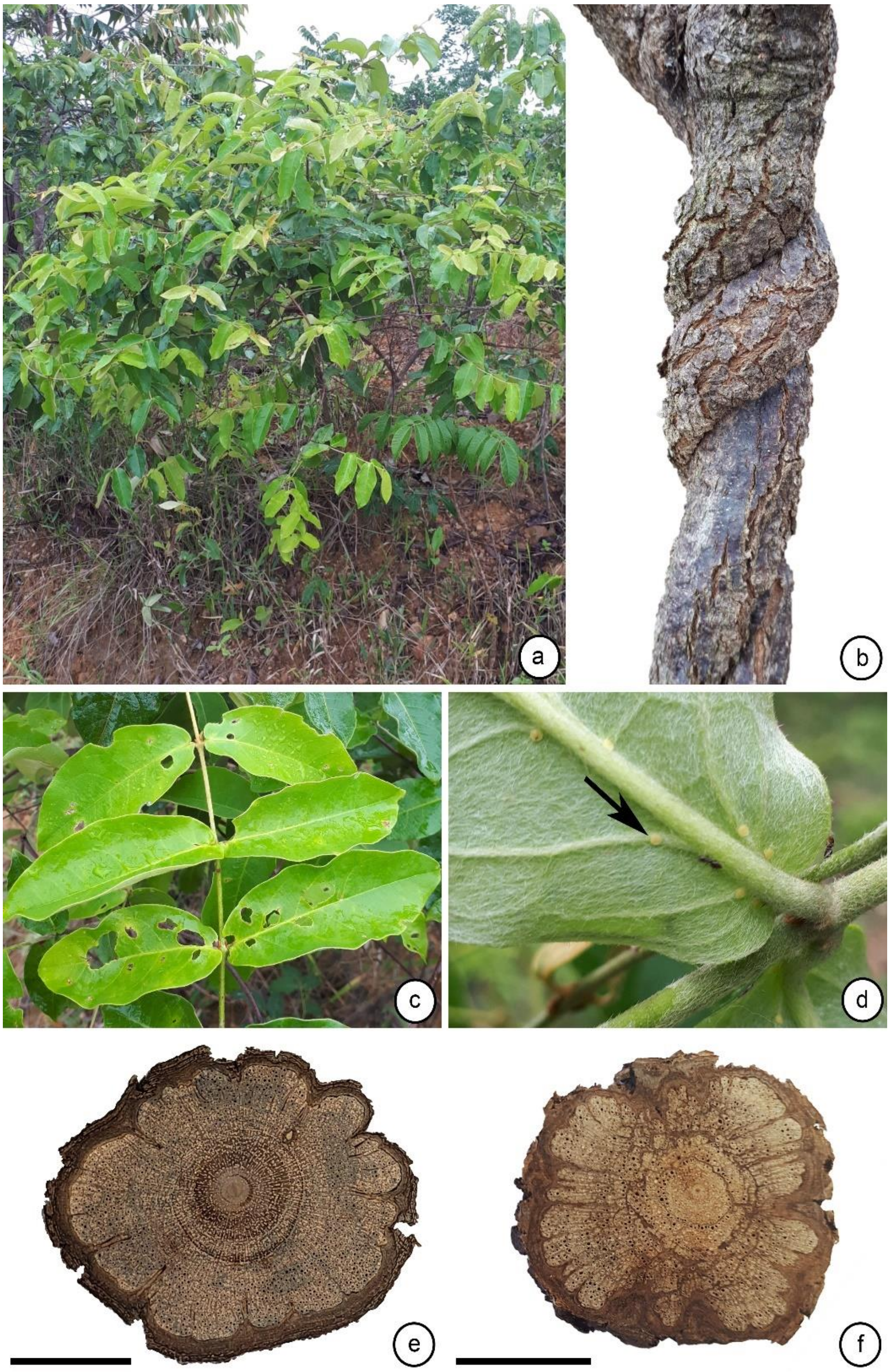

Figure 5. *B. laevifolia* macroscopic vegetative characteristics. a. Plant habit. b. Furrowed bark surface. c. Branch. d. Detail of the basilaminar leaf glands, usually on secondary veins, near the midrib. e-f. Macroscopic transverse sections with different stages of cambial variants. Scale bars: e-f=1 cm.

Figure 6. B. muricata macroscopic vegetative characteristics. a. Plant habit. b. Rough bark surface. c. Branch. Detail of basilaminar leaf glands, frequently on secondary veins d-e. Macroscopic transverse sections with different stages of cambial variants. f. Detail of semi-porous ring that may be found for this species. Scale bars: d-e=1 cm; f=0.5 cm.

4.2. Wood anatomy descriptions

A summarized description of the species with the main qualitative anatomical features are given in Table 2 for better comparison. A full description of each species can be found in Appendix 1. Detailed microscopic images of transverse, tangential and radial sections can be found on Fig. 7-9.

Table 2. Summarized description of qualitative anatomical features of studied species. X=presence in all individuals; +=presence in two individuals; *=presence in only one individual; blank=not present; Bl=*Banisteriopsis laevifolia*; Bm=*Banisteriopsis muricata*; Dp=*Diplopterys pubipetala*.

Anatomical features	**Species**		
	Dp	**Bl**	**Bm**
Growth rings well defined (Fig. 7a, c)	+		*
Growth rings poorly defined	*	*	*
Growth rings absent (Fig. 7b)		+	*
Wood diffuse-porous (Fig. 7a-b)	X	X	*
Semi-porous ring (Fig. 6f; 7c)			+
Small vessels in radial pattern (Fig. 7a-c)	X	X	X
Multiples and solitary vessels	X	X	X
Simple perforation plate	X	X	X
Intervessel pits alternate, circular (Fig. 9g)	X	X	X
Vestured pits (Fig. 9g)	X	X	X
Vessel-ray pits with distinct bordered pits, similar to intervessel pits in size and shape	X	X	X
Vessel dimorphism (Fig. 7a-c; 9b)	X	X	X
Tyloses in vessels		+	+
Deposits in vessels	X	X	X
Tracheids		*	+
Fibers with simple to minutely bordered pits	X	X	X
Septate fibers (Fig. 9e)	X	X	*
Fibers thin- to thick-walled	X	X	X
Gelatinous fibers (Fig. 9b, detail)	X	X	X
Apotracheal axial parenchyma diffuse	X	X	X
Apotracheal axial parenchyma diffuse-in-aggregate	+	X	X
Axial parenchyma scanty	X	X	X
Axial parenchyma vasicentric	X	X	X
Axial parenchyma confluent	+	X	X
Axial parenchyma in narrow bands or lines up to three cells wide (irregular)		X	X
Axial parenchyma in marginal bands	X	*	X
2 cells per parenchyma strand	+	+	X
3-4 cells per parenchyma strand	X	X	X
5-8 cells per parenchyma strand	+	+	
Non-lignified parenchyma (Fig. 7a-c; Fig. 9c-d)	X	X	X
Ray width 1-3 cells (Fig. 7d-f)	X	X	X
Larger rays commonly 4- to 10-seriate (Fig. 7f)	*	*	X
Ray height >1 mm (Fig. 7d-f)	X	X	X
Rays of two distinct sizes (Fig. 7f)			X

Anatomical features	Species		
	Dp	Bl	Bm
All ray cells procumbent			+
All ray cells upright and/or squares	X	X	X
Body ray cells procumbent with 1 rows of upright/squares marginal cells	X		X
Body ray cells procumbent with mostly 2-4 rows of upright/squares marginal cells	X		X
Body ray cells procumbent with over 4 rows of upright/squares marginal cells	X		
Ray with procumbent, upright and squares mixed throughout the ray (Fig. 9c)	X	X	X
Sheath cells	X	X	X
Perforated ray cells (Fig. 9h)	X	X	X
Disjunctive ray parenchyma cell walls (Fig. 9f)	X	X	X
Parenchyma cells with content (Fig. 7f; 9c-d)	X	+	X
Prismatic crystals in chambered upright/square ray cells (Fig. 7d; Fig. 8)	X		
Prismatic crystals in chambered axial parenchyma cells (Fig. 9d)	X	X	X
Prismatic crystals in non-lignified axial parenchyma cells	X	X	X
Cambial variants (Fig. 4e-f; 5e-f; 6d-e; 9a)	X	+	X

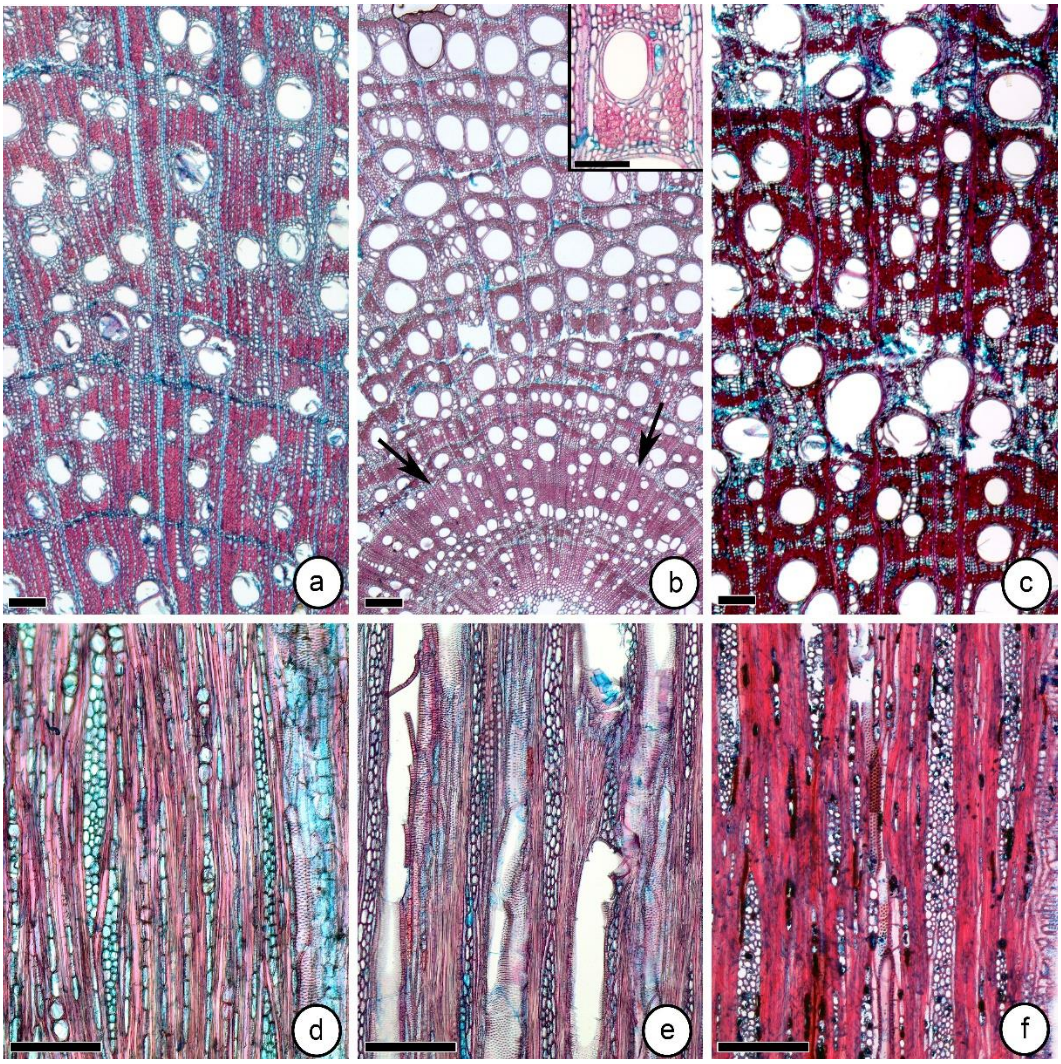

Figure 7. General microscopic wood view. a-c. transverse section. d-f. tangential section. a,d: *D. pubipetala*. b,e: *B. laevifolia*. c,f: *B. muricata*. Note self-supporting xylem (b-arrows), axial parenchyma alternating with gelatinous fibers (b-detail) and contend in ray cells (f) Scale bars: 200 µm.

