



CARLOS BERNARD MORENO CERQUEIRA SILVA

**DIVERSIDADE GENÉTICA MOLECULAR E REAÇÃO A DOENÇAS DE ACESSOS
SILVESTRES E COMERCIAIS DE MARACUJAZEIRO (*Passiflora* spp.) PRESENTES
EM BANCOS DE GERMOPLASMA**

***MOLECULAR GENETIC DIVERSITY AND DISEASE REACTION OF COMMERCIAL
AND WILD ACCESS OF PASSION FRUIT (*Passiflora* spp.) PRESENT IN
GERMPLASM BANKS***

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UNIVERSIDADE ESTADUAL DE CAMPINAS
Instituto de Biologia



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***MOLECULAR GENETIC DIVERSITY AND DISEASE REACTION OF COMMERCIAL AND WILD
ACCESS OF PASSION FRUIT (*Passiflora* spp.) PRESENT IN GERMPLASM BANKS***

Tese apresentada ao instituto de Biologia da
Universidade Estadual de Campinas como parte dos
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em Genética e Biologia Molecular na área de Genética
Vegetal e Melhoramento

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Genetic Breeding*

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Este exemplar corresponde à versão final da tese
defendida pelo aluno Carlos Bernard Moreno Cerqueira
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Anete Pereira de Souza

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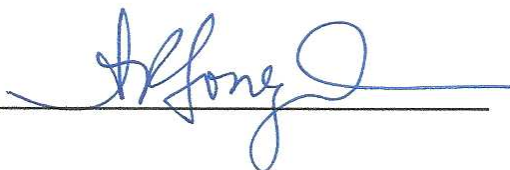
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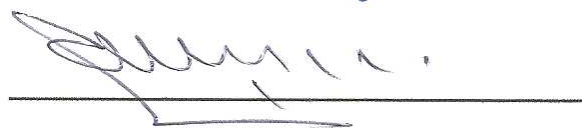
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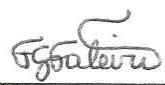
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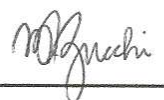
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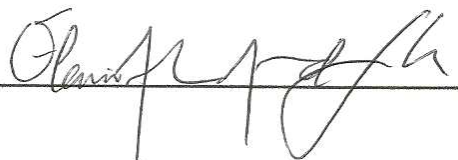
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Resumo

O gênero *Passiflora*, cujas espécies são popularmente conhecidas como maracujazeiros, destaca-se na família Passifloraceae tanto pelo número de espécies (aproximadamente 520) quanto pela importância econômica associada à parte destas espécies. Os maracujazeiros ocorrem em diferentes países, sendo sua diversidade amplamente representada nas Américas, onde a Colômbia e o Brasil se destacam com aproximadamente 170 e 150 espécies de *Passiflora*, respectivamente. Economicamente os maracujazeiros despertam interesse pela beleza de suas flores, presença de princípios ativos medicinais, extração de óleos essenciais para indústria de cosméticos, produção de frutos para consumo *in natura* ou produção de derivados. O Brasil se destaca como maior produtor de maracujá, embora a produtividade nacional seja baixa (média de 14 T/ha⁻¹ ano⁻¹), quando comparada ao potencial da passicultura (± 50 T/ha⁻¹ ano⁻¹). Em parte essa baixa produtividade é ocasionada pela ausência de cultivares adaptadas às diferentes regiões produtoras e pela suscetibilidade das cultivares às principais enfermidades que acometem a cultura. Embora crescente, os programas de melhoramento genético do maracujazeiro apresentam resultados modestos frente às demandas existentes. Entre os obstáculos enfrentados pelos melhoristas, está a reduzida representatividade do gênero *Passiflora* em bancos de germoplasma, bem como a escassez de informações biológicas e agrônômicas para maioria dos acessos. Assim, foi objetivo desta tese gerar informações genéticas e moleculares que contribuam para o melhoramento do maracujazeiro. Inicialmente foram construídas revisões críticas relacionadas aos avanços obtidos no melhoramento e na conservação das *Passiflora* com o uso de marcadores moleculares, bem como a cerca da principal doença que acomete a passicultura (virose do endurecimento dos frutos). Posteriormente foram obtidos, a partir de bibliotecas genômicas enriquecidas de microsatélites, 25, 17 e 52 novos pares de *primers* para *P. cincinnata*, *P. edulis* e *P. setacea* respectivamente, sendo observado um percentual de locos polimórficos inferior a 30% e um número médio de cinco alelos por loco. Testes de amplificação cruzada

foram realizados para 14 espécies de maracujazeiros, sendo observada uma média de 70% de amplificação cruzada. De posse destes marcadores, foi estimada a distância e quantificada a estrutura genética entre 116 acessos (representados por 364 plantas distribuídas em três espécies). A partir dos dados de genotipagem e das análises estatísticas (descritivas, frequentistas e Bayesianas) foi observado baixa diversidade entre os acessos, e níveis moderado a alto de estruturação (com $0,08 \leq G_{st} \leq 0,38$) e percentuais de alelos privados variando entre 20 e 40% entre os grupos sugeridos pelas estimativas Bayesianas (K=2 para *P. cincinnata*; K=3 ou 5, para *P. edulis*, e; K=2 ou 3 para *P. setacea*). Coleções nucleares representativas para até 100% da diversidade alélica amostrada foram sugeridas. Com base na caracterização de locos microssatélites e na avaliação de sintomas associadas à virose do endurecimento, verrugose e antracnose, observados em 36 acessos de *P. edulis* (amarelo e roxo), foi possível identificar grupos de acessos a serem priorizados em programas de (pré) melhoramento dedicados ao incremento de resistência em variedades cultivadas. Os resultados obtidos contribuem para o desenvolvimento dos programas de pré-melhoramento e melhoramento genético do maracujazeiro, além de auxiliarem no manejo e na conservação da variabilidade genética do gênero.

Abstract

The genus *Passiflora*, whose species are popularly known as passion fruits, stands out in the family Passifloraceae both by the number of species (about 520) as well as the associated economic importance of some of these species. Passion fruits occur in different countries, with their diversity widely represented in the Americas, where Colombia and Brazil stand out with approximately 170 and 150 species of *Passiflora*, respectively. Economic interest in passion fruit emerged from the beauty of their flowers, presence of active medicinal principles, extraction of essential oils for cosmetics industry, fruits production for fresh consumption or derivatives production. Brazil is considered the largest producer of passion fruit, although national productivity is low (average 14 T/ha⁻¹ year⁻¹) when compared to passion fruit culture potential ($\pm$ 50 T/ha⁻¹ year⁻¹). This low productivity is caused in part by the lack of adapted cultivars to the different production regions and due cultivars susceptibility to passion fruit major diseases. Despite an increasing rate, passion fruit breeding programs shows modest results versus existing demands. Among the obstacles faced by breeders, stand out the genus *Passiflora* reduced representation in germplasm banks, as well as the scarcity of biological and agronomic information for most accessions. Thus, the aim of this thesis was to generate information genetics and molecular that contributes to the passion fruit genetic breeding. Initially, critical reviews related to advances in *Passiflora* breeding and conservation using molecular markers, as along with information about the main disease affecting the passiculture (passion fruit woodiness disease) were presented. Novel primer pairs for *P. cincinnata*, *P. edulis* and *P. setacea*, in number of 25, 17 and 52 respectively, were subsequently obtained from microsatellite enriched genomic libraries, being observed a less than 30% polymorphic loci and an average number of five alleles per locus. Cross-amplification tests were performed for 14 species of passion fruit and an average of 70% cross-amplification was observed. Using these markers, genetic distance and structure were estimated among 116 accessions (represented by 364 plants distributed among three species). From genotyping and statistical analyzes data

(descriptive, frequentist and Bayesian), low genetic diversity among accessions was observed. Also, Bayesian estimated suggested groups (K=2 for *P. cincinnata*, K=3 or 5 for *P. edulis*, and, K=2 or 3 for *P. setacea*) showed moderate to high levels of structuring ($0.08 \leq G_{st} \leq 0.38$) along with private alleles percentage ranging from 20 to 40%. Representative core collections ensuring up to 100% of the sampled allelic diversity were suggested. Based on microsatellite loci characterization and symptoms evaluation of associated woodiness virus, scab and anthracnose, observed in 36 accessions of *P. edulis* (yellow and purple), it was possible to identify groups of accessions to be prioritized in (pre) breeding programs dedicated to resistance improving in cultivars. These results contribute to the passion fruit pre-breeding and breeding programs development, and assist the genus genetic variability management and conservation.

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Compartilho a opinião de muitos, no que tange a crer que dificilmente uma forma de ensino se mostra mais eficiente do que o próprio exemplo. Portanto, no meu caso, é mais que uma obrigação dedicar este trabalho de tese, e principalmente o que ele representa para a minha vida acadêmica, àquele que exemplifica para mim a figura de livre pensador (constante aprendiz e verdadeiro fã de uma boa leitura e de uma boa conversa, desde que construtivas).

Ao meu Pai, ***Antonio Cerqueira Silva***,

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“...Ao alcançarmos o meio dia da vida, assim como o Sol em seu meridiano, nos deparamos com metade dos anos já vividos e com a outra metade por viver, onde teremos ocasião de fazer o bem e sermos melhores...”

(autor desconhecido)

“O homem sábio, para ser feliz, olha abaixo de si e nunca acima, a não ser para elevar sua alma ao infinito”

(In. O Livro dos Espíritos)

Organização da Tese

Este manuscrito de tese está apresentado no formato de Capítulos. Por sua vez, as informações teóricas e práticas de cada capítulo, resultantes das ações de pesquisa realizadas durante as diferentes etapas do desenvolvimento deste trabalho, estão apresentadas no formato de artigo científico. Em cada Capítulo foram respeitadas as particularidades inerentes ao perfil dos objetivos traçados e dos consequentes resultados obtidos em cada etapa da pesquisa, resultando tanto em publicações de cunho teórico/crítico, quanto em publicações associadas ao desenvolvimento de técnicas/ferramentas. Resultados inéditos, decorrentes da aplicação do conhecimento teórico e prático gerado, também são apresentados e discutidos.