Some qualitative characteristics were found exclusively in one species. Semi-porous ring (Fig. 6f;7c), rays of two sizes and with all cells procumbent was present uniquely in *B. muricata*. On the other hand, *D. pubipetala* was the only one having rays with procumbent cells with over 4 rows of upright and/or square marginal cells and prismatic crystals in rays, usually in pairs in chambered enlarged upright cells (Fig. 8d). In some areas, these crystals line up in a unique tangential arrangement, sometimes alongside the marginal band (Fig. 8a-b). In Figure 8c, we can see how the cambium produces them concomitantly.

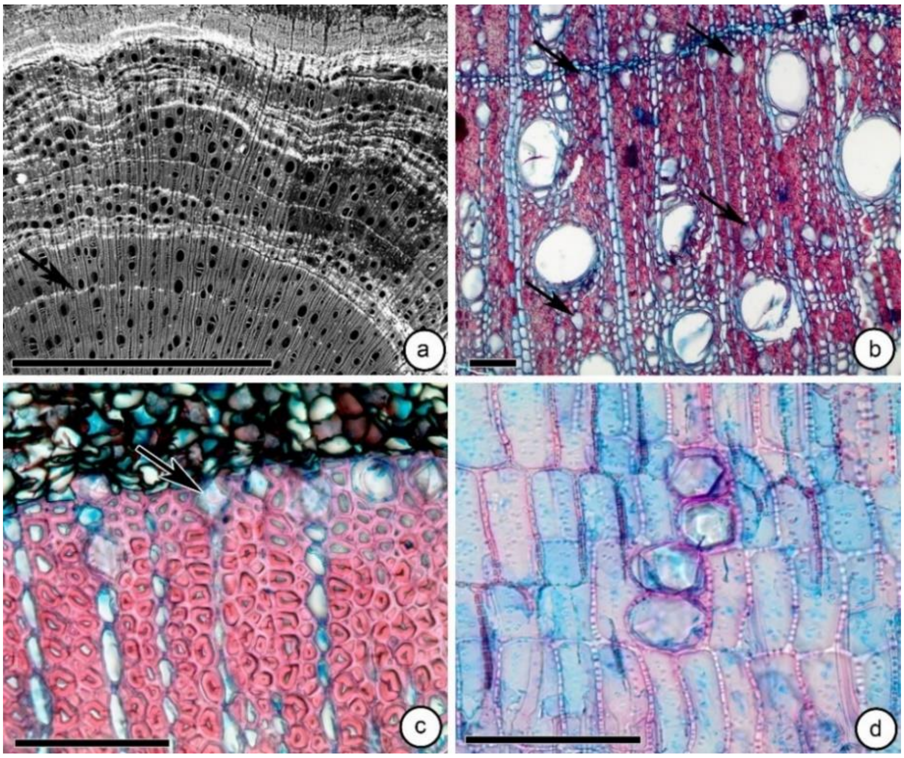

Figure 8. Prismatic crystals in *D. pubipetala* wood. a. radiographic image; b-c. transverse sections, d. radial section. a-b. Aligned crystals, following or not (arrow) the growth ring c. Concurrent crystals formation in different rays (arrow). d. Crystals in chambered upright ray cells. Scale bars: d-e=1 cm; b-d=100 µm.

Still, several traits are common for the three species, such as multiple and solitaries vessels with simple perforation plates; vessel dimorphism, with narrow ones in radial pattern (Fig. 9b); content in vessels and parenchyma cells (Fig. 7f; 9c-d); intervessel pits alternate and circular, vestured (9g); septate (Fig. 9e) and gelatinous fibers present (Fig. 9b, detail); prismatic crystals of distinct sizes in chambered axial (lignified or not) parenchyma cells (Fig. 9d); various types of axial parenchyma (Fig. 7a-c); tall rays (Fig. 7d-f), with all cells upright and/or squares as well as with procumbent, upright and squares mixed throughout it; perforated ray cells (Fig. 9h) connecting narrow vessel elements, disjunctive ray cells walls (Fig. 9f), sheath cells and cambial variants (Fig. 9a).

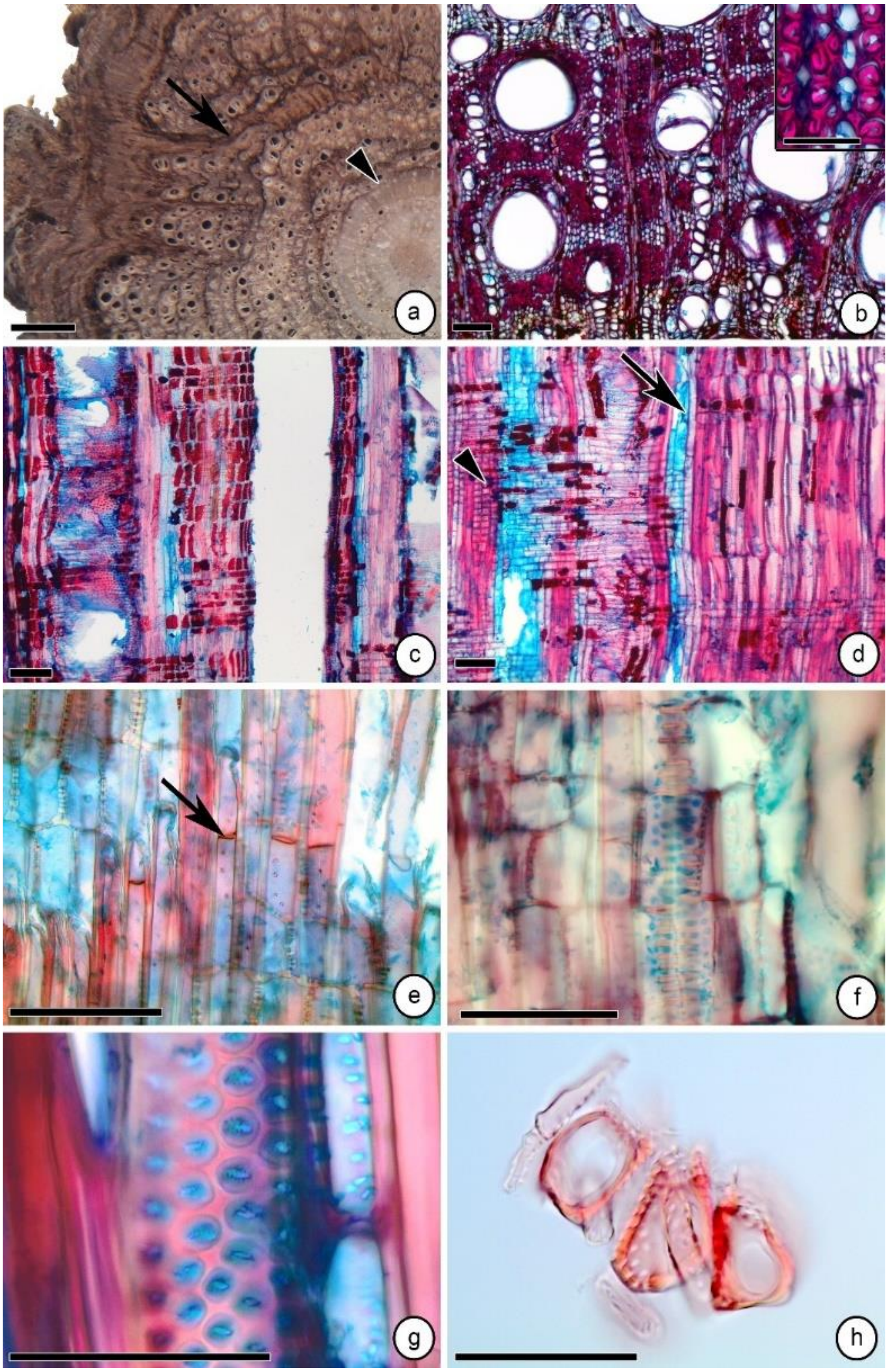

Figure 9. Common wood anatomical features for the studied species. a. *B. laevifolia*, b. *B. muricata*, transverse section. c-d. *B. muricata*, e-f. *D. pubipetala*, radial sections. g. *D. pubipetala*, tangential section. h. *B. muricata*, macerate. a. Cambial variation with interxylary phloem (arrow); note self-supporting secondary xylem (arrowhead) followed by secondary xylem with lianescent vascular syndrome. b. Vessel dimorphism, with narrow vessels in radial pattern; note different types of axial parenchyma. Detail: gelatinous fibers. c. Heterocellular rays; parenchyma cells with content. d. Non-lignified axial parenchyma (arrow); prismatic crystals in chambered axial parenchyma (arrowhead); simple perforation plate. Note the alignment of narrow vessel elements. e. Septate fibers. f. Disjunctive ray parenchyma cell walls. g. Vestured pits. h. Perforated ray cells. Scale bars: a=1cm; b-f, h=100 μm; b (detail), g=50 μm.

Statistically significant differences between species were found for all measured quantitative parameters (Table 3).

Table 3. Statistical data with analysis of variance (ANOVA) of quantitative measurements of studied species. A=Average; SD=Standard Deviation; Fdf value and p refer to the analysis of variance (ANOVA). Equal letters indicate statistical similarities among the species to each variable according to Tukey's test at 5%, variants in bold.

Characteristic	Species	A	±	SD	*Tukey*	$F_{2.267}$	p
Fiber length (µm)	*D. pubipetala*	554.62	±	146.6	a	28.53	< 0.001
	B. laevifolia	557.10	±	153.0	a		
	B. muricata	796.05	±	278.5	**b**		
Wide vessel element length (µm)	*D. pubipetala*	275.11	±	74.9	a	37.32	< 0.001
	B. laevifolia	212.55	±	44.2	**b**		
	B. muricata	275.22	±	43.5	a		
Narrow vessel element length (µm)	*D. pubipetala*	320.03	±	56.7	**a**	50.67	< 0.001
	B. laevifolia	233.09	±	53.0	**b**		
	B. muricata	292.18	±	50.3	**c**		
Intervessel pits diameter (µm)	*D. pubipetala*	7.90	±	1.1	a	161.2	< 0.001
	B. laevifolia	5.63	±	1.1	**b**		
	B. muricata	8.20	±	0.9	a		
Ray-vessel pits diameter (µm)	*D. pubipetala*	4.50	±	0.6	**a**	339.4	< 0.001
	B. laevifolia	3.90	±	0.8	**b**		
	B. muricata	6.74	±	0.9	**c**		
Wide vessel lumina diameter (µm)	*D. pubipetala*	143.48	±	39.9	a	43.57	< 0.001
	B. laevifolia	128.30	±	43.7	a		
	B. muricata	200.86	±	50.0	**b**		
Narrow vessel lumina diameter (µm)	*D. pubipetala*	20.20	±	7.9	**a**	35.85	< 0.001
	B. laevifolia	15.89	±	5.0	**b**		
	B. muricata	23.66	±	6.6	**c**		
Fiber wall thickness (µm)	*D. pubipetala*	7.05	±	3.2	**b**	9.82	< 0.001
	B. laevifolia	5.12	±	1.3	a		
	B. muricata	5.84	±	2.0	a		
Rays/mm	*D. pubipetala*	16.68	±	4.1	**a**	271.6	< 0.001
	B. laevifolia	6.44	±	1.9	**b**		
	B. muricata	9.56	±	2.5	**c**		
Vessels density (mm^2)	*D. pubipetala*	88.41	±	31.0	a	24.14	< 0.001
	B. laevifolia	66.04	±	26.9	**b**		
	B. muricata	94.49	±	35.2	a		
Ray height (µm)	*D. pubipetala*	472.79	±	318.3	a	21.48	< 0.001
	B. laevifolia	954.68	±	937.1	**b**		
	B. muricata	407.87	±	326.0	a		
Ray width (µm)	*D. pubipetala*	18.59	±	10.9	**b**	8.03	< 0.001
	B. laevifolia	24.22	±	11.4	a		
	B. muricata	27.82	±	16.5	a		
Solitary vessels (%)	*D. pubipetala*	31.93	±	13.4	**b**	15.15	< 0.001
	B. laevifolia	40.21	±	10.2	a		
	B. muricata	40.03	±	10.8	a		

Although *B. laevifolia* shared most qualitative aspects with at least one of the other two species, this was the most distinctive species regarding quantitative traits. It had smaller measurements for wide vessel elements length, intervessel pits diameter and vessels density, whereas its rays were more than twice as tall as for the other two species. *D. pubipetala* was distinguished due to its thicker fiber walls, thinner rays and less occurrence of solitary vessels. Finally, fiber length and wide vessel lumina diameter were bigger for *B. muricata*. The remaining features had significant differences between all species: narrow vessel elements length and diameter (both smaller in *B. laevifolia*); ray-vessel pits diameter (quite bigger in *B. muricata*); and rays per millimeter (substantially higher for *D. pubipetala*).

Regarding vessel groupings, radial multiples of 4 or more are rather common for all three: *D. pubipetala*-29%; *B. laevifolia*-24% and *B. muricata*-21%, reaching incredible 34 vessels in *B. laevifolia* and 20 in the remaining; still, multiples of 2 to 4 are more predominant (49%, 44% and 47%, respectively), usually tangentially arranged in wider vessels and radially oriented in narrow ones.

Additionally, quantitative data used in PCA (Fig. 10) divided them well within two factors that altogether explain 75% of the variance (Table 4). The first axis separated *B. laevifolia* from the others, being substantially influenced by its smaller measurements for both wide and narrow vessel elements length and lumina diameter for the later, explaining 47% of the variance. The second factor, accounting for 28% of the variance, best segregated the other two, as the most influential traits were ray width and solitary vessels density, both significantly lower in *D. pubipetala*, followed by *B. muricata*'s longer fibers.

Table 4. High value data of principal component analysis. Features that contributed most for axes are in bold. C1=Component 1; C2=Component 2.

Features	C1	C2
Fiber length (µm)	0.22	**0.36**
Wide vessel element length (µm)	**0.36**	-0.04
Narrow vessel element length (µm)	**0.35**	-0.14
Intervessel pits diameter (µm)	0.31	-0.02
Vessel-ray pit diameter (µm)	0.27	0.33
Wide vessel lumina diameter (µm)	0.27	0.31
Narrow vessel lumina diameter (µm)	**0.34**	0.05
Fiber wall thickness (µm)	0.25	-0.25
Rays/mm	0.27	-0.33
Vessels/mm²	0.26	0.11
Ray height (µm)	-0.32	-0.02
Ray width (µm)	-0.03	**0.50**
Solitary vessels/mm²	-0.17	**0.45**

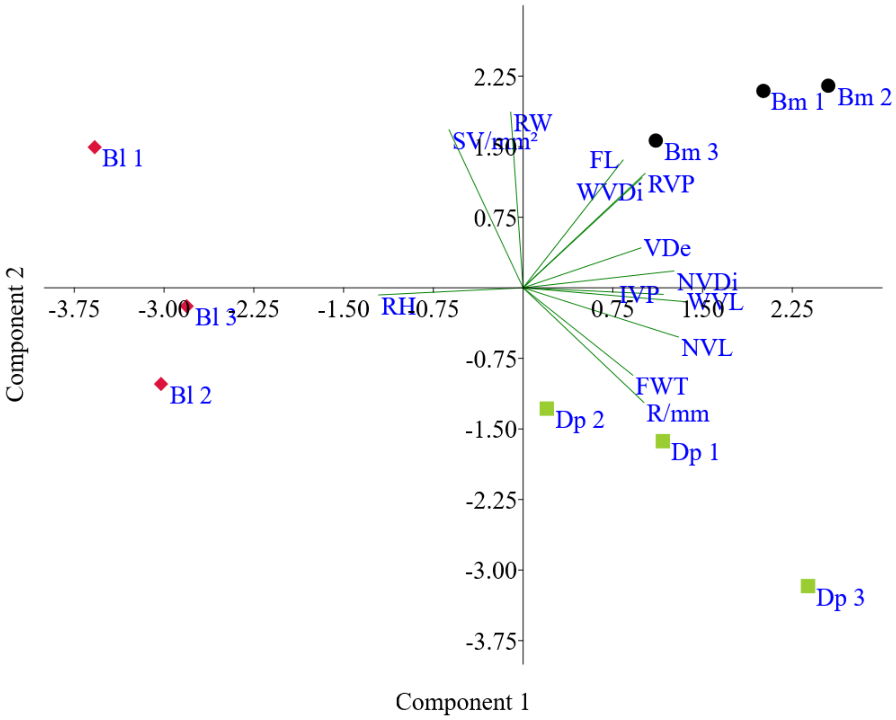

Figure 10. Principal component analysis. ■ Dp: *D. pubipetala;* ♦ Bl: *B. laevifolia*; ● Bm: *B. muricata*; VDe: vessels density; WVL: wide vessel element length; NVL: narrow vessel element length; WVDi: Wide vessel lumina diameter; NVDi: narrow vessel lumina diameter; FL: fiber length; FWT: fiber wall thickness; R/mm: rays/mm; IVP: intervessel pits diameter; RVP: ray-vessel pit diameter; RH: ray height; RW: ray width; SV/mm²: solitary vessels/mm².

Cluster analysis with quantitative and qualitative traits grouped the specimens into the three expected species (Fig. 11). In addition, *D. pubipetala* segregated first, leaving *Banisteriopsis* species in a separate group.

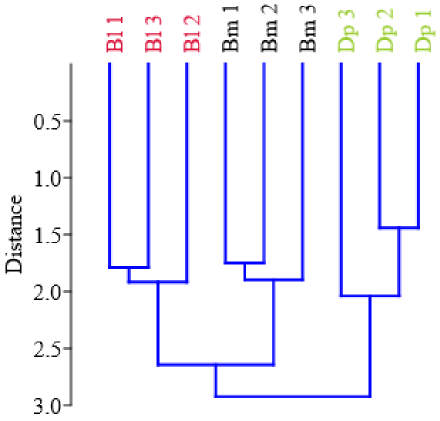

Figure 11. Cluster analysis for wood features (Euclidean distance. UPGMA method). Bl=*B. laevifolia*; Bm=*B. muricata*; Dp=*D. pubipetala*.

Histochemical tests for the stem (bark and wood) of *B. caapi*, *B. laevifolia*, *B. muricata* and *D. pubipetala* are shown in Fig. 12-13. Alkaloids seem to be mostly in parenchymatous tissues in the inner bark and secondly in xylem parenchymatic cells in the three species as well as in *B. caapi* (Fig. 12a-f, controls in 12g-h), being remarkably present in *B. muricata* (Fig. 12e). For the latter, we can see that younger wood has significantly less amounts of this compound (Fig. 12c, compare to 12a). The same results (i.e. presence in parenchymatous tissues) were found for tannins (Fig. 13a-b), phenolic compounds (Fig. 13c-d), terpenoids (mostly in non-lignified parenchyma and near both interxylary and regular phloem; Fig. 13e) and lipids mostly near the cambium area (Fig. 13h), except for young *B. caapi*, which showed no phenols in the wood and still no tannins at all, and for *D. pubipetala*, which had lipids mostly in the vessels and companion cells (Fig. 13g, 13h-detail). Pectin is present at different intensities in all cell walls, being noticeable lower in fibers, sometimes slightly marked in their inner wall (Fig. 13f). Saponins were slightly evident in all bark and wood samples, being only abundant in *B. muricata*'s bark.

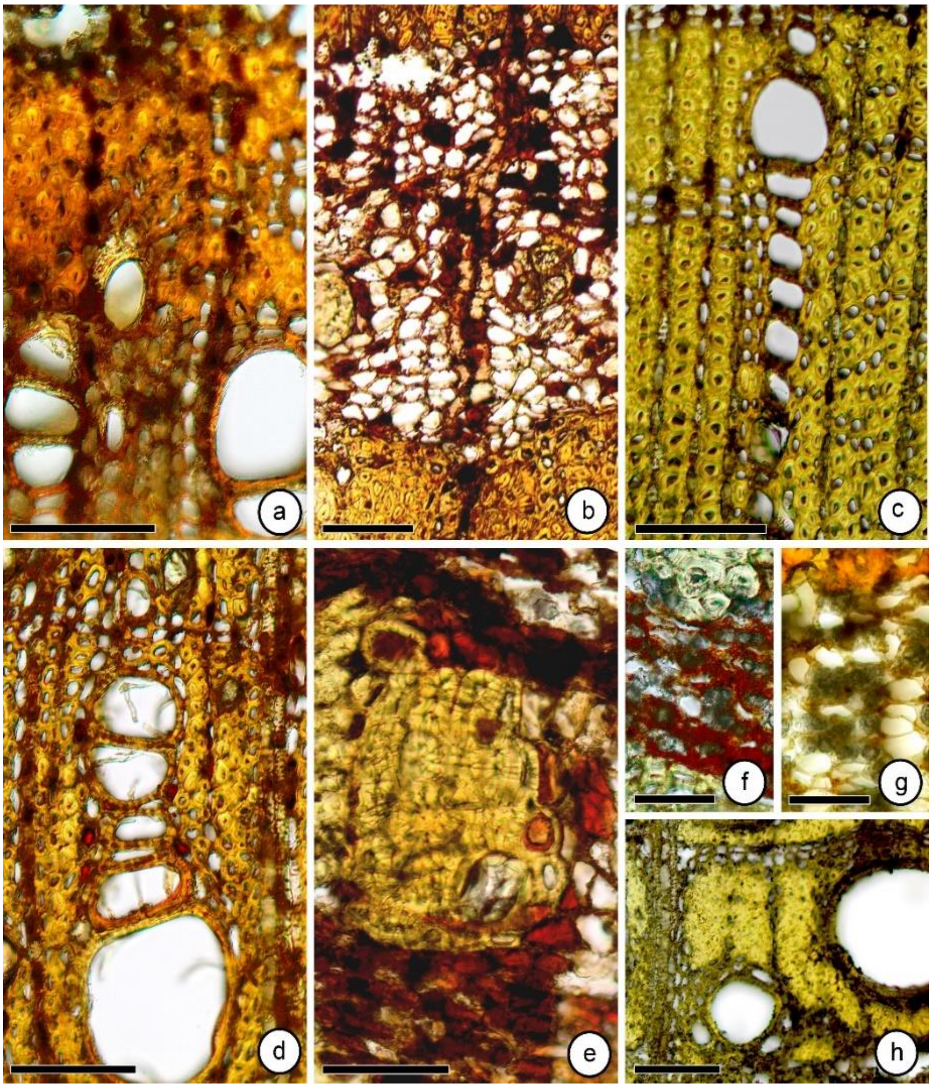

Figure 12. Alkaloid histochemical tests (a-f) and controls (g-h) in transverse sections of wood (a, c, d, h) and inner bark (b, e-g). a-c: *B. caapi*, "c" in young stage; d, f-g: *D. pubipetala*, g control; e: *B. muricata*; h: *B. laevifolia*, control. Wagner reagent: a, b, c, d, f; Dittmar reagent: e. Alkaloids are colored in reddish-brown. Scale bars a-e, h=100 µm; f, g=50 µm.