Inicialmente é apresentada uma introdução geral que antecede os resultados dispostos ao longo dos Capítulos. O primeiro destes Capítulos apresenta a revisão de literatura, sendo composto por dois artigos científicos. O primeiro deles, intitulado *Genetic Breeding and Diversity of the Genus Passiflora: Progress and Perspective of Molecular and Genetic Studies* foi publicado no periódico *International Journal of Molecular Science* em agosto de 2014 (doi: 10.3390/ijms150814122) e apresenta desde um contexto histórico associado a cultura do maracujazeiro até os recentes progressos obtidos com o auxílio da biologia molecular, enfatizando-se o conhecimento e uso da variabilidade do gênero como recurso genético indispensável aos programas de melhoramento genético. Neste manuscrito o crescente emprego dos marcadores moleculares em estudos genéticos do gênero *Passiflora* é também discutido e perspectivas são apresentadas. O segundo artigo deste Capítulo, intitulado *A history of passion fruit woodiness disease with emphasis on the current situation in Brazil and prospects for Brazilian passion fruit cultivation* foi publicado no periódico *European Journal of Plant Pathology* em fevereiro de 2014 (doi: 10.1007/s10658-014-0391-z) e apresenta detalhes a respeito do patossistema maracujazeiro vs. virose do endurecimento. Neste artigo são discutidos desde estratégias clássicas para identificação e convívio com a doença, inclusive o uso da biologia molecular para

diagnóstico, combate e controle dessa que é a principal enfermidade associada a cultura do maracujazeiro.

O segundo Capítulo da tese versa sobre o desenvolvimento de marcadores microssatélites para três espécies de maracujazeiro e encontra-se dividido em dois artigos científicos. O primeiro destes artigos, intitulado *Development and characterization of microsatellite markers for the wild south american Passiflora cincinnata (Passifloraceae)* foi publicado em abril de 2012 no periódico *American Journal of Botany* (doi: 10.3732/ajb.1100477) e apresenta os primeiros marcadores microssatélites desenvolvidos para o maracujazeiro do mato (*P. cincinnata* Mast.). O segundo artigo deste Capítulo, intitulado *New Microsatellite Markers for Wild and Commercial Species of Passiflora (Passifloraceae) and Cross-Amplification* foi publicado em fevereiro de 2014 no periódico *Applications in Plant Sciences* (<http://dx.doi.org/10.3732/apps.1300061>) e apresenta os primeiros marcadores microssatélites desenvolvidos para maracujazeiro do sono (*P. setacea* DC.), bem como um novo conjunto de marcadores para o maracujazeiro amarelo (*P. edulis* Sims.) e para o maracujazeiro do mato.

No terceiro Capítulo desta tese são apresentados os resultados obtidos a partir da caracterização da diversidade genética de acessos do Banco Ativo de Germoplasma (BAG) da EMBRAPA Mandioca e Fruticultura (Cruz da Almas, Bahia) e da coleção de maracujazeiro da Universidade Estadual do Sudoeste da Bahia (*campus* Itapetinga, Bahia), sendo a semelhança dos Capítulos anteriores dividido em dois artigos científicos. O primeiro artigo científico, intitulado *Molecular genetic variability of commercial and wild accessions of passion fruit (Passiflora spp.) to assist ex situ conservation and genetic breeding* foi submetido para o periódico *International Journal of Molecular Science* e apresenta estimativas de diversidade e estrutura genética de 364 plantas de maracujazeiros distribuídos em 116 acessos de três espécies de *Passiflora*. Neste artigo são também apresentados resultados de amplificação cruzada de 109 locos microssatélites entre 14 espécies de *Passiflora*. O segundo artigo deste capítulo, intitulado *Characterization and selection of passion fruit (yellow and purple) accessions based on molecular markers and disease reactions for use in breeding*

programs foi publicado em agosto de 2014 no periódico *Euphytica* (doi: 10.1007/s10681-014-1235-9), sendo apresentada estimativas de diversidade genética com base em 23 locos microssatélites, bem como a reação a três importantes doenças da cultura do maracujazeiro, a partir de 36 acessos de maracujazeiro (com frutos de casca amarela e roxa). Neste artigo são apresentados grupos de acessos a serem priorizados, devido a diversidade genética presente e os percentuais de sintomas observados, em programas de melhoramento genético dedicados ao incremento de resistência em variedades cultivadas e, ou, na formação de novas cultivares.

Após os três Capítulos, são apresentados como resultados complementares estimativas preliminares de diversidade e estrutura genética interespecífica entre três importantes espécies para os programas de melhoramento do maracujazeiro (a saber: *P. cincinnata*, *P. edulis* e *P. setacea*), cuja variabilidade intraespecífica foi caracterizada no Capítulo 3. Logo após, são apresentadas as considerações finais, as conclusões e as perspectivas traçada para o curto, médio e longo prazo.

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INTRODUÇÃO GERAL

Os maracujazeiros, como são popularmente denominadas as espécies do gênero *Passiflora* (Família Passifloraceae), caracterizam-se como plantas trepadeiras que se apresentam como ervas e arbustos (VANDERPLANK, 1996). O gênero *Passiflora* possui aproximadamente 520 espécies (MACDOUGAL e FEUILLET, 2004), sendo esta estimativa variável, visto que é constante a descrição de novas espécies (WETZEL et al., 2011). As diferentes espécies de maracujazeiros são comumente observadas ao longo da América Latina, especialmente na América do Sul, sendo países como a Colômbia e o Brasil (com aproximadamente 170 e 150 espécies de maracujazeiro, respectivamente) considerados centros de diversidade do gênero (BERNACCI et al., 2013; OCAMPO et al., 2010; FAJARDO et al., 1998). Embora em menor número de espécies, os maracujazeiros são também observados fora das Américas, como por exemplo na Índia, China, Austrália e em algumas ilhas do Pacífico (BERNACCI et al., 2005).

A importância do gênero *Passiflora* não é restrita aos aspectos ecológicos inerentes à diversidade e dispersão de suas espécies, visto que muitas destas são reconhecidas como recurso natural devido ao valor (ao menos potencial) agregado a suas folhas, flores e frutos. A beleza e a diversidade observada nas folhagens e flores dos maracujazeiros despertam interesse econômico para o uso ornamental de muitas espécies, já tendo sido registrado a nível mundial mais de 400 híbridos ornamentais (ABREU et al., 2009; PEIXOTO, 2005). O interesse econômico devido ao potencial uso das passifloras como planta medicinal e fonte de princípios ativos para indústria farmacêutica é também largamente reconhecido (COSTA e TUPINAMBA, 2005; DHAWAN et al., 2004). Além do que, o uso de óleos e extratos obtidos a partir de maracujazeiros tem sido também registrado pela indústria de cosméticos e na fabricação de cremes, sabonetes, xampus (FALEIRO et al., 2011; ZERAIK et al., 2010).

Embora diversificado, conforme apresentado anteriormente, o valor econômico agregado ao gênero *Passiflora* decorre principalmente da produção e comercialização

de frutos. Neste contexto, embora sejam reconhecidas ao menos 70 espécies produtoras de frutos comestíveis (COPPENS D'EECKENBRUGGE et al., 2001; SOUZA e MELETTI, 1997), a produção comercial de maracujá decorre principalmente da espécie *P. edulis* Sims, popularmente conhecida como maracujazeiro amarelo ou azedo, sendo seguida pela espécie *P. alata* Curtis, popularmente denominada de maracujazeiro doce (WETZEL et al., 2011; FALEIRO et al., 2011). Neste contexto, estima-se que no mínimo 90% da área destinada a passicultura seja cultivada com o *P. edulis*, contemplando tanto a produção de frutos de casca amarela quanto roxa (FALEIRO et al., 2011; MELETTI et al., 2005).

O Brasil é reconhecido como maior produtor mundial de maracujá, tendo produzido em 2012 aproximadamente 776 mil toneladas de maracujá em uma área de aproximadamente 59 mil hectares (Figura 1). A produção brasileira, embora presente nas diferentes regiões do país, está concentrada na região nordeste que responde por aproximadamente 75% da área plantada e da produção nacional (Figura 1) (IBGE, 2012).

Embora incipiente, em um contexto de produção nacional, o cultivo e consumo de frutos e derivados de espécies silvestres como *P. cincinnata* Mast., *P. nitida* HBK., *P. quadrangularis* L. e *P. setacea* estão registrados na literatura (MELETTI et al., 2005; SOUZA e MELETTI, 1997). Além do que, por apresentarem resistência a fatores de estresse bióticos e abióticos, tais espécies apresentam importância para os programas de melhoramento genético do maracujazeiro (FALEIRO et al., 2011; JUNQUEIRA et al., 2005).

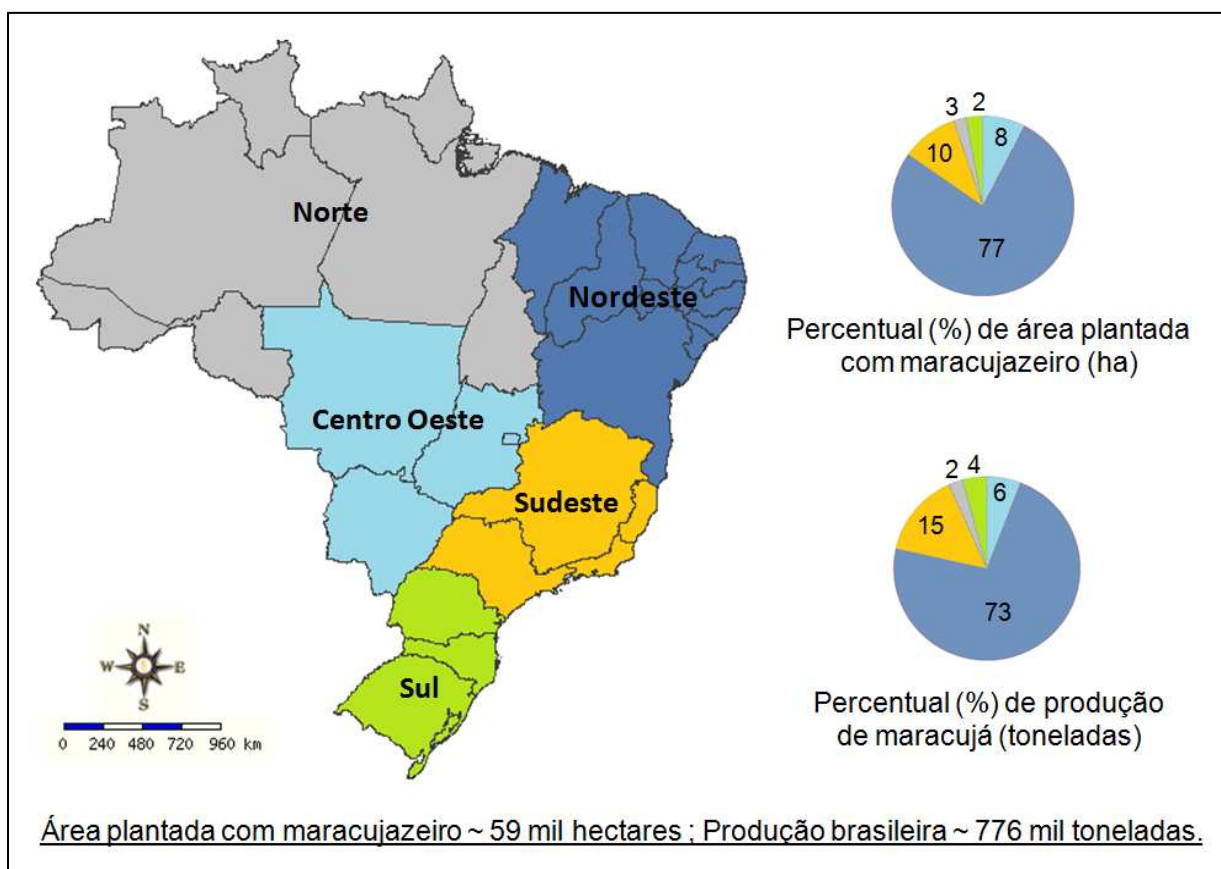


Figura 1. Apresentação da produção de maracujazeiro no território brasileiro, com descrição quantitativa referente as áreas de cultivo e produção de frutos nas cinco macrorregiões do território brasileiro. Mapa construído com a ferramenta SpeciesMapper tool (<http://splink.cria.org.br/>). Fonte de dados: http://biblioteca.ibge.gov.br/visualizacao/periodicos/66/pam_2012_v39_br.pdf