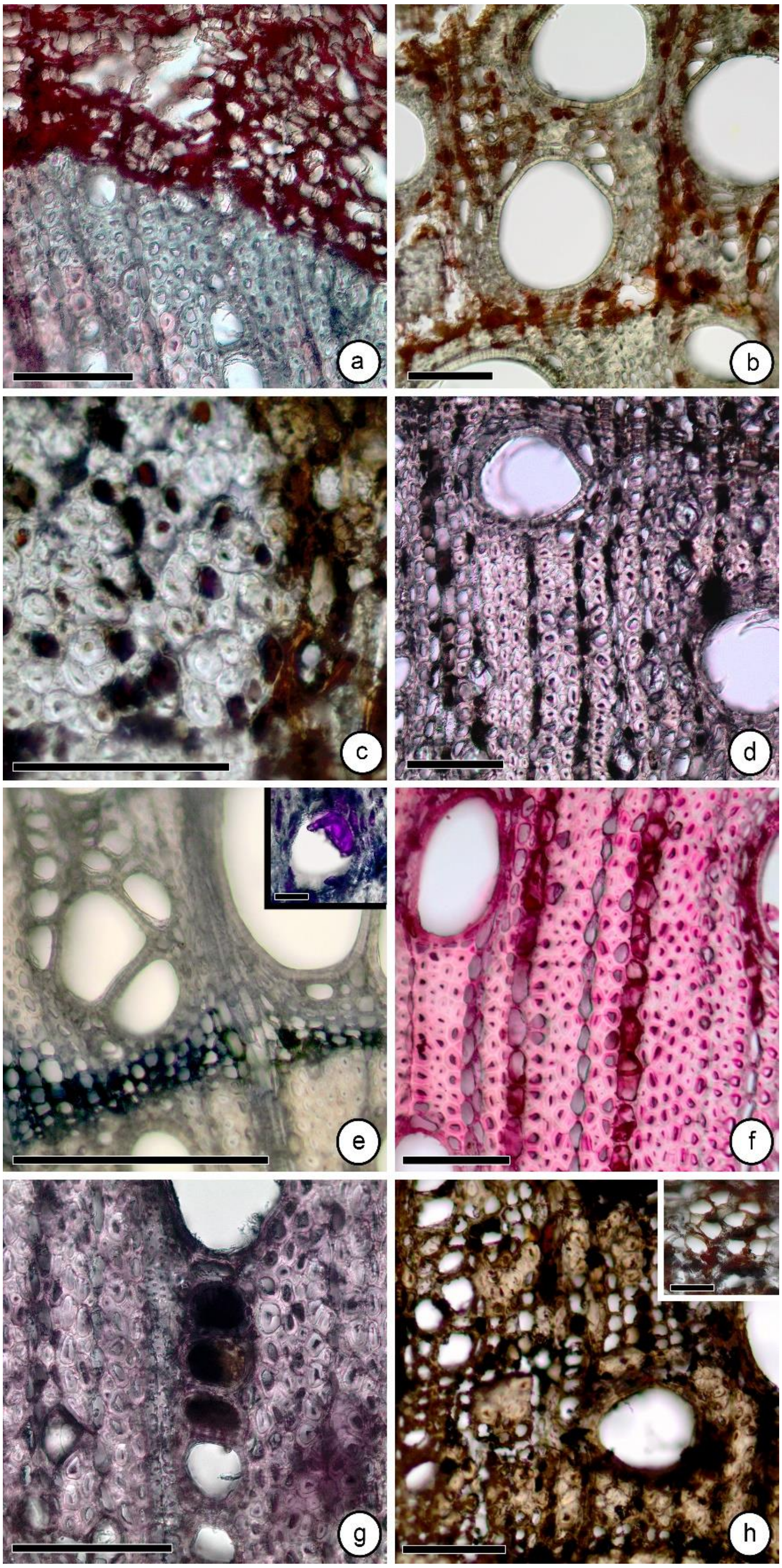

Figure 13. Histochemical tests in transverse sections of inner bark (a, c, h-detail) and stem (b, d-h). a, d, f-g, h-detail: *D. pubipetala*; b-c, e: *B. laevifolia*; e-detail: *B. caapi*; h: *B. muricata*. a-b. Vanillin-hydrochloric acid test for tannins, in red. c-d. Ferric chloride test for phenolic compounds, in dark. e. NADI test for essential oil, in dark blue. Detail of vessel content, with both essential oil and resin acid, in purple. f. Ruthenium red test for pectin, in intense pink. g-h. Sudan Black test for lipids, in black; detail of colored companion cells. Note tannins in orange brown. Scale bars: a-h =100 µm; e, h details=50 µm.

4.3. Leaf morpho-anatomy descriptions

Main morphological features of leaves of *B. laevifolia*, *B. muricata* and *D. pubipetala* are given in Table 5. Some general morphological traits are not included, as they were already mentioned in a previous section.

Most venation features are extremely similar between the three species. All of them had festooned brochidodromous leaves, varying basically in major secondary angle to midvein and areolation, on which *B. muricata* exhibited lower frequency and development of areoles, as well as some marginal ultimate veins incomplete. Thus, the majority of differences were found on the remaining traits, which can be checked on the leaf descriptions (Appendix 2) and Table 5.

Table 5. Leaf morphological features of studied species. X=present; blank=absent; -=immensurable; mean±standart deviation; Dp=*D. pubipetala*; Bl=*B. laevifolia*; Bm=*B. muricata*; HO=highest order; MU=marginal ultimate venation; l:w=length/width; bold=variants according to Tukey's test (5%) in analysis of variance tests, equal letters indicate statistical similarities.

Character			**Species**		
			Dp	**Bl**	**Bm**
Petiole	Cross section	plane-convex		X	
		concave-convex	X	X	X
	Length (cm)		**0.71a**±0.1	**0.24b**±0.1	**1.17c**±0.4
Lamina	Size	area (cm²)	**78.2a**±12.6	57.4b±3.3	54.3b±4.5
	Length/width ratio	2:1	X	X	X
		3:1		X	
	Shape	elliptic to narrow elliptic		X	
		elliptic to large elliptic	X		
		narrow ovate to lanceolate		X	
		ovate to wide ovate			X
	Color	discolor	X	X	X
	Midrib	prominent on both faces	X	X	X
Apex	Angle	acute	X	X	X
		obtuse	X		
	Shape	strongly acuminate with tip drip	X		
		straight to slightly acuminate		X	X
Base	Angle	acute	X	X	X
		obtuse	X		X
	Shape	convex to rounded	X		X
		cordate		X	
Glands	Position	basilaminar		X	X
		marginal	X		
		abaxial	X	X	X
	Size	length (µm)	**247a**±64	**816b**±82	**499c**±45
		width (µm)	**203a**±60	**624b**±88	**409c**±29

Character			Species		
			Dp	Bl	Bm
Indumentum	Adaxial	sparse pubescent			X
		glabrous	X		
		glabrescent		X	
	Abaxial	dense pubescent		X	
		sparse pubescent			X
		glabrescent	X		
	Malpighiaceous hair	size: arms (µm)	300a±41	**1482b**±25	296a±22
		size: stalk (µm)	-	53±25	-
		Significative difference between arms' length	X		X
		arms shape straight	X		X
		arms shape twisted		X	
Major 2º veins	Framework	Festooned	X	X	X
	Spacing	irregular to decreasing proximally	X	X	X
	Angle to midvein variation	inconsistent			X
		smoothly decreasing	X		
		abruptly increasing		X	
	Attachment	decurrent	X	X	X
Inter2º veins	Proximal course	parallel to major 2º	X	X	X
	Distal course	perpendicular to a	X	X	X
	Frequency	<1 per intercostal area	X	X	X
		~1 per intercostal area	X		
3º veins	Intercostal 3º vein fabric	percurrent mixed	X	X	X
	Intercostal 3º angle	decreasing exmedially	X	X	X
	Epimedial 3º fabric	percurrent mixed	X	X	X
	Exterior 3º course	looped	X	X	X
4º veins	Fabric	percurrent alternate	X	X	X
5º veins	Fabric	reticulate irregular	X	X	X
Areolation	Development	well-developed	X	X	
		moderately developed			X
	Density	areoles/mm² (mean)	39a±5	41a±3	**12b**±3
	Freely ending veinlets	mostly unbranched or with one branch	X	X	X
HO			6º	6º	6º
MU	Present	looped	X	X	X
		incomplete loops			X

Table 6 summarizes leaf anatomy data for the studied species. These characteristics were less constant among them. *Diplopterys pubipetala*, for instance, could be distinguished by some features, such as: greater differences in thickness between adaxial and abaxial wall+cuticle layers (Fig. 15e); straight anticlinal walls in adaxial epidermis (Fig. 15f); mesophyll height and layers of spongy parenchyma; druses in mesophyll; absence of collenchymatic sheath extension on secondary vascular bundles; and lobate bundle in the petiole, which can have concentric amphicribal accessory bundles (Fig. 15c).

Banisteriopsis laevifolia, in its turn, was the only one with bilayered epidermis in some regions (Fig. 16e), palisade parenchyma occupying almost half of the mesophyll and sclerenchyma sheath in the petiole vascular region (Fig. 16c).

Lastly, *B. muricata* had a smaller difference between adaxial and abaxial wall and cuticle thickness and stomata under the line of commons cells (Fig. 17e); less stomata per square millimeter, with no ledges, and thinner mesophyll with less spongy parenchyma layers (Fig. 17e); plane-convex midvein, with no druses in phloem (Fig. 17d); and divided vascular bundle on the petiole with non-lignified sclerenchyma (Fig. 17c).

Table 6. Leaf anatomical features of of *B. laevifolia*, *B. muricata*, and *D. pubipetala*. X=present; blank=not present; mean±standart deviation. Bl=*Banisteriopsis laevifolia*; Bm=*Banisteriopsis muricata*; Dp=*Diplopterys pubipetala*; bold=variants according to Tukey's test (5%) in analysis of variance test, different letters indicate statistical dissimilarities, * = character not tested due to small sampling.

Character			**Species**		
			Dp	**Bl**	**Bm**
Epidermis	Wall+Cuticle thickness	adaxial=1-1.4 abaxial			X
		adaxial=1.5-2 abaxial		X	X
		adaxial ≥2.1 abaxial	X	X	
	Adaxial cells shape	straight walls	X		
		straight to curved walls		X	X
	Abaxial cells shape	straight to curved walls	X	X	X
	Layers	bilayered on some adaxial regions		X	
	Stomata	paracytic hypostomatic	X	X	X
		under the level of common cells			X
		at the same level of common cells	X	X	
		density (mm^2)*	464±74	504±74	233±62
		ledges	X	X	
Mesophyll	Type	dorsiventral	X	X	X
	Height (µm)		**159a**±1	**111b**±18	**79c**±7
	Palisade parenchyma	ratio to mesophyll height	1:3	1:2	1:3
		layers number	1	1	1
	Spongy parenchyma	layers number	5-7	4-5	3-5
	Braciform parenchyma	present	X	X	X
	Idioblasts	present	X	X	X
Mineral inclusions	Druses occurrence	palisade and spongy parenchyma	X		
		midvein phloem	X	X	
		midvein parenchyma	X	X	X
	Prismatic crystals occurrence	palisade parenchyma		X	X
		spongy parenchyma	X		
		midvein parenchyma		X	

	Character		Species		
			Dp	Bl	Bm
2º bundles	collateral		X	X	X
	fibers on major bundles		X	X	X
	sheath	parenchymatic	X	X	X
	sheath extension	collenchymatic		X	X
Midvein	Shape	biconvex	X	X	
		plane-convex			X
	Vascular bundle	one continuous open arc	X	X	X
	Sclerenchyma	bicollateral	X	X	X
	Colenchyma	bicollateral, angular to annular	X	X	X
	Epidermis	smaller than on mesophyll	X	X	X
Petiole	Open arc vascular bundle	continuous	X	X	
		divided			X
		lobate	X		
	Sclerenchyma sheath	absent	X		
		lignified		X	
		non-lignified			X
	Accessories bundles	number	2-4	2-5	2-3
		collateral	X	X	X
		concentric amphicribal	X		

Cluster analyses with all leaf assessments corroborated what was found for wood analysis (Fig. 14). ANOVA tests (Table 5 and 6) show significative differences (with $p \leq 0.001$) for all three species relatively to both palisade parenchyma and mesophyll height, petiole length and glands size. On the other hand, hair length was considered equal for *D. pubipetala* and *B. muricata*. In addition, their hairs had a significant difference between shorter and longer arms on t-test (p=0,002 for *D. pubipetala* and p=0.0002 for *B. muricata*), which was non-significant for *B. laevifolia* (p=0;92).

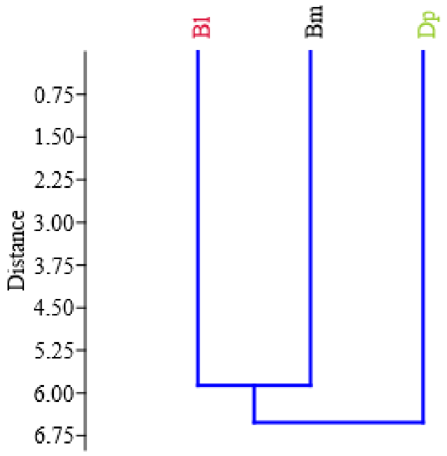

Figure 14. Cluster analysis for leaf features (Euclidean distance. UPGMA method). Dp=*D. pubipetala*; Bl=*B. laevifolia*; Bm=*B. muricata*.

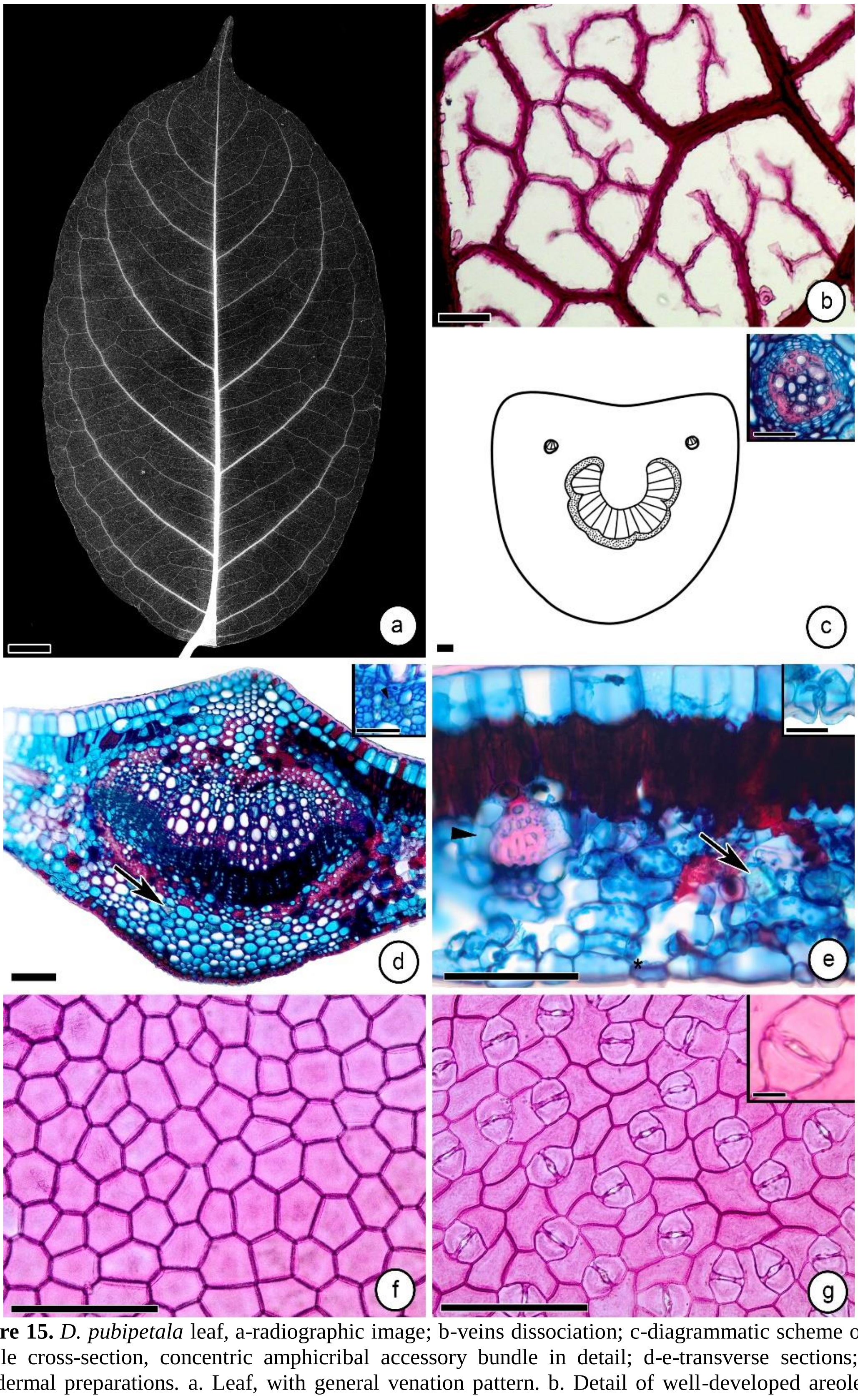

Figure 15. *D. pubipetala* leaf, a-radiographic image; b-veins dissociation; c-diagrammatic scheme of the petiole cross-section, concentric amphicribal accessory bundle in detail; d-e-transverse sections; f-g-paradermal preparations. a. Leaf, with general venation pattern. b. Detail of well-developed areoles. d. Midrib: druses in the cortex (arrow) and phloem (arrowhead, detail). Note abundant idioblasts. e. Leaf blade: mesophyll with prismatic crystals and druses (arrows), vascular bundles with parenchymatic sheath (arrowhead), stomata in adaxial epidermis, with ledges (*, detail). Note abundant idioblasts and adaxial epidermis 2-3 times taller than abaxial. f. Adaxial epidermis: straight walls. g. Abaxial epidermis: straight to curved walls, paracytic stomata (detail). Dot-filled areas=phloem; stripped areas=xylem. Scale bars: a=1cm; b-g=100 µm; c detail=50 µm; d-e detail=50 µm; g detail=10 µm.

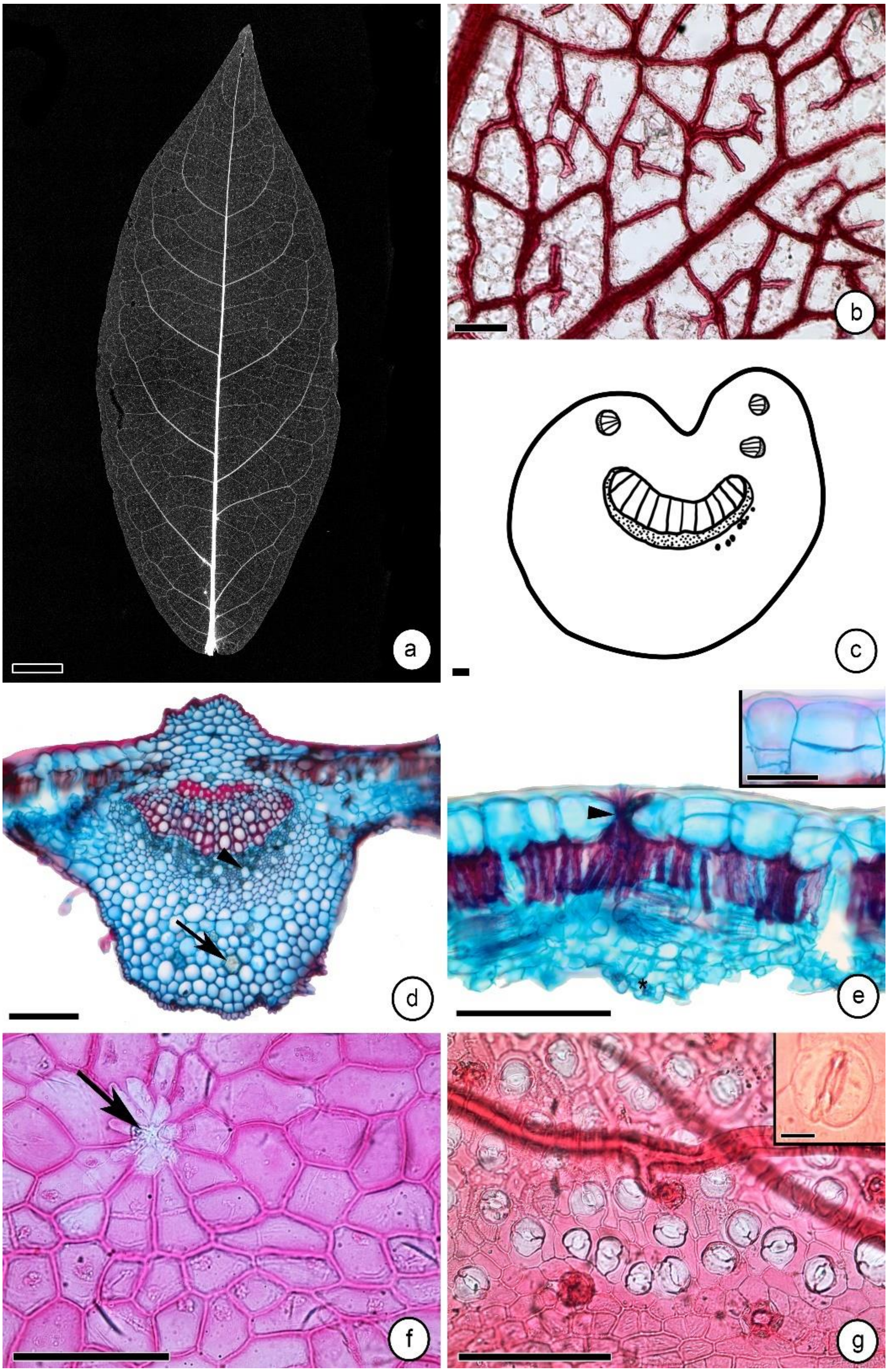

Figure 16. *B. laevifolia* leaf, a-radiographic image; b-veins dissociation; c-diagrammatic scheme of the petiole cross-section, d-e-transverse sections; f-g-paradermal preparations. a. Leaf, with general venation pattern. b. Detail of well-developed areoles. d. Midrib: druses in the cortex (arrow) and phloem, idioblasts in the cortex (arrowhead). e. Leaf blade: mesophyll, with stomata in adaxial epidermis (*) and lignified hair attachment (arrowhead). Note abundant idioblasts. Detail: region of bistratified epidermis. f. Adaxial epidermis: straight walls, trichome insertion region with radiate pedal cells (arrow). g. Abaxial epidermis: straight to curved walls, paracytic stomata (detail). Dot-filled areas=phloem; stripped areas=xylem; dark areas=fibers. Scale bars: a=1cm; b-g=100 µm; d detail=50 µm; g detail=10 µm.

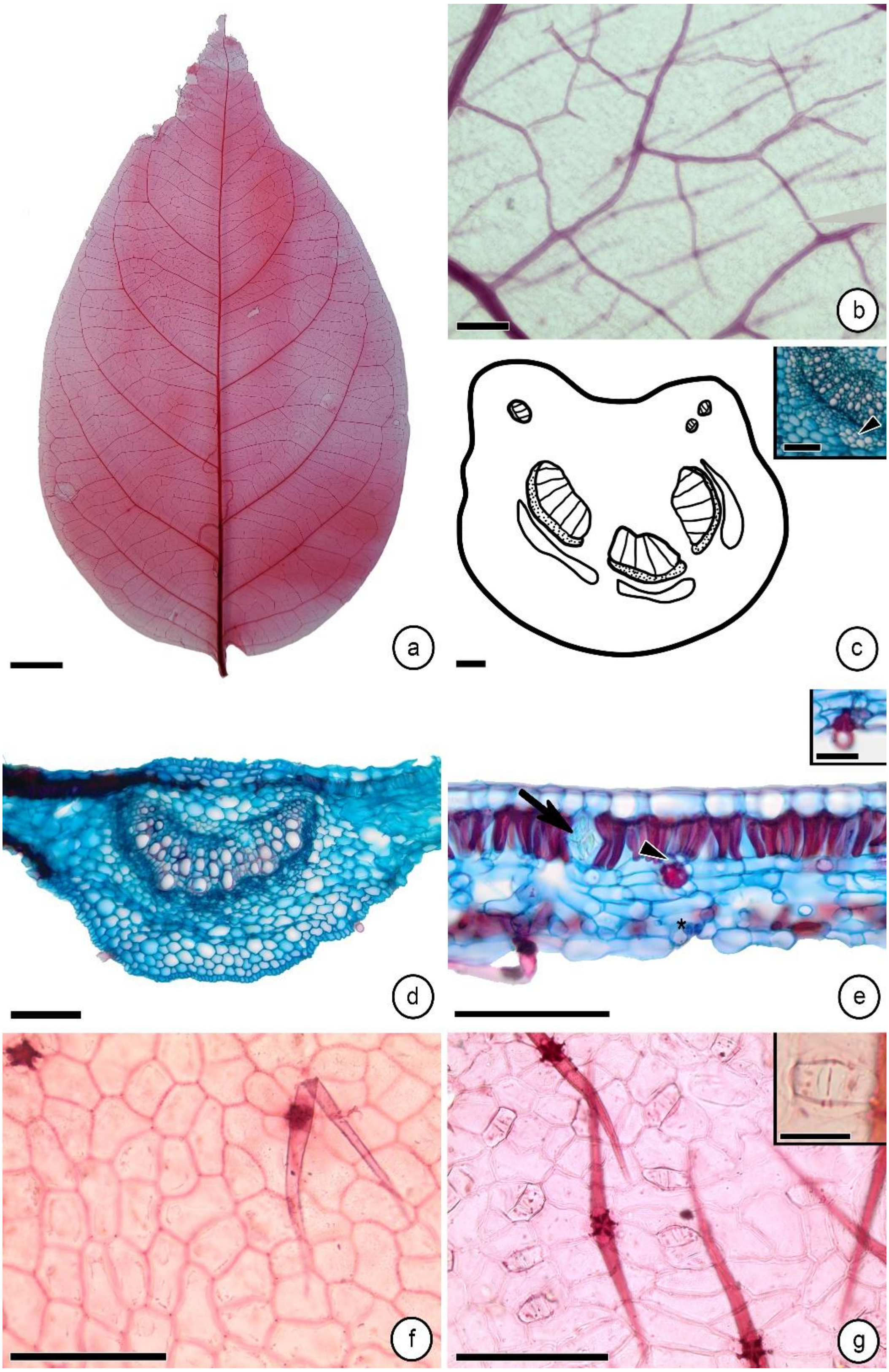

Figure 17. *B. muricata* leaf, a-b-diaphanized leaf; c-diagrammatic scheme of the petiole cross-section, d-e-transverse sections; f-g-paradermal preparations. a. Leaf, with general venation pattern. b. Detail of moderately-developed areoles. Note parallel pattern in trichomes. d. Midrib. e. Leaf blade: mesophyll with prismatic crystals (arrow), vascular bundles with parenchymatic sheath (arrowhead), stomata in adaxial epidermis (*) Detail: lignified insertion of hair with virtually no stalk. Note idioblasts. Adaxial epidermis: straight to curved walls, sparse trichomes g. Abaxial epidermis: straight to curved walls, sparse trichomes, paracytic stomata (detail). Dot-filled areas=phloem; stripped areas=xylem; blank area=non-lignified sclerenchyma. Scale bars: a=1cm; b-g=100 µm; d detail=50 µm; g detail=10 µm.