A despeito da alta produção brasileira e do posto de maior produtor mundial de maracujá, é importante reconhecer que esse destaque não decorre necessariamente da eficiência na produtividade dos pomares e sim da extensão de terra destinada a passicultura. Pesquisas atestam que a produtividade da cultura pode chegar a valores entre 40 e 50 T/ha⁻¹ ano⁻¹ (NEVES et al., 2013; MELETTI et al., 2005; MELO et al., 2001), número bem superior a média nacional dos últimos 10 anos que foi de 14 T/ha⁻¹

ano⁻¹. Ainda nesse contexto, é possível observar, a partir dos dados de Produção Agrícola Municipal (disponível no *site* do IBGE, <http://downloads.ibge.gov.br/>) que o crescimento observado para a produção nacional decorre muito mais da expansão da área plantada do que do crescimento da produtividade dos pomares (Figura 2), sendo observado um coeficiente de regressão muito superior para associação entre os dados de produção e área plantada ($R^2 = 0,98$), em comparação ao coeficiente observado entre produtividade e produção ($R^2 = 0,53$). Ambas as regressões são significativas ($p < 0,05$) e obtidas mediante análise de regressão linear.

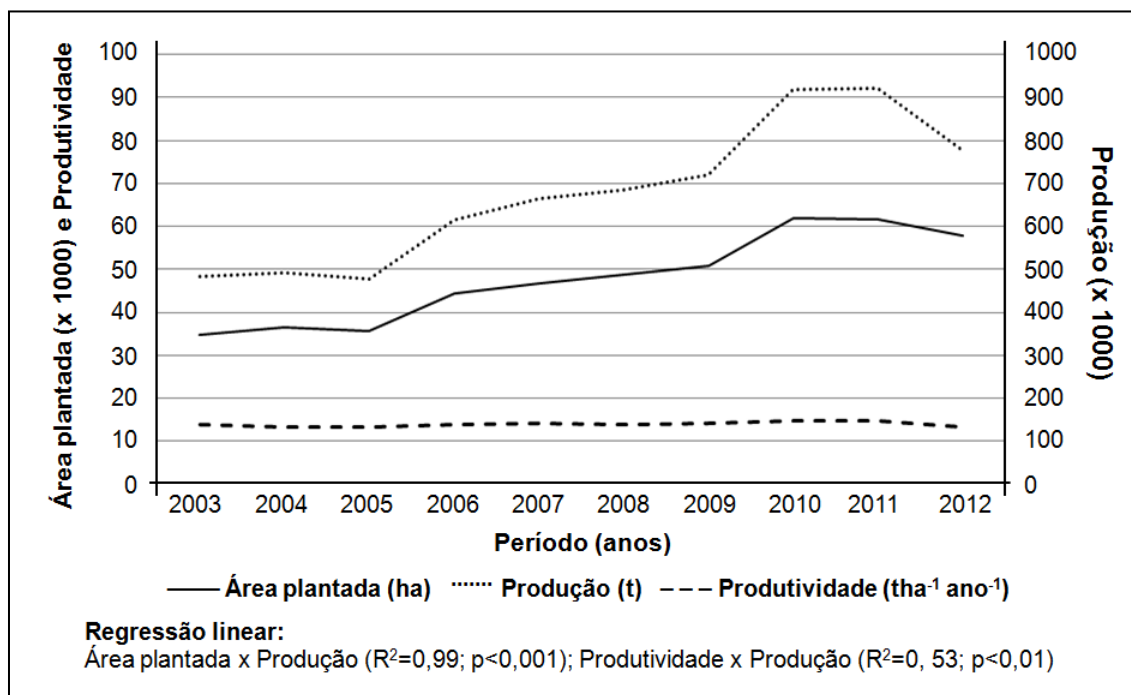


Figura 2. Gráficos de linhas indicando a tendência de três variáveis relacionadas ao cultivo do maracujazeiro no Brasil (área plantada, produção e produtividade), durante o período de 2003 a 2012. A associação de causa e efeito entre a área planta e a produtividade, em relação produção é também apresentada. Fonte dos dados de cultivo:

http://biblioteca.ibge.gov.br/visualizacao/periodicos/66/pam_2012_v39_br.pdf

Ao menos em parte, a baixa produtividade observada para a passicultura é justificada pela ocorrência de fatores de estresse abióticos (a exemplo da baixa disponibilidade de água, característica de determinadas regiões brasileiras) e bióticos (a exemplo das perdas ocasionadas por pragas e patógenos). Considerando que as perdas ocasionadas devido à ação de patógenos, em grande parte, não são solucionadas com adoção de técnicas de cultivo e uso de defensivos agrícolas, é necessária atenção especial dos pesquisadores para os patossistemas associados à passicultura.

Dentre as doenças que acometem o maracujazeiro destacam-se, devido a ocorrência generalizada no território nacional, e, ou, devido as dificuldades de combate, controle e convívio: a antracnose (*Glomerella cingulata*, anamorfo: *Colletotrichum gloeosporioides*), a verrugose (*Cladosporium cladosporioides* e *C. herbarum*), a fusariose (*Fusarium oxysporum* f. sp. *passiflorae*), a bacteriose (*Xanthomonas axonopodis* pv. *passiflorae*) e a virose do endurecimento dos frutos (*Cowpea aphid-borne mosaic virus*) (FISCHER e RESENDE, 2008; JUNQUEIRA et al., 2005b; MELETTI et al., 2005). Estes autores discutem que embora sejam observados estudos relacionados a estes patossistemas, a grande maioria dos resultados é associada à identificação de patógenos e aspectos epidemiológicos, mas não são observados programas de pesquisa integrados que possibilitem contínua avaliação e gerenciamento das doenças. Para FISCHER e RESENDE (2008), essa realidade é também justificada pelo fato da produção do maracujá ser associada a pequenos produtores que por vezes possuem outras prioridades de cultivo, não dando o devido valor a realização e, ou, aos resultados de pesquisas.

Por sua vez, o número de cultivares disponíveis no mercado é limitado e insuficiente para atender de maneira adequada a diversidade de ambientes de cultivo presentes no território brasileiro (FALEIRO et al., 2011; MELETTI et al., 2005). Além do que, baixos níveis de resistência a doenças (BATISTTI et al., 2013; OLIVEIRA et al., 2013a; SILVA et al., 2013; FISCHER et al., 2010; MACIEL et al., 2009; CERQUEIRA-SILVA et al., 2008; JUNQUEIRA et al., 2003) e pouca variabilidade genética (ORTIZ et

al., 2012; CERQUEIRA-SILVA et al., 2010a; VIANA et al., 2003), tem sido observada entre as cultivares e, ou, genótipos comerciais de *P. edulis*. Neste contexto, o uso de espécies silvestres como fonte de genes de resistência e diversidade, é reconhecido por diferentes autores como fundamental para ampliação da base genética e do progresso em programas de melhoramento genético, contribuindo para o sucesso no desenvolvimento de variedades superiores (FALEIRO et al., 2011; WETZEL et al., 2011; JUNQUEIRA et al., 2003).

Embora seja considerado um dos centros de diversidade do gênero, o uso desta diversidade genética em programas de melhoramento, ou mesmo em etapas de pré-melhoramento (a exemplo da prospecção, manutenção e caracterização de germoplasma silvestre) é bastante limitado para os maracujazeiros (CASTELLEN et al., 2005; FERREIRA, 2005). Além do que, tem-se registrado a redução no número de acessos presentes em algumas coleções, ou mesmo a extinção destas coleções em centros de pesquisa e universidades (FALEIRO et al., 2011). Estima-se que o acervo total de germoplasma, referente aos maracujazeiros mantidos pelos centros de pesquisa e universidades em todo o Brasil, seja composto por aproximadamente 700 acessos (representando apenas 67 espécies) (FERREIRA et al., 2005), o que representa menos de 15% da diversidade de espécies descritas para o gênero *Passiflora*.

Considerando a ocorrência de doenças, as dificuldades técnicas e financeiras para manutenção de acessos em Bancos Ativos de Germoplasma (BAGs), bem como a escassez de caracterizações biológicas e agronômicas, é esperado que o número de acessos efetivamente disponíveis para programas de melhoramento genético seja bem inferior ao total de acessos registrados nos BAGs. A falta de informações relacionadas aos acessos mantidos nas coleções e BAGs é considerada por diferentes autores como a principal dificuldade para o maior aproveitamento da variabilidade genética disponível em programas de melhoramento (NASS, 2011; DUVICK, 1984).

Embora o cultivo comercial do maracujazeiro no Brasil seja realizado a mais de 40 anos e variedades regionais estejam disponíveis desde a década de 1990 (a exemplo das variedades Maguary, Golden Star, Composto IAC-27, Amafrutas), as primeiras cultivares de maracujazeiro foram lançadas há aproximadamente 15 anos (MELETTI, 2011; FALEIRO et al., 2011) e a primeira cultivar silvestre somente foi lançada em 2013. Atualmente, embora existam 31 entradas catalogadas no sistema de registro nacional de cultivares do Ministério da Agricultura, Pecuária e Abastecimento - MAPA, associados ao termo maracujá (consulta realizada em 24 de maio de 2014, http://extranet.agricultura.gov.br/php/snpc/cultivarweb/cultivares_registradas.php), menos da metade destes registros dizem respeito a cultivares efetivamente disponíveis para os produtores, ou avaliadas pela comunidade científica. Na Tabela 1 são apresentadas 13 cultivares, para as quais estão disponíveis informações de divulgação e comercialização, bem como dados de avaliações científicas para produção e reação a doenças.