5. DISCUSSION

5.1. Wood Morfoanatomy

<u>Common features</u>

Many qualitative features common to *B. laevifolia*, *B. muricata* and *D. pubipetala* conform to the expected for Malpighiaceae family: simple perforation plate; narrow vessel in radial disposition; vestured pits; septate fibers; abundant prismatic crystals in axial parenchyma; tall heterocellular mixed rays and perforated ray cells (Solereder, 1908; Metcalfe & Chalk, 1950; Domingues, 2008; Amorim *et al.*, 2017; Cabanillas, Pace, & Angyalossy, 2017; Pace *et al.*, 2018). Other common attributes are well documented for lianas in general, consistent with the lianescent vascular syndrome proposed by Angyalossy *et al*. (2015), such as vessel dimorphism and tall rays. We speculate that these last traits are not usually reported for the whole family because lianas are derived within Malpighiaceae (Davis & Anderson, 2010; Pace, 2016).

Simple perforation plates are admittedly dominant in tropical areas as Brazil (see (Dickison, 1989; Alves & Angyalossy-Alfonso, 2000), being understood as an adaptation to greater conductivity in dryer and warmer climates (Baas & Schweingruber, 1987). Thus, it is not surprising that this trait would be found in species studied here and, further, be so prevalent in a family with such a tropical distribution as Malpighiaceae. Nevertheless in the same family, Sonsin *et al.* (2014) report foraminate perforation plate in three *Byrsonima* Rich. ex Kunth species collected in Cerrado. Still, Carlquist (1991) showed that lianas often bear simple plated vessels even when included in lineages which have close relatives with multiple plated vessels. This makes sense, if we acknowledge that simple plates offer less resistance to water flow, which is so important in lianas as water has to reach long distances through their lengthy stems (Christman & Sperry, 2010; Isnard & Field, 2014).

Multiple vessels may spread embolism when it occurs, as verified by Knipfer *et al*. (2015) in *Juglans microcarpa* Berland. wood under drought stress and by Fukuda *et al*. (2015) in radial series of vessels found in *Betula platyphylla* var. *japonica* (Miq.) Hara. One could think that this could be a problem for the species studied here, since at least 60% of their vessels are multiple, sometimes reaching, in a single cluster, more than 20 vessels. However, Fukuda *et al*. (2015) also concluded that the susceptibility of multiple vessels of suffering cavitation is significantly reduced if there are more vessels of small diameter in *J. microcarpa*. This might be applied to the species studied here as well, since these large clusters are always of narrow vessels. Furthermore, other mechanisms are expected to take part to avoid this problem.

It is long accepted that bordered pits are an optimal and effective construction, which aid passage of water while restricting air bubbles (Carlquist, 1988; Baas & Wheeler, 1991; Jansen, Smets, & Baas, 1998; Choat, Cobb, & Jansen, 2008). Further, the most likely theory for vestured pits is that they may assist this avoidance of embolism by increasing amounts of water near the wall, in addition to allowing it to endure higher tensions (Carlquist, 1982; Jansen *et al.*, 1998), although Jansen *et al.* (2003) question if this is also applied for plants in dryer and warmer climates. Still, the presence of bordered and vestured pits in the studied species may compensate the vulnerability to embolism that their wide vessel elements are subject to.

Still regarding water transport, it has been suggested that perforated ray cells are important in efficiently maintaining it in different periods of the year (Machado & Angyalossy-Alfonso, 1995). Here, the perforated ray cells were only found connecting in narrow vessels; again, this water transport maintenance is even more crucial in the long stems of lianas (Isnard & Field, 2014).

In this matter, vessel dimorphism is also of vast importance in lianas, as narrow vessels ensure water conduction when wider vessels undergo embolism, while the latter offer lower resistance to water to flow for long distances (Carlquist, 1985; Ewers, 1985). As discussed by Carlquist (1985), larger diameter in vessels may compensate for the relatively small cross-sectional area of the lianas, as it decreases the friction of water with vessel wall and exponentially increases the internal flow, rendering water conduction extremely efficient.

The horizontal alignment of vessel elements as viewed on radial section reported here for the three species (Fig. 9d) does not seem to have been acknowledged or described in previous works (as a storied structure is determined from the tangential section only, IAWA Commitee, 1989). Even so, this aspect can be found in different degrees in many species which have radial pattern, both within Malpighiaceae and in other families (see, for instance, *Byrsonima basiloba* A.Juss., *Baccharis trimera* (Less.) DC. and *Styrax ferrugineus* Nees & Mart. in Sonsin *et al.*, 2014). Ontogenetic studies may provide a better understanding of this characteristic, on which radially adjacent vessel elements seem to have a synchronized development from radially aligned fusiform initials. As this feature may be relevant in descriptions, we here suggest reporting it as “radially storied vessel elements”. Further analysis should be done, but we expect that this trait also aids conduction, as the occurrence of perforation plates at the same level may cause adjacent vessels elements to function as one, but avoiding the risks of being susceptible to cavitation such as for wide vessels.

Gelatinous fibers were also found in abundance on every observed specimen (Fig 9b-detail). This may have a close relation with their lianescent habit, as, according to Bowling & Vaughn (2009), the fast swelling of the gelatinous layer allows twining in vines stems, producing similar tension as in reaction wood in trees. Indeed, Fisher & Blanco (2014) also shown that the stem curvature in *Bauhinia glabra* Jacq. liana was only possible with alternate regions with gelatinous fibers on concave sides of undulations. Furthermore, these authors speculated that these undulations are of adaptative valor, aiding flexibility of the vine. The studied lianas might be benefited in the same manner. Also, *B. laevifolia* seems to have developed an even more specialized organization of gelatinous fibers, on which each axial parenchyma lines (lignified or not) are commonly followed by an external area of gelatinous fibers, which in turn is adjacent to an area with no gelatinous fibers just before the next line (Fig.7b-detail). We speculate that this alternate arrangement of these fibers – found similarly in *B. caapi* by J. Sonsin-Oliveira, C. W. Fagg, N. Nagamine-Pinheiro, J. A. Pereira, R. C. Oliveira (unpubl. data.) – grants great deals of flexibility, which is so indispensable in the habit.

Relative high amounts of parenchymatous tissues in the studied species may also reflect this chase for flexibility, which we habitually reckon in lianas. This would explain, for instance, why all nine specimens have rays with more than 1 mm high, which is not expected for the family (see Metcalfe & Chalk, 1950; Amorim *et al.*, 2017; Pace *et al.*, 2018), but was also found by Cabanillas *et al*. (2017) in lianas of *Callaeum* Small genus.

Regarding axial parenchyma, the studied plants have scanty, vasicentric and diffuse axial parenchyma as most dominant types, which was also found for two variants of *B. caapi* used for Ayahuasca (Sonsin-Oliveira *et al*., 2016). Also, they all have non-lignified parenchyma, which, according to Angyalossy *et al.* (2015) and Carlquist (1985), retains its meristematic capacity and allows the secondary xylem to undergo structural changes. It can also aid the repair of injuries to which the lianas are exposed and increases their flexibility and storage capacity (Carlquist, 1985; Ewers & Fisher, 1991). Additionally, it seems that bands of non-lignified parenchyma in the studied species acquire cambium activity to form secondary phloem and perhaps even secondary xylem, similarly as the interxylary cambium seen in Pace *et al*. (2018), but further ontogenetic analysis are to be performed to truly understand it.

Still, it was possible to acknowledge distinct cambial variants in *B. laevifolia, B. muricata* and *D. pubipetala*, which may provide special advantages over a regular cambium. Indeed, anomalous secondary growth with interxylary phloem has been reported as common for Malpighiaceae (Metcalfe & Chalk, 1950; Pace, 2016). It can benefit not only conduction in lianas but provide greater flexibility and protection for their stem, as it usually intercalates soft and stiff tissues (Pace, Lohmann, & Angyalossy, 2009; Isnard & Field, 2014; Angyalossy *et al.*, 2015).

Distinctive features

Other features were better to separate the species, supported by ANOVA tests and PCA analysis. For *Diplopterys pubipetala*, the most distinctive characteristic from *Banisteriopsis* species was perhaps the presence of prismatic crystals in upright and enlarged ray cells, usually in pairs. Prismatic crystals in ray cells are not rather uncommon in Malpighiaceae (see Andrade, 1997) and crystals in marginal parenchyma have already been acknowledged marking ring boundaries by Gourlay (1995) in acacias. Nonetheless, their tangential alignment in ray cells as described here has not been yet reported. The reasons underlying this arrangement and exact mechanisms of development shall still be explored in a separate study.

In addition, this species was separated by its thinner rays, most with procumbent cells with over 4 rows of upright and/or square marginal cells; thicker fiber walls; and less frequency of solitary vessels. In turn, both *Banisteriopsis* species had tracheids, tyloses in vessels and irregular lines of axial parenchyma, characteristics not found on *D. pubipetala*. These differences summed with our cluster analysis corroborate the placement of this species in a clade distinct from *Banisteriopsis*, proposed by Anderson & Davis (2006).

Banisteriopsis laevifolia could be distinguished from the other two species mainly by its rays about twice as tall, narrower vessels, smaller pits and lower density of vessels, besides the already mentioned gelatinous fibers arrangement. One particular cambial variant found on *B. laevifolia* seems to derive from a close variation of the interxylary cambia cited by Pace *et al*. (2018), on which cambial tissue appears within the xylem, but not in a scale-like pattern as the authors described. As they found on *B. nummifera* group, *B. laevifolia* of this study seems to exhibit a centripetal differentiation from non-lignified axial parenchyma bands near the regular cambium to the center, but further investigative analysis is still being performed.

As for *B. muricata*, the rays were of distinct sizes, the wider commonly having more than 4 cells in width. All ray cells procumbent was also found only on this species. Additionally, analysis of variance isolated this species for its longer fiber and wider vessels, which led them to the last class of IAWA Committee (1989) for vessel lumina (mean tangential diameters greater than 200 µm). *B. muricata* specimens were the ones with greater development and most remarkable lianescent habit, when compared with the other two species, which were smaller and sometimes still seeking for better subtracts to climb on. Possibly, some of their distinctive features are related to a better adaption for this habit. Larger rays in lianas, for instance, increases their mechanical flexibility, allowing twisting and adjustments to the different rates of growth, which prevents tissue from rupturing, in addition to increased regeneration capacity when external damage occurs (Carlquist, 1985). This could explain why rays with more than 4 cells in width were so much more common in this species. Wider vessels may too be related to their developed habit, as they are also very characteristic of lianas (Carlquist, 1985; Angyalossy *et al.*, 2015).