Estudos dedicados à avaliação de estratégias adequadas para potencializar ganhos de seleção, bem como para quantificar a variabilidade disponível para características de interesse à passicultura, embora ocorram a mais de 30 anos (OLIVEIRA, 1980), foram apenas intensificados nos últimos anos, tanto para ganhos de quantidade e qualidade de produção (NEVES et al. 2013; REIS et al. 2012; CERQUEIRA-SILVA et al., 2009; ARAÚJO et al., 2008), quanto para busca de resistência a doenças (OLIVEIRA et al., 2013a; SILVA et al., 2013; CERQUEIRA-SILVA et al., 2008; SANTOS et al., 2008; JUNQUEIRA et al., 2003). Diante deste contexto, iniciativas de pesquisas dedicadas ao entendimento dos patossistemas relacionados ao maracujazeiro, bem como a caracterização da reação de acessos silvestres e comerciais às principais doenças que afetam a cultura, têm sido realizadas (JUNQUEIRA et al., 2005) e indicadas como ações prioritárias para os programas de melhoramento genético do maracujazeiro (FALEIRO et al., 2011; FALEIRO et al., 2006).

Tabela 1. Apresentação dos principais cultivares de maracujazeiro (variedades e híbridos) disponíveis para a passicultura brasileira.

Identificação	Empresa Responsável	Ano de referência*	Informações complementares
Monte Alegre (IAC-273)	Instituto Agrônomo de Campinas	1999	http://www.iac.sp.gov.br/publicacoes/agronomico/maracuja_amarelo.php
Maravilha (IAC-275)			
Joia (IAC-277)			
IAC Paulista		2005	http://www.iac.sp.gov.br/publicacoes/agronomico/pdf/v58_Maracuja_Roxo.pdf
CPATU casca fina	Embrapa Amazônia Oriental	2002	http://dx.doi.org/10.1590/S0100-29452003000100052
FB 200 – <i>Yellow Master</i>	Viveiro Flora Brasil	2008	http://www.viveiroflorabrasil.com.br/site/produtos-2/
FB 300 - Araguari			
BRS Sol do Cerrado	EMBRAPA Cerrados	2007	www.campinas.spm.embrapa.br/seresmap/maracujas/
BRS Gigante Amarelo			
BRS Ouro Vermelho		2012	http://www.cpac.embrapa.br/lanca mentoperola/
BRS Rubi do Cerrado			
BRS Perola do Cerrado		2013	
Sul Brasil	AFRUEC / Fundo Passiflora	2010	-----

*como ano de referência foi utilizado o ano de lançamento (desde que disponível material de informativo) ou o ano de registro da cultivar junto ao Ministério da Agricultura, Pecuária e Abastecimento, sendo considerado a primeira ocorrência.

Desde a publicação dos trabalhos de FAJARDO et al. (1998) e SEGURA et al. (1998), tem-se observado um crescimento no número de artigos publicados a partir do emprego de marcadores moleculares em espécies do gênero *Passiflora* (Figura 3). Também são observados o crescimento e a diversificação dos objetivos traçados para o uso dos marcadores moleculares nas pesquisas envolvendo maracujazeiros silvestres e

comerciais. Especificamente no contexto do melhoramento genético, é notória a contribuição e, ou, a necessidade de uso dos marcadores para: (i) caracterizações de diversidade genética entre acessos de espécies silvestres (CERQUEIRA-SILVA et al., 2012a; PÉREZ-ALMEIDA et al., 2010; CERQUEIRA-SILVA et al., 2010bc; JUNQUEIRA et al., 2007) e comerciais (ORTIZ et al., 2012; CERQUEIRA-SILVA et al., 2010a; BELLON et al., 2009; FONSECA-TRUJILLO et al., 2009; BELLON et al., 2007), presentes em coleções e bancos de germoplasma; (ii) monitoramento de variabilidade e recuperação do genoma do genitor ao longo de ciclos de seleção recorrente (REIS et al., 2012; REIS et al., 2011; FONSECA et al., 2009); (iii) mapeamento de genes e regiões genômicas associados a características de interesse (PENHA et al., 2013; OLIVEIRA et al., 2008; LOPES et al., 2006; CARNEIRO et al., 2002); (iv) confirmação de híbridos (CONCEIÇÃO et al., 2011; JUNQUEIRA et al., 2008); e inclusive no (v) monitoramento de variedades lançadas/comercializadas, como discutido por FERREIRA e RANGEL (2011).

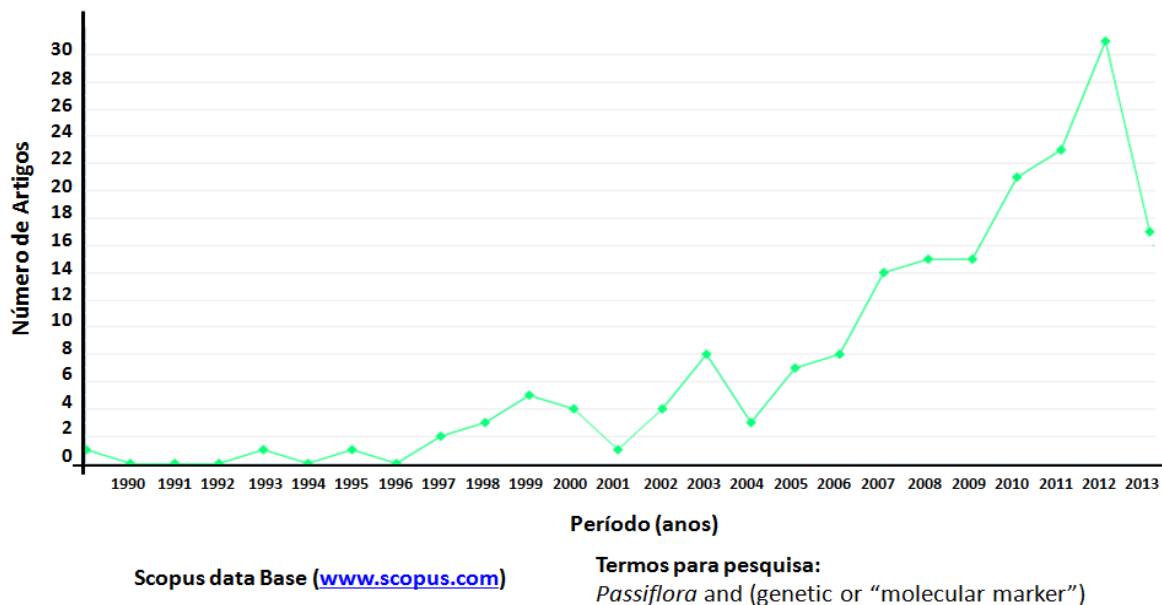


Figura 3. Gráfico de tendência relativo ao número de artigos publicados com o emprego de marcadores moleculares em espécies silvestres e comerciais de maracujazeiro (*Passiflora* spp.).

Contudo, a despeito dos avanços apresentados com o uso dos marcadores moleculares em estudos genéticos das passifloras, a grande maioria dos artigos publicados está associada ao uso de marcadores dominantes (a exemplo dos marcadores *Random Amplified Polymorphic DNA* - RAPD, *Inter-Simple Sequence Repeat* – ISSR e *Amplified Fragment Length Polymorphisms* - AFLP). O emprego de marcadores co-dominantes como os microssatélites (*single sequence repeats* - SSR) e os SNPs (*single nucleotide polymorphisms*), são bastante limitados e, em linhas gerais, restritos a pesquisas de identificação e caracterização de locos (PENHA et al., 2013; CAZÉ et al., 2012; PENHA, 2012; PEREIRA, 2010; OLIVEIRA et al., 2005; PÁDUA et al., 2005), sendo seu emprego limitado a inserção de marcas em mapas genéticos previamente construídos (PEREIRA et al., 2013; OLIVEIRA et al., 2008) ou a caracterizações genética envolvendo poucos acessos (OLIVEIRA et al., 2013b; PAIVA, 2013).

Contudo, o volume potencial de informação gerada com base no polimorfismo destes marcadores (SSR e SNP) deverá contribuir a curto e médio prazo para inferências associadas desde a conservação até o uso efetivo dos recursos genéticos disponíveis em bancos de germoplasma e populações naturais (FERREIRA e RANGEL, 2011).

Considerando a importância econômica da passicultura, os desafios inerentes à presença de patógenos nas áreas de cultivo, à necessidade de incremento na quantidade e qualidade de frutos produzidos, bem como a disponibilidade cada vez mais acentuada de técnicas moleculares passíveis de aplicação nos programas de melhoramento genético, é ao menos esperado, bem como imprescindível, que nos próximos anos ocorra um significativo crescimento na geração de informações e produtos tecnológicos relacionados às passifloras.

OBJETIVO GERAL

Disponibilizar novos marcadores microssatélites e caracterizar a diversidade genética molecular e reação a doenças de acessos silvestres e comerciais de maracujazeiro (*Passiflora* spp.) presentes em banco de germoplasma com vistas a contribuir para o melhoramento genético do maracujá.

OBJETIVOS ESPECÍFICOS

Disponibilizar novos marcadores microssatélites para espécies silvestres e comerciais de maracujazeiro (*Passiflora* spp.);

Estimar a estrutura e a diversidade genética molecular de acessos de maracujazeiros comerciais e silvestres mantidos em banco de germoplasma;

Identificar acessos de maracujazeiro (com frutos de casca amarela e roxa) a serem priorizados em programas de melhoramento genético dedicados ao incremento de resistência a doenças.

CAPÍTULO I

Revisão de Literatura

ARTIGO 1.

C.B.M. Cerqueira-Silva; O.N. Jesus; E.S.L. Santos; R.X. Corrêa; A.P. Souza (2014). **Genetic Breeding and Diversity of the Genus *Passiflora*: Progress and Perspective of Molecular and Genetic Studies.** *International Journal Molecular Science*. DOI 10.3390/ijms150814122

ARTIGO 2.

C.B.M. Cerqueira-Silva; L.D.H.C.S. Conceição; A.P. Souza; R.X. Corrêa (2014). **A history of passion fruit woodiness disease with emphasis on the current situation in Brazil and prospects for Brazilian passion fruit cultivation.** *European Journal of Plant Pathology*. DOI 10.1007/s10658-014-0391-z

CAPÍTULO I

Revisão de Literatura

O presente capítulo tem por objetivo apresentar uma visão geral a respeito das pesquisas realizadas com espécies silvestres e comerciais do gênero *Passiflora*, dando-se ênfase aos estudos relacionados tanto com a caracterização da diversidade genética e conservação, quanto aos estudos dedicados às ações de pré-melhoramento e melhoramento genético do maracujazeiro. Neste contexto, é apresentado e discutido o cenário das pesquisas realizadas com marcadores moleculares, ficando evidente o crescimento no número de estudos realizados desde o início do século XXI, bem como a necessidade de novas pesquisas, haja visto a inexistência de estudos genéticos básicos para a grande maioria das espécies silvestres que compõem o gênero *Passiflora*. Especificamente no contexto do melhoramento genético, é apresentado e discutido os avanços tanto no número de programas quanto no número de cultivares lançadas e registradas. Contudo, desafios como a obtenção de cultivares resistentes às principais doenças do maracujazeiro (a exemplo da fusariose, verrugose, antracnose e virose do endurecimento dos frutos) permanecem como um campo aberto e desafiador para os programas de melhoramento genético. Discussão detalhada a respeito do patossistema maracujazeiro vs. virose do endurecimento também é realizada. Para este patossistema, o avanço no uso de estratégias clássicas para identificação e convívio com a doença, bem como a aplicação da biologia molecular para diagnóstico, combate e controle dessa enfermidade são apresentadas e contextualizadas.

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Review

Genetic Breeding and Diversity of the Genus *Passiflora*: Progress and Perspectives in Molecular and Genetic Studies

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Abstract: Despite the ecological and economic importance of passion fruit (*Passiflora* spp.), molecular markers have only recently been utilized in genetic studies of this genus. In addition, both basic genetic researches related to population studies and pre-breeding programs of passion fruit remain scarce for most *Passiflora* species. Considering the number of *Passiflora* species and the increasing use of these species as a resource for ornamental, medicinal, and food purposes, the aims of this review are the following: (i) to present the current condition of the passion fruit crop; (ii) to quantify the applications and effects of using molecular markers in studies of *Passiflora*; (iii) to present the contributions of genetic engineering for passion fruit culture; and (iv) to discuss the progress and perspectives of this research. Thus, the present review aims to summarize and discuss the relationship between historical and current progress on the culture, breeding, and molecular genetics of passion fruit.

Keywords: active germplasm banks; genetic diversity; genetic engineering; molecular marker; passion fruit breeding; population genetics

1. Introduction

1.1. General Characteristics of the Genus *Passiflora*, Family *Passifloraceae*

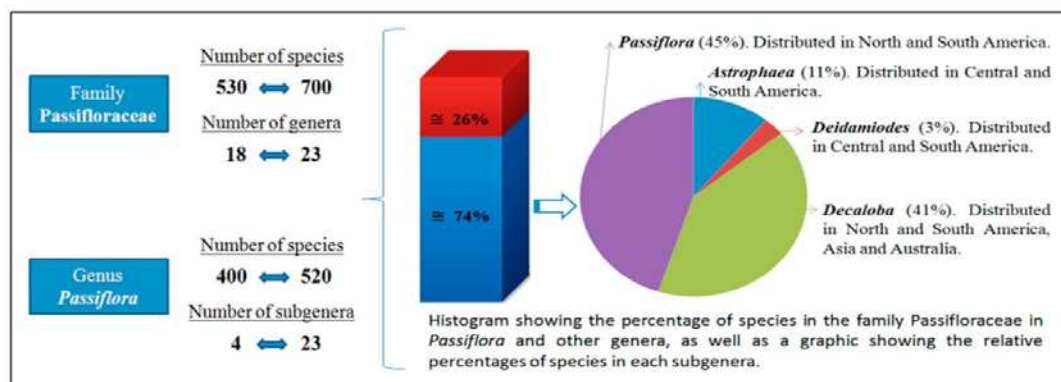
The family *Passifloraceae* Juss. ex DC. belongs to the order Violales, class Magnoliopsida, and phylum Magnoliophyta. Although records of plants that belong to this family have been in existence since 1553, there have been disagreements regarding the number of genera and species in the family since the first literary record of a plant in this group was written in Colombia [1]. Over the past few centuries, several scientific studies have been published describing the species and the organization of the species into genera and subgenera. Estimates of the number of species in the *Passifloraceae* family vary between 700 [2] and 520 [3–5]. Such variations are the result of taxonomical uncertainties, the use of synonyms, and descriptions of new species. There is also no consistency in the number of genera, which varies between 18 [2,3] and 23 [6].

Despite taxonomical uncertainties, *Passiflora* is highly diverse, with approximately 520 species [5]. Approximately 96% of the species are distributed in the Americas, although there are records of species in India, China, Southeastern Asia, Australia, the Pacific islands, and neighboring regions (examples of species include *Passiflora aurantia*, *Passiflora cinnabarina*, *Passiflora herbertiana*, *Passiflora cupiformis*, *Passiflora henryi*, *Passiflora jugorum*, *Passiflora moluccana*, and *Passiflora siamica*). Brazil and Colombia, in particular, are centers of diversity; approximately 30% of *Passiflora* species are found in these countries (approximately 150 in Brazil and 170 in Colombia), including 89 that are endemic to Brazil [7–10].

Based on the progress in taxonomic and phylogenetic studies using molecular markers and sequencing techniques, particularly over the last 10 years, the taxonomic classification of *Passiflora* species has been organized into four subgenera: *Astrophaea*, *Decaloba*, *Deidamiodes*, and *Passiflora* [11,12]. Although this infrageneric organization into only four subgenera diverges from the hypothesized 22 or 23 subgenera [13,14], research based on phylogenetic approaches, morphometric data, genotypic data of microsatellite markers [15] and DNA fragments from seven regions of the passion fruit genome (the *rbcL* and *rps4* genes, the *trnL* intron and *trnL-F* intergenic spacers from the plastid genome, the *nad1* b/c and *nad5* d/e introns from the mitochondrial genome, and a portion of the 26S gene from the nuclear ribosomal genome) [16] supports this simpler division of the genus. Together, the information available for passion fruit supports the infrageneric classification of *Passiflora* based on studies of molecular phylogeny that are corroborated by morphological and ecological characteristics [16–18].

Figure 1 presents a summary of the data discussed above. Additional information on the family *Passifloraceae*, the group's taxonomy, and the occurrence of species is reported in the List of Species of Brazilian Flora [10] and in the book *Passiflora: Passionflowers of the world* [19].

Figure 1. Schematic illustrating general information regarding the *Passifloraceae* family (the number of genera, species, and distribution within different taxonomic levels) and details of the occurrence of *Passiflora* in Brazil. The data presented were summarized from the results of previous studies [2–4,9,12].



Molecular studies of passion fruit based on the combined and individual analysis of DNA sequences from the nuclear, plastid, and mitochondrial genomes also contribute directly to (i) estimates of the origin of the genus *Passiflora* and the diversification of its subgenera obtained through the analysis of molecular dating [16]; (ii) the identification and understanding of different organellar inheritance patterns [20]; and (iii) the demonstration of the potential use of fragments from the internal transcribed spacer (ITS) regions of nuclear ribosomal sequences and variations in the plastidial sequences to study inter- and intraspecific variability in *Passiflora* species [21,22]. Furthermore, the contributions to molecular phylogeny may be observed in in-depth studies [16–18].

With the expansion of the genomic information (sequences) available in public databases as well as the availability of genetic material in collections, germplasm banks, and herbaria, the use of short orthologous DNA sequences (DNA barcodes) to identify species can contribute considerably to the characterization and utilization of biological diversity [23,24]. In particular, for groups such as the genus *Passiflora*, for which taxonomic uncertainties exist and new species are continuously being discovered (approximately five species per year) [25], the definition and use of DNA barcodes can contribute directly to germplasm characterization and the evaluation of diversity in natural populations.

The inherent variability of *Passiflora* is not restricted to its morphological characteristics [26,27] and the physical and chemical compositions of the fruit and flowers [28–31] but includes traits important for its survival in natural environments and/or cultivation conditions, such as the resistance of wild species to biotic (fungi, bacteria, nematodes, and viruses) and abiotic stresses (variations in the edaphoclimatic characteristics of macro- and microregions in which *Passiflora* species naturally occur or are cultivated) [32–34]. Such variability may be useful for breeding programs with different end results.

Results from cytogenetic research on the genus *Passiflora* are scarce and restricted to less than 20% of the species [35]. Although such studies are increasing [36,37], this area of knowledge remains open to basic investigation. Despite this scarcity of cytogenetic information, research has contributed to our understanding of the group's evolutionary relationships and has facilitated direct interspecies crosses and thus the genetic breeding of passion fruit. Certain species, particularly those of economic interest

(due to fruit production and ornamentation, such as *Passiflora edulis* Sims, *Passiflora alata* Curtis, *Passiflora coccinea* Aubl, and *Passiflora incarnate* L.), are described by cytogenetic information available at different levels, including data from molecular cytogenetic techniques, such as fluorescent *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) [38–41]. Details outlining the use of cytogenetic techniques in *Passiflora* are available [36,37]. Molecular data were also recently made available from the construction and initial characterization of a genomic library of *P. edulis* using the bacterial artificial chromosome (BAC) technique [42,43], which should contribute to the characterization of the germplasm, construction of chromosome maps of *P. edulis* and neighboring species, and identification of interspecies hybrids.

In relation to estimates related to the genome size of passion fruit species, as is discussed in the review presented by Souza *et al.* [37], the studies are very limited, and for most species, the information was derived from works carried out in the last 10 years with use of flow cytometry. This methodology may contribute to the estimation of DNA content as an aid in the analysis of ploidy, the identification of hybrids and the establishment of correlations between the genome sizes and biological and agronomic characteristics [37,44]. The few studies investigating the genome sizes of passion fruit have indicated significant interspecific variation, such as the results reported by Souza *et al.* [37] for eight species (with 2C nuclear DNA content ranging from 1.83 pg in *Passiflora suberosa* to 5.36 pg in *Passiflora quadrangularis*) and the more recent results from Yotoko *et al.* [44] for 50 species (indicating variation of 2C content from 0.42 pg in *P. organogenesis* to 4.41 pg in *P. alata*).