Furthermore, both intervessel and ray-vessel pits had higher measurements for *B. muricata* then for the remaining species. This finding is possibly related to the environment on which they were collected (Amazon Forest-*B. muricata*; Cerrado-*D. pubipetala* and *B. laevifolia*). Carvalho *et al.* (2015) shown that lianas from dryer environments have smaller pits than those from moist forest in order to resist to cavitation, which may apply for the species studied here.

Semi-ring-porous woods, found in *B. muricata*, have been also reported to *B. oxyclada* and are present in at least one stage of development in most Bignoniaceae lianas (Andrade, 1997; Lima, Pace, & Angyalossy, 2010). Indeed, IAWA Committee (1989) state that it is not unusual for many plants to range from semi-ring-porous to diffuse-porous. Furthermore, this porosity can follow the same logic as described for ring-porosity and vessel dimorphism in general, on which efficiency of wide vessels are combined with safety provided by numerous narrow vessels at diverse stages of development (Baas & Schweingruber, 1987). This could accordingly explain the high occurrence of semi-ring porosity in some liana groups (eg. Brandes, Lisi, & Barros, 2011).

Histochemistry

The active principles in *B. caapi* are the β-carboline alkaloids harmine, harmaline and tetrahydroharmine (Callaway, Brito, & Neves, 2005). So, our finding of great quantities of alkaloids in *B. laevifolia*, *B. muricata* and *D. pubipetala* was expected, based on the fact that they all are used in Ayahuasca analogs, and preliminarily supports this alternative use. *B. muricata*'s notable high concentrations may explain why this species is one of the most frequently reported as used in replacement of *B. caapi*, being even called *falso jagube* ("false jagube; jagube=*B. caapi*") in Acre (Oliveira, R.C., personal communication). Also, results of the present work contradicted what was found for *D. pubipetala* by Santos *et al.* (2015), who did not detect alkaloids in its bark.

Higher concentrations of alkaloids in the bark than in wood were also anticipated. In Wang *et al.* (2010)'s study, bioactive markers in *B. caapi* were mostly found in dried bark and, secondly, in whole dried stems. This discrepancy could even be used to improve management plans of extraction, on which the plant could be spared in the beverage's preparation with techniques that only removes parts of the bark. This method has been reported by Davis & Yost (1983) referring to Waorani Indians using *B. muricata* and could likewise be extended to the usage of other species.

However, these findings by Wang *et al.* (2010) applied to matured stems only. Indeed, young *B. caapi* stems exhibited low concentrations of alkaloids in our work, both in bark and wood. Still, Callaway *et al.* (2005) found that older plants (with around 8 years old) also had low levels of harmala alkaloids. Thus, there might be an optimum stage when these plants concentrate highest amounts of alkaloids, suggesting that they should be collected at this age for a best yield. In addition, the β-carboline alkaloids in these species could be more deeply explored in the medical field, for they seem to have a broad antimicrobial effects (de Frias, 2012).

This presence of alkaloids does not seem to be so constant among the whole Malpighiaceae family, being more restricted to some liana groups such as *Banisteriopsis*, *Diplopterys, Tetrapterys* and *Stigmaphyllon* (see Ghosal & Mazumder, 1971; de Frias, 2012; Guilhon-Simplicio *et al.*, 2013; Queiroz *et al.*, 2015; Guimarães *et al.*, 2016). The broad prevalence of alkaloids in *Banisteriopsis* species and close relatives might explain why there is such a variety of alternative species used for Ayahuasca. We believe that the common presence of such alkaloids in these genera summed with the difficulties of discrimination between species within them entail people to collect different plants that look alike but bring on similar effects, with little concern to precisely identify and use solely *B. caapi*. Yet, supplementary phytochemical analysis is needed, and caution should be taken to avoid intoxications, as some alternative species may contain additional toxic alkaloids such as bufotenine (eg. *Tetrapterys mucronata* Cav. in Queiroz *et al.*, 2015).

In a cellular level, vessel contents had positive reactions only for lipids in *D. pubipetala* and for essential oil and resin acid in *B. caapi*. Most chemical compounds (alkaloids, tannins, phenolic compounds, essential oil and lipids) were found in parenchymatous tissues both in secondary xylem and phloem. This is not surprising, as these cells are alive and not infrequently harbor diverse substances in them (Appezzato-da-Glória & Carmello-Guerreiro, 2006).

Some of these compounds may be also present in leaf idioblasts, which were abundant in all species studied here. Indeed, the same harmine alkaloids previously mentioned was found in lower concentrations in *B. caapi*'s and *B. muricata*'s leaves (Ghosal & Mazumder, 1971; Wang *et al.*, 2010). On the other hand, this chemical class was not found neither in *B. laevifolia*'s leaves - which had flavonoids, terpenoids, phenols, steroids and saponins instead – nor in *D. pubipetala*'s leaves – which, in its turn, had positive tests for tannins and also flavonoids, terpenoids, steroids and saponins (Sacramento *et al.*, 2014; Santos *et al.*, 2015; Nunes, 2016). Nunes (2016) speculates that this absence of alkaloids would be related to climate and soil differences, but we believe that further studies, perhaps comparing metabolites in leaves and stems or in plants in different stages of development and at different times of the year, could better elucidate this matter.

Also, Andrade (1997) verified a dense cytoplasm in companion cells of *B. oxyclada* and other Malpighiaceae species. This could partly refer to lipids, which were located in these cells of *D. pubipetala* in this work. Also in relation to *D. pubipetala*, our findings of tannins, phenols and saponins conforms to what was found by Santos *et al.* (2015) in its bark. These metabolites, also present in the other studied species, can be of great value for human use.

Phenolic compounds, for instance, have antifungal properties and may be related to avoidance of herbivory (Oki, 2005). They were extensively found in the phloem of Malpighiaceae species, such as in *Niedenzuella acutifolia* (Cav.) W.R.Anderson by Andrade (1997), in *Byrsonima japurensis* by Guilhon-Simplicio *et al.* (2013) and in *Banisteriopsis nummifera* by Pace *et al.* (2018), this last abundantly in its phloem and secondly in the wood. The phenolic compounds that we found in our species might be largely composed of tannins, since these metabolites are broadly found in Malpighiaceae family and their location did coincided (Cronquist, 1988).

Positive tests of saponins in the studied species may be promising, as they are also important for having bactericide and fungicide properties (Santos *et al.*, 2015). Their presence, in particular at relative high levels in *B. muricata*, raises the question on how would they be interacting with harmine alkaloids. Milugo *et al*. (2013) showed that alkaloids and saponins appeared to have an antagonist effect, exhibiting lower antioxidant activity when combined. If this is the case for the species used in Ayahuasca such as the ones we studied, the effects of the beverage may even be masked at some level.

Essential oils were found in the bark and in the wood near the cambium mostly in axial parenchyma cells. They also can have many useful effects, ranging from antibacterial and antifungal activity to insecticidal and cosmetic applications (Bakkali *et al.*, 2008; Miller *et al.*, 2015). Not many tests have been performed to investigate essential oils in different organs of *Banisteriopsis* and related species. Rocha *et al.* (2018) investigated it in flowers of *B. campestris*. In *B. laevifolia*, Araújo & Meira (2016) revealed that these essential oils are not secreted by leaf glands, and Nunes *et al.* (2015) revealed that its leaves have an extremely low yield for these compounds. We here suggest that some focus should also be given to the bark when studying essential oils in these species.

One type of cell that had almost no reaction and staining was gelatinous fibers. Mellerowicz & Gorshkova (2012) explain that the crystalline cellulose in the G-layer of gelatinous fibers justifies why it hardly ever gets stained. In our ruthenium red test, the slight pink staining of this layer corroborated their speculation that spaces between these cellulose macrofibrils were occupied by pectins and proteins. In addition, the abundance of gelatinous fibers in these lianas could be of great value if we acknowledge the novel technologies that may use then, such as in biofuel making, saccharification processes and development of biomimetic materials (Mellerowicz & Gorshkova, 2012).

It is well accepted that *Banisteriopsis* species have sundry bioactive metabolites that can be useful for human health (Rodrigues & Carvalho, 2001; Frias, Costa, & Takahashi, 2011; de Frias, 2012; Souza & Grael, 2014). The histochemical tests that we performed here supports that the studied species contain compounds of potential pharmacological use. *D. pubipetala* is already used in wound treatment and itchiness (Sacramento *et al.*, 2014). *B. laevifolia*'s roots are used as anti-inflammatory and branches with leaves and flowers in renal problems (Rodrigues & Carvalho, 2001). Nunes (2016) shown the potential antioxidant and antifungal activity of its leaves, but the essential oil had a low yield when extracted from these organs. So, we believe that further studies of bark composition of these species could allow better results and perspectives for their popular medicinal use.

5.2. Leaf Morphoanatomy

Regarding leaf features, the most distinctive traits were its general shape as well as aspects and distribution of trichomes and glands. These last have been factually mentioned as prevalent in Malpighiaceae family and important for taxonomical identification of genus and species within it (Metcalfe & Chalk, 1950; Gates, 1982; Elias, 1983; Cronquist, 1988).

While *D. pubipetala* and *B. laevifolia* gland's shape confirmed the described ones in previous works, there seems to be no accordance in relation to *B. muricata* (Possobom, 2008; Araújo, 2014). Araújo (2014) claims that they are sessile as found in the present work; nevertheless, Nery et al. (2017) describe them as pedunculated. At UB Herbarium, both morphotypes can indeed be found, raising the query of whether this would be just an expected variation, as it has a wide distribution among different biomes, or if two distinct plants are being called by the same name. In addition, it has been described by the same authors and by Gates (1982) that *B. muricata* may have petiolar glands, although they were not found in our study. This may be a variation according to environment, since their absence is also reported for this species.

In Malpighiaceae, these leaf glands are so called extrafloral nectaries and are prevalent in most liana species of the family (Elias, 1983; Possobom, 2008). It has been suggested that these nectaries have a key role in attracting ants, which would offer the plant some defense against herbivory, even for the three species studied here, as pointed by Nery *et al.* (2017), Possobom, (2008) and Araújo (2014). This latest author worked only with herborized material, thus could not verify this hypothesis in field; we can, however, corroborate it as ants could indeed be observed visiting *B. laevifolia* (Fig. 5d).

As previously mentioned, *D. pubipetala* leaf glands are significantly smaller than for *Banisteriopsis* species. Elias & Gelband (1976) suggested that minutes glands can be compensated for their abundance, conferring an advantage as they increase the area visited and protected by ants. This might be the case for *D. pubipetala*, what could be corroborated by the fact that these glands are all along the leaf border in this species. In addition, Levin (1973) concluded that pubescence in leaves can hamper the passage of insects; this could to some extension explain the position of glands near the margin and near the base on studied species and other Malpighiaceae, as these areas would be more easily accessible.

In the matter of trichomes, Gates (1982) defines “malpighiaceous” hairs as unicellular and medifixed, often slightly eccentrically attached in *Banisteriopsis*. In the present work, only for *B. laevifolia* was the difference between hair arms not statistically significant. Additionally, while trichomes in this species were strictly T-shaped with twisted arms and very long stalks, *B. muricata* and *D. pubipetala* had almost no stalk. This morphological variance is expected within *Banisteriopsis* genus, which used to include *Diplopterys* as well (Metcalfe & Chalk, 1950; Cronquist, 1988; Gates,1982). Also, *B. laevifolia* have a peculiar radiate arrangement of pedal cells, which might be of taxonomic value.

The difference in overall pubescence, can further be influenced by the environment in which the specimens were collected. While *B. muricata,* collected in the shade of an Amazon forest, have a sparse pubescence, *B. laevifolia* from an open Cerrado even exhibits a discolor pattern associated with the abundant trichomes on its abaxial surface. It is well supported that these structures can be important and more prevalent on dryer environments, as they reduce air movement on leaf’s surface and, thereafter, decrease transpiration rates, besides reflecting radiation to prevent overheating and reducing insect herbivory (Ehleringer & Mooney, 1978; Fahn & Cutler, 1992).

Brochidodromous frameworks are common in several genera of Malpighiacae, particularly frequent in Cerrado species (Mamede, 1993). While general venation pattern seems to be quite stable, variation of major secondary angle to midvein and areoles development was not the same for the studied specimens, which could be helpful in other investigative works on the family.