The available cytogenetic information on passion fruit species describes four different chromosome numbers distributed among the four subgenera of *Passiflora*: $x = 6$ in *Decaloba*, $x = 9$ and $x = 10$ or 11 in *Passiflora*, $x = 10$ in *Astrophaea*, and $x = 12$ in *Deidamiodes* [5,39]. This distribution is largely in accordance with the infrageneric classification supported by molecular phylogeny for the genus *Passiflora*. Although there is no precise estimation of the basic number of chromosomes across the genus, studies have suggested 6 or 12 as the precise number [35,38]. In the case of polyploidy, most *Passiflora* species are diploid, with $2n = 12$, 18, or 20 chromosomes. Tetra-, hexa-, or octoploid species are rare [36,39]. The most commercially important species is diploid ($2n = 18$), which favors genetic breeding to obtain interspecies hybrids [36,43,44].

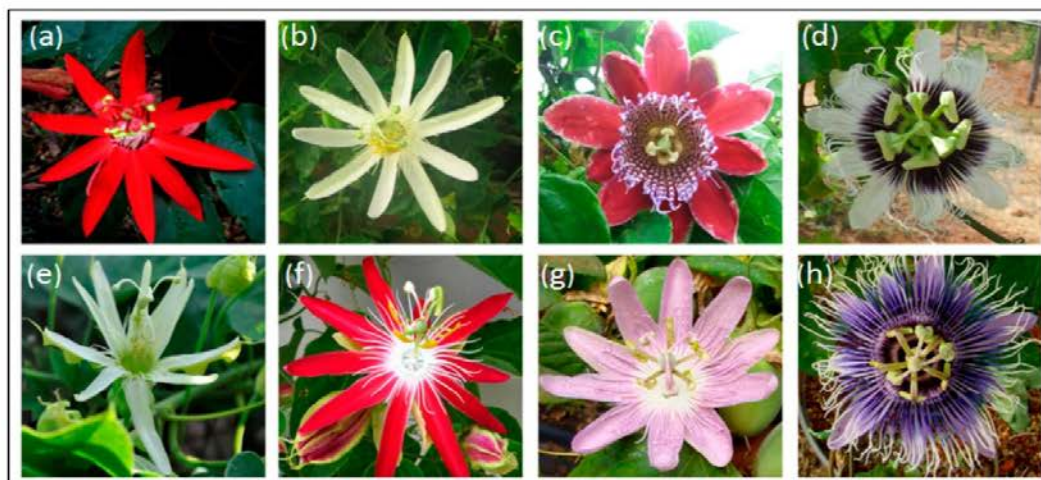
1.2. Economic Importance of Passion Fruit Species

Several segments of society display a great interest in passion fruit plants as ornamental and medicinal plants, as a source of oils for the cosmetic industry, and for their fruits and fruit derivatives. The use of passion fruit as an ornamental plant is justified because of the diversity and beauty of its leaves, flowers, and fruit, and more than 400 ornamental hybrids exist worldwide [45] (Figure 2). However, the use of passion fruit as an ornamental plant has only recently begun in Brazil, as the first hybrids for ornamental use were developed and launched in the last decade: the “Brasil (BRS) Estrela do Serrado” (N.Ref. RNC-MAPA 21717) was obtained from a cross between *P. coccinea* and *Passiflora setacea*, “BRS Rubiflora” (N.Ref. RNC-MAPA 21718) was obtained from a cross between the F₁ hybrid (*P. coccinea* and *P. setacea*) with *P. coccinea*, and “BRS Roseflora” (N.Ref. RNC-MAPA 21715) was obtained from the cross between the F₁ hybrid (*P. coccinea* and *P. setacea*) with *P. setacea*.

These ornamental hybrids were developed at Embrapa Cerrados, Planaltina, Brazil (images and details are available at [46]). Ornamental hybrids have also been produced in Brazil's northeast region, including "*Passiflora alva*", "*Passiflora priscilla*" and "*Passiflora aninha*" all of which were obtained from a cross between *Passiflora palmeri* and *Passiflora foetida*. These ornamental hybrids were developed at the Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil [47] (images and details are available at [48]).

The identification of species with morphological and adaptive characteristics for ornamental use has increased in Brazil, particularly in research centers, such as the Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil; Embrapa Cerrados, Embrapa Mandioca e Fruticultura, Cruz das Almas, BA, Brazil; and the Instituto Agrônomo, Campinas, São Paulo, Brazil. Among the various passion fruit species with ornamental potential, the characterization and utility of ornamental hybrid generation of the following species is distinctive, at least at the institutions noted above: *P. coccinea*, *P. foetida*, *Passiflora morifolia*, *Passiflora mucronata*, *P. palmeri*, *P. setacea*, *P. suberosa*. For readers interested in a deeper understanding of the subject, we suggest reading the specific research of Abreu *et al.* [49] and Costa *et al.* [50].

Figure 2. Panel illustrating flowers from passion fruit species of ornamental ((a) *Passiflora coccinea*; (b) *Passiflora mucronata*; (c) *Passiflora alata*) as well as examples of ornamental passion fruit hybrids ((d) BRS Rubi do Cerrado; (e) BRS Pérola do Cerrado; (f) BRS Estrela do Cerrado; (g) BRS Rosea Púrpura; (h) BRS Céu do Cerrado). Credits: F.G. Faleiro & NTV Junqueira, researchers from the Embrapa Cerrado, Brazil.



The use of *Passiflora* species as medicinal plants has been previously documented. The leaves, flowers, roots, and fruit of wild and commercial species are known to fight diseases, such as helminth infestations, gastric tumors, and stress, and are an integral component of the cultural tradition of many populations [50]. Passion fruit is a rich source of minerals and Vitamins A, C, and D [51] as well as a source of alkaloids, flavonoids, and carotenoids that are beneficial to human health [52]. Passion fruit seeds are sources of essential fatty acids (55%–66% linoleic acid, 18%–20% oleic acid, and 10%–14% palmitic acid), which may be used in the food and cosmetic industries [53]. The potential commercial

use of passion fruit also includes the extraction of oils for the manufacturing of soaps, creams, shampoos, and other products by the cosmetic industry [54]. Compounds in passion fruit plants with anxiolytic, antihypertensive, sedative, and analgesic properties are well known [55,56]. Considering the research presented above, the following species stand out among the various passion fruit species with potential medicinal properties: *P. alata*, *Passiflora caerulea*, *P. edulis*, *P. foetida*, *Passiflora incarnata*, *Passiflora laurifolia*, and *Passiflora maliformis*.

Despite the ecological importance and different potential uses of *Passiflora* as a genetic resource, it is the production of fruit for food and commercially produced juice that justifies the cultivation of the passion fruit plant [57] and that has led to increasing academic and economic interest. Brazil is the largest producer and consumer of passion fruit [58,59]. National production reached approximately 776,000 tons in 2012 [60]. Although the cultivation of passion fruit occurs in all regions of the country, northeastern Brazil produces approximately 73% of the national product [60]. Approximately 90% of the area designated for passion fruit culture is cultivated with *P. edulis*, and *P. alata* is the second most cultivated species of the genus [54,61]. Although they represent a small proportion of national production, the cultivation and consumption of fruits from wild species, such as *Passiflora cincinnata*, *Passiflora nitida*, *P. quadrangularis*, and *P. setacea*, have been reported in the literature [31,57,61–63].

Brazil's position as the largest passion fruit producer is due to the large size of the area cultivated rather than its productivity (Table 1). Data from the Brazilian Institute of Geography and Statistics, shown in Table 1, indicate an increase in the area devoted to passion fruit cultivation and fruit production but do not indicate a corresponding increase in productivity. The change in productivity of passion fruit cultivation was no more than 4% over the last 10 years despite the available natural diversity and progress in genetic breeding techniques. Furthermore, the recent mean productivity (14 tha^{-1}) in Brazil (Table 1) is less than 30% of the potential productivity rate estimated for passion fruit cultivation (50 $\text{tha}^{-1}\cdot\text{year}^{-1}$) with the use of improved cultivars and techniques [61].

The major factors that limit productivity are the absence of productive varieties that are adapted to local conditions, a lack of resistance to major diseases, and the use of seeds obtained by open pollination from plants in the planted area, which generates low-quality fruit and inconsistent production [30,32,64]. These conditions primarily occur because the available improved genotype selections for producers are scarce [61,64,65]. The *Passiflora* genetic resource exhibits variability in resistance to major passion fruit diseases and a high morphological variability, which together may contribute to disease control or the diversification of products. Although the potential for wild and commercial species is remarkable, basic studies involving the agronomic characterization of the species must be initiated.

Because molecular biology in general and the use of molecular markers in particular provide a wide range of useful tools for basic and applied research associated with genetic breeding at different stages (from the prospect of germplasms to the characterizations required for pre-breeding and the protection of cultivars in the post-breeding period), success is expected for passion fruit breeding programs in the future. Therefore, the current review will address the available knowledge on this subject (with tables and diagrams to represent the data quantitatively and qualitatively) and discuss the relevant studies and resulting knowledge, although this review does not pretend to end the discussion on the current and potential use of molecular markers to characterize the genus *Passiflora* and for plant breeding.

Table 1. Descriptive presentation of the Brazilian agricultural production * of passion fruit between 2003 and 2012.

Period (Years)	Planted Area (ha × 1000)	Production (t × 1000)	Productivity (t/ha)
2003	34.9	485.3	13.9
2004	36.6	491.6	13.4
2005	35.8	479.8	13.4
2006	44.4	615.2	13.9
2007	46.8	664.3	14.2
2008	48.7	684.4	14.0
2009	50.8	718.8	14.2
2010	62.0	920.2	14.8
2011	61.6	923.0	14.9
2012	57.8	776.1	13.4
Average (CV)	48 (21%)	676 (24%)	14 (4%)

* Original data were obtained from the Municipal Agricultural Production presented by the Instituto Brasileiro de Geografia e Estatística [60] between 2003 and 2012; CV = coefficient of variation.

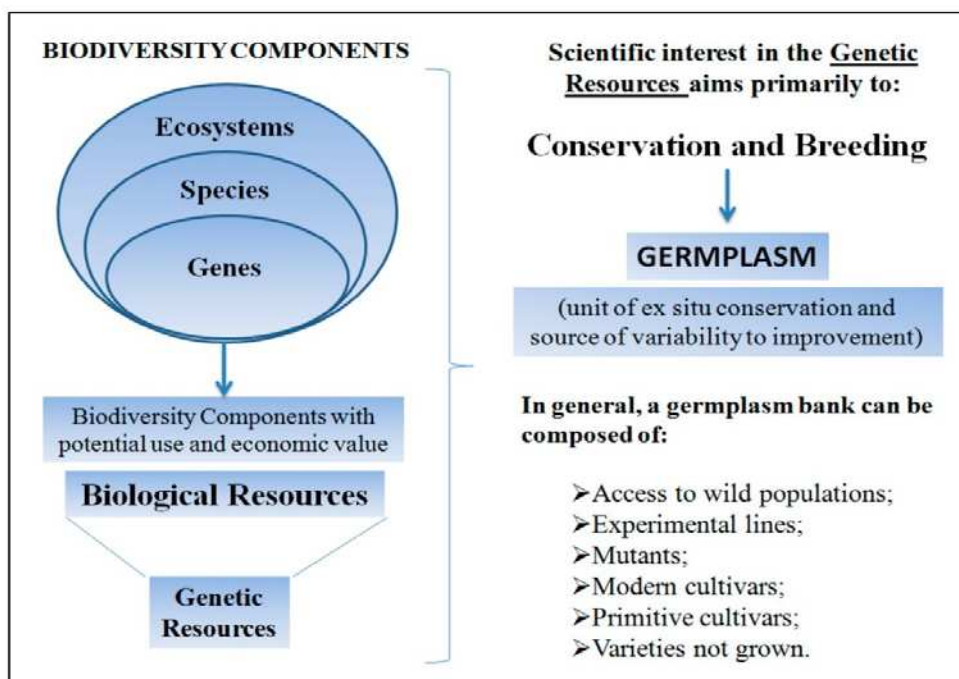
2. Contributions of Molecular and Genetic Studies for the Characterization and Use of *Passiflora* Biodiversity

2.1. Biodiversity, Conservation, and Breeding of Passion Fruit

The concept of biodiversity may vary according to the context in which it is employed. Biodiversity may be defined as the totality of genes, species, and ecosystems of a specific region or the planet as a whole. This totality is the result of evolutionary processes [66,67]. The components of biodiversity with an immediate and/or potential interest for humans constitute a biological resource that may, in turn, be classified as a genetic resource [67] including the genetic variability of social and economic assets. In general, genetic resources are considered sources of variability (natural raw material) both for breeding programs and to support conservation strategies. However, several characterizations that involve different methodological approaches are required to use genetic resources for the conservation of biodiversity and genetic breeding. We have devised an illustrative scheme to facilitate the understanding and visualization of the relationships among the presented concepts (Figure 3). However, for readers interested in expanding on this theme, we also recommend the reading of specialized texts [68].

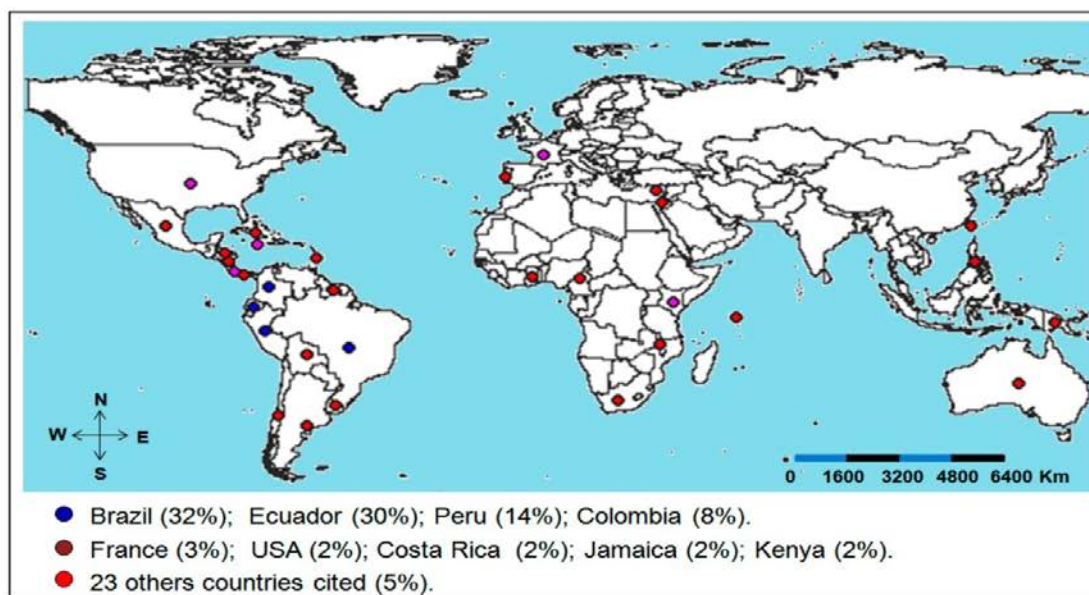
Although *Passiflora* has a high species diversity, and despite the dispersion of this genus within different tropical biomes, information on the intra- and extraspecies genetic diversity is rare for most species. Research available in the primary databases of scientific publications (Scopus [69] and Web of Science [70]) includes characterizations of genetic diversity based on molecular markers of fewer than 60 species (details are provided in Table 2 and discussed in the following sections). Nearly all these studies were performed using dominant markers, which do not allow for the maximum exploitation of genetic information because it is impossible to distinguish heterozygote genotypes, and were based on a few commercial genotypes with only local interest and/or a few wild accessions per species. Therefore, these studies cannot be characterized as population genetics studies.

Figure 3. Schematic illustrating the different components of biodiversity and the classification of those components that have generated immediate interest or are of potential use for society, scientific interests, or conservation and breeding. The data presented were summarized from the results of previous work [66,67].



Similar to the lack of information available on the cytogenetics (discussed in the introduction of this review) and genetic diversity of *Passiflora*, the number of accessions present in collections and active germplasm banks (BAGs) of passion fruit are considered incipient in Brazil and across the world [27,54,59]. At least 1200 accessions of *Passiflora* spp. are estimated and are preserved in 50 collections of germplasms distributed in approximately 32 countries (Figure 4) [54,59]. Among these countries, we highlight Brazil, Ecuador, Peru and Colombia, which account for approximately 84% of accessions. Although it is likely that the exact number of accessions kept in these collections has varied since the compilations made by Ferreira [59], the predominance of accessions in South American collections present in tropical countries is expected because this region is considered as a center of diversity of the genus *Passiflora* [7–10]. In addition, these same countries are recognized as leading producers of passion fruit. [59]. Although Brazil is considered an important center of genus diversity, extant collections contain fewer than 70 species of the genus, or approximately 50% of the Brazilian *Passiflora* species and less than 15% of all species within the genus [59]. Furthermore, the majority of accessions in BAGs lack biological, genetic, and molecular characterizations. This dearth of information on accessions is partially responsible for the lack of passion fruit breeding programs and may have also resulted in the reduced productivity of existing programs.

Figure 4. Map showing the countries where worldwide collections of passion fruit (*Passiflora* spp.) are located. The countries with the most representative collections are identified with blue circles (four countries, representing approximately 84% of accessions), followed by countries identified with magenta circles (five countries, representing approximately 11% of accessions) and countries identified with red circles (23 countries, representing approximately 5% of accessions). Note: The percentages of accessions were calculated from the data presented by Ferreira [59].



The vast majority of passion fruit accessions are maintained in Brazilian institutions, highlighted by the collections and germplasm banks maintained by Embrapa (Centro de Pesquisa Nacional de Mandioca e Fruticultura, and Centro de Pesquisa Agropecuário do Cerrado), the Instituto Agrônomo de Campinas (IAC), and the Instituto Agrônomo do Paraná (IAPAR) as well as the Universidade Estadual Paulista (UNESP), Universidade Estadual do Norte Fluminense (UENF), Universidade Federal do Rio de Janeiro (UFRJ), and Escola Superior de Agricultura Luiz de Queiroz (ESALQ/USP) [25]. The current estimate is that these eight institutions have 65 passion fruit species represented in 640 accessions, approximately 25% of which are related to the two species of greatest commercial interest, namely, *P. edulis* (with 105 accessions) and *P. alata* (with 65 accessions) [25,59].

The discrepancy between the natural variability inherent to the genus and the reduced variability represented in BAGs should be remedied as quickly as possible because it hinders the establishment and progression of *Passiflora* genetic breeding programs [59]. Similarly, utilization of the available genetic variability for *Passiflora* has been neglected during cultivar generation [61]. Thus, although several wild passion fruit species are resistant to diseases and pests, derived cultivars do not have the same genetic resistance. In fact, these cultivars are prone to contracting the major diseases of cultivated passion fruit. Furthermore, the literature suggests that different cultivars exhibit low variability in their levels of resistance [32].

The above conditions are justified, at least partially, because the first studies in the 1980s [28,71] that contributed to the establishment of *Passiflora* breeding programs prioritized production (quantitatively and qualitatively) and not necessarily the other aspects of genetic diversity, such as resistance, ornamentation, and the production of potential medicinal compounds.

In addition to the potential for commercialization because of the natural beauty of their flowers and the taste of their fruits, wild passion fruit species have desirable characteristics for the yellow passion fruit, including resistance to pathogens and pests (*P. caerulea*, *P. cincinnata*, *P. coccinea*, *Passiflora gilberti*, *P. laurifolia*, *P. setacea*, and *P. suberosa*), self-compatibility (*P. tenuifolia*, *P. elegans*, *P. capsularis*, *P. villosa*, *P. suberosa*, *P. morifolia*, and *P. foetida*), and variability in the season of production compared with commercial species (*P. setacea* and *P. coccinea*) [33,54]. For some species, such as *P. caerulea*, *P. cincinnata*, *P. coccinea*, and *P. setacea*, interspecific hybrids are being developed and characterized [33,54,72] and estimates of genetic diversity and confirmation of the hybrids based on molecular markers are being carried out [54,62,63].

According to these studies, the genetic base of the yellow passion fruit is restricted with respect to disease resistance and tolerance. In fact, the authors favor the use of genetic resources from wild species in breeding programs. Several authors have emphasized research on the description, characterization, and use of germplasm as a priority for passion fruit production to obtain more productive passion fruit cultivars with increased resistance to diseases [54,73]. The joint use of molecular markers and classical breeding procedures has been suggested as a necessary strategy to accelerate the production of fruit varieties that are adapted to different Brazilian regions [64].

Although the realities of existing cultivars, such as low variability and susceptibility to disease, still endure for cultured passion fruit, an increasing number of studies devoted to the characterization of resistance in wild species and commercial accessions are being performed [32,34,74,75]. Interspecies crosses that aim to incorporate the resistance genes of wild species into commercial species are also being implemented and evaluated [54,76,77]. The following sections discuss the applications and contributions of molecular markers to our understanding of the genetic diversity of *Passiflora* and to the various stages of breeding programs.