Petiole aspects were also quite distinctive between species, as found by Araújo (2014). Metcalfe & Chalk (1979) agree that petiole anatomy is stable and, thus, reliable for differing taxa. Despite the vascular system on *D. pubipetala* and *B. laevifolia* is divided at its base, it is only in *B. muricata* that it remains in separated bundles, which could then be a diagnostic feature (Fig. 17c). In addition to Araújo (2014)’s description, we bring that *B. laevifolia* can have both plane-convex and concave-convex outlines. Petiole length can also be quite distinctive, as claimed by Gates (1982), specially the short ones in *B. laevifolia*. It is also relevant to mention that *D. pubipetala*’s petiole shown a greater degree of lignification. This would be predictable, as their leaves were thicker and broader, requiring more rigid tissues, which would cause them to be more erect. Although the number of accessory bundles on petiole is often considered of taxonomic value, their exact number may not be rather reliable, as it can vary within the species of *Banisteriopsis* and *Diplopterys* (Araújo, 2014). In Chrysobalanaceae, accessory bundles in the petiole seem to distinguish species, but there are other reports of their inconsistency within the same species (Shaheen, 2007; Corrêa, Araújo, & Scudeller, 2018). So, in addition to what da Silva *et al.* (2015) suggested as essential to distinguish species (mostly midrib traits), there are many different and useful features in the petiole.

Anatomical characteristics proven to be more distinctive than morphological ones. For instance, differences of wall and cuticle thickness on adaxial and abaxial epidermis were greater for *D. pubipetala* and *B. laevifolia*. Nevertheless, it may not be a constant taxonomic trait, as *B. muricata* was collected in a much more humid environment (Amazon forest, in contrast to Cerrado) and, thus, adaxial epidermis does not suffer the same pressure to be as thick as if it was on a dryer ambient, since thickness of epidermal cell wall and cuticle is inversely proportional to epidermal water loss (Ristic & Jenks, 2002). In addition, these large adaxial epidermal cells are not uncommon in the family (Metcalfe & Chalk, 1950).

Several other traits that we found followed descriptions for Malpighiaceae, such as dorsiventral lamina (which is only rarely isobilateral), druses or solitary-prismatic crystals in the mesophyll, paracytic stomata in the lower surface, arc-shaped vascular strand with accessory ones in the petiole and idioblasts (Watson & Dallwitz, 2018; Metcalfe & Chalk, 1950). Druses, for instance, were also found by Andrade (1997) in palisade parenchyma of *B. oxyclada*, which seems to be very common in the genus.

Ledges formed by the cuticle on the guard cells were also described by Araújo (2014). They were found on *D. pubipetala* and *B. laevifolia* (Fig. 15e-detail), indicating that they are aiding ostiole closure as an additional sealing and maybe retaining water, in order to avoid water loss in this frequently dry environment (Mauseth, 1988; Fricker & Willmer, 1996). Also, we can see that the guard cells have a colored content (which may refer to the presence of chloroplasts), while vacuoles are the predominant organelles in the subsidiary cells, which exhibit translucent aspect of the protoplast.

Two layered epidermis in some regions has been registered for *Tetrapteris*, *Janusia* and other *Banisteriopsis* species, sometimes related to mucilaginous secretion (Andrade, 1997; Araújo, 2014; Metcalfe & Chalk, 1950). Their presence in *B. laevifolia* confirms what was found by Araújo (2014) for this species. It is important to mention, though, that this characteristic was not so clear at first: folding walls could give the impression that what we observed as horizontal lines was from the same cell; bilayered condition could only be endorsed as transverse sections with no staining treatment were analyzed and content in only one of the two cells was identified.

Almost half of *B. laevifolia*'s parenchyma was palisade, while it represented 1/3 of the other mesophyll for the others. Fahn & Cutler (1992) affirm that it can conduct water to the epidermis more efficiently than spongy; a greater area of this parenchymatous type could be an adaptative advantage in biomes like Cerrado, increasing photosynthetic area. We can also see that this parenchyma almost invades the midvein for all three species, indicating a putative tendency to increase this area.

Leaf features of species from the present study seem to coincide with what was found for most *B. caapi* ethnotypes (Mystery, Ourinho, and Tucunacá), such as only one vascular bundle in the midrib and one palisade parenchyma layer with narrow cells (Araújo *et al.*, 2016).

6. CONCLUSIONS

Our findings reveal some new morpho-anatomical and histochemical data for understanding different lianas used in Ayahuasca analogues. To sum up, we conclude that:

- it is possible to separate *B. laevifolia*, *B. muricata* and *D. pubipetala* both by wood and leaf morpho-anatomy, and their descriptions can support identification in vegetative state;
- the main qualitative differences in wood anatomy were related to growth rings definition, porosity, presence of narrow bands of axial parenchyma, presence of prismatic crystals in ray cells and ray composition; quantitative measurements had significant differences for every character, vessel element length and ray width being particularly relevant in the PCA;
- novel descriptions in wood anatomy are presented: the tangential alignment of prismatic crystals in ray cells in *D. pubipetala*, sometimes following a growth ring; and the horizontal alignment of vessel elements as viewed on radial section, i.e. "radially storied vessel elements";

- the distinctive taxonomic characters for leaf morphology referred to: petiole length and shape; lamina shape; glands position, size and shape; hairs position, size, shape and insertion; angle of major secondary veins to midvein variation; areolation; and marginal ultimate venation;
- regarding leaf anatomy, distinctive traits were related to: epidermis wall+cuticle thickness; adaxial cells shape; epidermis layers; stomata position, density and ledges; palisade and spongy parenchyma heights and proportions; mineral inclusions; sheath extension of secondary bundles; midvein shape; and petiole characteristics;
- cluster analysis using different morphological and anatomical characters of the stem and leaf reinforces the proposal of Anderson & Davis (2006), on which *Diplopterys* and *Banisteriopsis* appear as distinct groups;
- histochemical analysis revealed the presence of important compounds of potential pharmacologic use: alkaloids, saponins, essential oils, lipids, pectins, tannins and general phenolic compounds, mostly in parenchymatous tissues in the bark and, secondly, in the wood (except for lipids in *D. pubipetala*, which were found inside vessels and in companion cells, and for the presence of resin acids and essential oils in vessel contents of *B. caapi*;
- *B. laevifolia*, *B. muricata* and *D. pubipetala* seem to be reliable alternatives to *B. caapi* in Ayahuasca due to the presence of alkaloids in the stem, but further studies are needed to ensure its safety of use;
- innovative extraction techniques which uses solely strips of bark in Ayahuasca preparations could be an option specially when used in small quantities, avoiding the death of the vine and possibly improving forest management related to the production of this brew.

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APPENDIX 1

Wood Descriptions

Diplopterys pubipetala (A.Juss.) W.R.Anderson & C.Davis

Growth rings: poorly defined, marked by lines of marginal axial parenchyma or, more rarely, radially flattened latewood cells.

Vessels: diffuse porosity; vessels of two distinct sizes (vessel dimorphism); vessels solitary (32%), multiple of 2 to 4 (49%) and up to 20 narrow vessels radially arranged or in clusters; simple perforated plates; intervessel pit alternate, circular and vestured; ray-vessel pits similar to intervessel in shape and size; few vessels with an amber colored content.

Fibers: with simple to minutely bordered pits, thin to thick-walled; septate fiber; abundant thick gelatinous fibers.

Tracheid: absent.

Axial parenchyma: vasicentric, scanty paratracheal, confluent, marginal lines of non-lignified parenchyma, diffuse, diffuse-in-aggregate; mostly 2 to 4, up to 8 cells per parenchyma strand.

Ray: width 1 to 3 cells, up to 5; more than 1 mm tall; body ray cells predominantly with procumbent, square and upright cells mixed throughout the ray and with procumbent with over 4 rows of upright and/or squares marginal cells, some with 1-2 rows, and also all ray cells upright and/or squares; sheath cells; perforated ray cells; disjunctive ray cells; ray cells may have with content.

Storied structure: absent, only radially storied vessel elements (when viewed in radial section).

Mineral inclusion: prismatic crystals in chambered axial parenchyma cells (lignified and non-lignified) and in enlarged cells of axial parenchyma; prismatic crystals in ray cells, usually in pairs in chambered enlarged upright cells, sometimes tangentially aligned in transverse section.

Banisteriopsis laevifolia (A.Juss.) B.Gates

Growth rings: absent to poorly defined

Vessels: diffuse porosity; vessels of two distinct sizes (vessel dimorphism); vessels solitary (40%), multiple of 2 to 4 (47%) and up to 20 narrow vessels in clusters or radially arranged; wider vessels sometimes tangentially oriented; simple perforated plates; intervessel pits alternate, circular and vestured; some polygonal intervessel pits in narrow vessels; ray-vessel pits similar to intervessel in shape and size; tyloses (rare); few vessels with amber to yellow deposits.

Fibers: with simple to minutely bordered pits; thin to thick-walled; septate fibers; abundant thick gelatinous fibers, normally alternating with axial parenchyma.

Tracheid: very rare.

Axial parenchyma: in lines (some of non-lignified parenchyma), discontinuous lines, vasicentric, scanty, confluent, few diffuse and diffuse in aggregate; mostly 2 to 4 up to 8 cells per parenchyma strand.

Ray: width 1 to 3 cells, up to 4; much more than 1 mm tall; body ray with square and upright cells mixed throughout the ray, some with all cells upright and/or square; perforated ray cells; sheath cells; few disjunctive ray cells; ray cells may have with content.

Storied structure: absent, only radially storied vessel elements (when viewed in radial section).

Mineral inclusion: prismatic crystals in chambered axial parenchyma cells; prismatic crystal in ray cells observed just once.

Banisteriopsis muricata (Cav.) Cuatrec

Growth rings: often poorly defined, marked by marginal bands of axial parenchyma and/or semi-pouros ring.

Vessels: diffuse porosity or semi-porous ring; vessels of two distinct sizes (vessel dimorphism); vessels solitary (40%), multiple of 2 to 4 (44%) and up to 34 narrow vessels radially arranged or, less frequently, in clusters; simple perforated plates; intervessel pits alternate, circular and vestured, some polygonal intervessel pits in narrow vessels; ray-vessel pits are similar to intervessel in shape and size; tyloses in few vessels; whitish deposits in few vessels.

Tracheids: rare.

Fibers: with simple to minutely bordered pits; very thick-walled, very thin and thin to thick-walled only near the pith; very rare septate fibers.

Axial parenchyma: in lines, sometimes irregular, non-lignified parenchyma sometimes adjacent to regular lines), vasicentric, scanty, few confluent, few diffuse and diffuse in aggregate, may have marginal bands; 2 to 4 per parenchyma strand.

Ray: width 1 to up to 8 cells; rays of 2 distinct sizes; more than 1 mm tall; some aggregate rays; body ray cells procumbent with one to 4 rows of upright and/or square marginal cells and, rays with procumbent, square and upright cells mixed throughout the ray, and few all ray cells upright and/or square; few sheath cells; few disjunctive cells; perforated ray cells; ray cells often with content.

Storied structure: absent, only radially storied vessel elements (when viewed in radial section).

Mineral inclusion: in chambered axial parenchyma cells in radial chains, in enlarged cells of axial parenchyma.

APPENDIX 2

Leaf Descriptions

Diplopterys pubipetala (A.Juss.) W.R.Anderson & C.Davis
Leaf simple; opposite; elliptic to large elliptic; margin entire; lamina ratio 2:1; sometimes discolor; apex acute to obtuse, strongly acuminate, tip drip 1.53±0.32 length; base acute to obtuse, convex to rounded; adaxial face glabrous, abaxial face glabrescent; venation festooned brochidodromous; petiole 0.71±0.13, concave-convex.

Banisteriopsis laevifolia (A.Juss.) B.Gates
Leaf simple; opposite; elliptic to narrow elliptic or narrow ovate to lanceolate; margin entire; lamina ratio 2-3:1; discolor; apex acute, straight to slightly acuminate; base acute, cordate; adaxial face glabrescent, abaxial face densely pubescent; venation festooned brochidodromous; petiole 0.71±0.13, plane-convex to concave-convex.

Banisteriopsis muricata (Cav.) Cuatrec
Leaf simple; opposite; ovate to wide ovate; margin entire; lamina ratio 2:1; discolor; apex acute, straight to slightly acuminate; base acute, convex to rounded; adaxial and abaxial faces sparsely pubescent with parallel hairs; venation festooned brochidodromous; petiole 1.17±0.44, concave-convex.