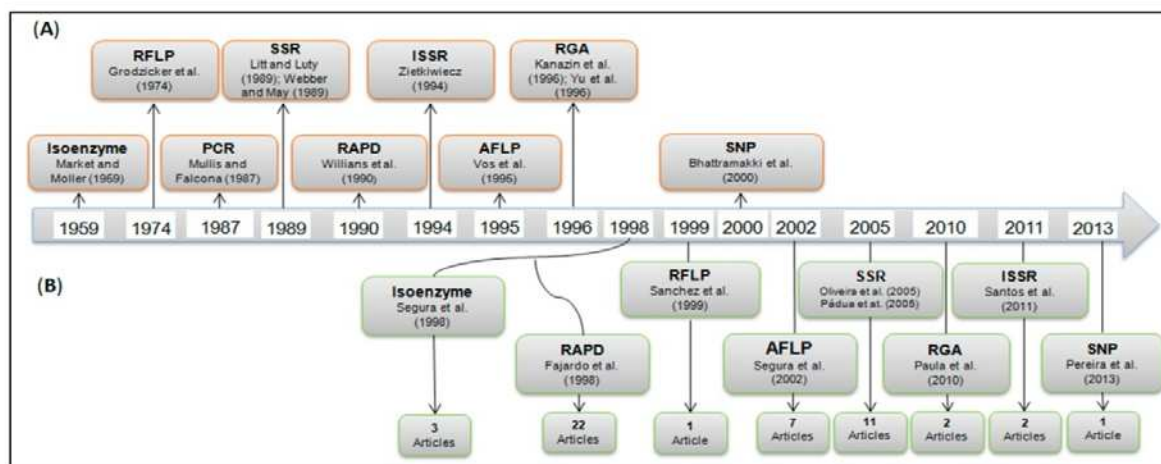
2.2. Application of Molecular Markers for the Characterization of *Passiflora* Diversity

The use of molecular markers for genetic characterization projects with a variety of objectives for a range of organisms is routine in many laboratories. Although molecular biology techniques have become prevalent in recent decades, leading to a reduction in the cost of most molecular markers, the availability of resources and background information are still determining factors in the choice of methods and markers to be used. In recent decades, the increase in the number of molecular markers associated with the growth of related fields of study, such as population genetics, genetic breeding, statistics, and bioinformatics, has generated immense progress in the knowledge applicable to conservation and biodiversity manipulation.

Genetic studies associated with molecular markers are becoming standard for the *Passiflora* genus. These studies range from the use of dominant markers to the development and employment of co-dominant markers and single-nucleotide polymorphisms (SNPs). Figure 5 provides a general chronological overview of the evolution of major molecular markers and their use in studies of

Passiflora. A simple quantitative evaluation of published papers, the data from which are shown in Figure 5, indicates that the use of molecular markers in *Passiflora* research began approximately 15 years ago with the use of the random amplified polymorphic DNA (RAPD) markers to study the diversity of 52 accessions from 14 *Passiflora* species [7].

Figure 5. Chronological schematic illustrating the main molecular markers and their initial reference (A) as well as the published articles using molecular markers in the genus *Passiflora*, their initial reference, and the total number of papers (B).



Despite the availability of approximately 4000 fragments of DNA and RNA sequences from *Passiflora* (data from the NCBI nucleotide database [78]), genetic studies employing widely used molecular markers in different stages of breeding programs and for estimates of diversity (for example, restriction fragment length polymorphism (RFLP), RAPD, amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), and SNP markers) have typically been limited to a few *Passiflora* species. However, additional species are currently being studied. Table 2 (and the supplementary Information) provides a summary of existing research using molecular markers during different phases of genetic breeding (from pre- to post-breeding) regardless of the study's scope. Table 2 includes all of the information about these studies, ranging from the investigation goals to the techniques and species used. The data in Table 2 confirm the absence of genetic and molecular information reflected by the genetic diversity of *Passiflora*; few species have been studied (fewer than 60), and most of the research has focused on *Passiflora* species or accessions with previously established commercial interest. Research on ecological justifications and aims coupled with species conservation have not been performed, although brief discussions on the characterization and *ex situ* conservation have been published.

Most molecular genetic studies (approximately 65% of all papers, 85% of which are based on genetic diversity) are based on the use of dominant markers. Although dominant markers are useful for genetically distinguishing species (for tests to confirm hybrids and the direction of convergent and divergent breeding), they have limitations, such as low repeatability and suboptimal genetic

information profiles (homozygote and heterozygote specimens cannot be distinguished), that are highly relevant for population studies.

Despite the limitations inherent in the use of dominant markers, genetic diversity estimates based on wild and commercial accessions have contributed to the understanding of the genetic variability of passion fruit. In this context, RAPD and AFLP markers have been used to demonstrate high genetic variability among accessions of *P. edulis* [58,79] as well as a lack of correlation between the estimated genetic distances and geographical origins of the accessions evaluated [79]. However, estimates of variability using RAPD markers performed with commercial accessions of *P. edulis* indicated low genetic variability [8,80]. The authors of these studies have suggested that the apparent contradiction between the low genetic diversity identified with molecular markers and the large variability observed in commercial plantations is due to the major influence of the environment on morphological characteristics. Greater genetic distance was observed among accessions of purple and yellow fruits [8,58], and the accessions of purple and yellow fruits are considered genetically distinct despite belonging to the same species [58].

The use of RAPD markers has also contributed to estimates of diversity in wild species of passion fruit, such as *P. cincinnata*, *P. nitida*, and *P. setacea* [62,63,81]. In these studies, large genetic variability was observed, with percentages of polymorphic loci ranging from 64% in *P. nitida* [81] to 93% in *P. setacea* [63]. The fact that many studies, especially those conducted on wild species, only address accessions collected in the same geographic region illustrates the need to conduct further studies with a broader representation of accessions to more completely determine the variability and possible genetic structure of passion fruit.

Results on the characterization of genetic variability and the identification of potential regions related to resistance genes, phylogenetically conserved in plants, were first published for the genus *Passiflora* in 2010 [82] (Figure 5 and Table 2). This study used six degenerate combinations of resistance gene analogs (RGA) primers, originally designed to anneal with the NBS (nucleotide binding site) motive present in several classes of resistance genes. The results presented by Paula *et al.* [82] show wide interspecific variability among the amplicons generated, enabling the use of these markers for variability studies in germplasm banks as well as in genetic mapping. Although these are preliminary results, the authors also observed amplicons containing genomic segments of type NBS-LRR (leucine-rich protein and the nucleotide binding sites) present in different plant species.

Since the publication of these results, studies of intra-and interspecific diversity performed with use of RGA markers are being conducted at the Universidade Estadual do Sudoeste da Bahia (UESB, Itapetinga, Bahia), and the preliminary results confirm the efficiency of these markers for the characterization of genetic variability in commercial and wild species of passion fruit [83]. RGA markers have also contributed to linkage analysis and the construction of a recent genetic map for *P. alata* [84].

Table 2. Descriptive and quantitative presentation of papers published using different molecular markers in the genus *Passiflora*.

Molecular Markers	General Aim of the Article	Evaluated Species	No. of Species	No. of Articles
Isozymes	Estimation of diversity (germplasm)	<i>P. ampullacea</i> , <i>P. antioquiensis</i> , <i>P. bracteosa</i> , <i>P. cumbalensis</i> , <i>P. manicata</i> , <i>P. mixta</i> , <i>P. pinnatistipula</i> , <i>P. tarminiana</i> , <i>P. tripartita</i>	10	3
RFLP	Estimation of diversity (germplasm)	<i>P. edulis</i> , <i>P. ligulares</i> , <i>P. maliformis</i> , <i>P. caerulea</i> , <i>P. mollissima</i> , <i>P. sp. india</i> , <i>P. cumbalensis</i> , <i>P. antioquiensis</i> , <i>P. pinnatistipula</i> , <i>P. x rosea</i> , <i>P. adenopoda</i> , <i>P. coriacea</i>	12	1
RAPD	Estimation of diversity (germplasm)	<i>P. adenopoda</i> , <i>P. alata</i> , <i>P. amethystina</i> , <i>P. antioquiensis</i> , <i>P. caerulea</i> , <i>P. capsularis</i> , <i>P. cincinnata</i> , <i>P. coccinea</i> , <i>P. coriacea</i> , <i>P. cumbalensis</i> , <i>P. edulis</i> , <i>P. foetida</i> , <i>P. gibertii</i> , <i>P. laurifolia</i> , <i>P. ligularis</i> , <i>P. macrocarpa</i> , <i>P. malacophylla</i> , <i>P. maliformis</i> , <i>P. micropetala</i> , <i>P. mollissima</i> , <i>P. morifolia</i> , <i>P. mucronata</i> , <i>P. nitida</i> , <i>P. palmeri</i> , <i>P. pinnatistipula</i> , <i>P. serrato</i> , <i>P. setacea</i> , <i>P. spinosa</i> , <i>P. suberosa</i> , <i>P. subpeltata</i> , <i>P. trintae</i> , <i>P. vitifolia</i> , <i>P. xrosea</i>	33	15
	Characterization and confirmation of hybrids	<i>P. alata</i> , <i>P. gardneri</i> , <i>P. gibertii</i> , <i>P. foetida</i> , <i>P. subanceolata</i> , <i>P. watsoniana</i>	6	3
	Genetic mapping	<i>P. edulis</i>	1	1
	Other	<i>P. actinia</i> , <i>P. alata</i> , <i>P. amethystina</i> , <i>P. caerulea</i> , <i>P. coccinea</i> , <i>P. eichleriana</i> , <i>P. edulis</i> , <i>P. galbana</i> , <i>P. glandulosa</i> , <i>P. gibertii</i> , <i>P. laurifolia</i> , <i>P. mucronata</i> , <i>P. nitida</i> , <i>P. sidaefolia</i> , <i>P. setacea</i>	15	2
AFLP	Estimation of diversity (germplasm)	<i>P. alnifolia</i> , <i>P. ampullacea</i> , <i>P. antioquiensis</i> , <i>P. bracteosa</i> , <i>P. cumbalensis</i> , <i>P. edulis</i> , <i>P. fimbriatistipula</i> , <i>P. gracilens</i> , <i>P. ligularis</i> , <i>P. mixta</i> , <i>P. manicata</i> , <i>P. parritae</i> , <i>P. pinnatistipula</i> , <i>P. popenovii</i> , <i>P. tarminiana</i> , <i>P. tenerifensis</i> , <i>P. tiliaefolia</i> , <i>P. tripartita</i> , <i>P. trifoliata</i> , <i>P. trinervia</i>	20	3
	Genetic mapping	<i>P. alata</i> , <i>P. edulis</i>	2	2

Table 2. Cont.

Molecular Markers	General Aim of the Article	Evaluated Species	No. of Species	No. of Articles
SSR	Estimation of diversity (germplasm)	<i>P. capsularis</i> , <i>P. edulis</i> , <i>P. rubra</i>	2	4
	Characterization and confirmation of hybrids	<i>P. foetida</i> , <i>P. subanceolata</i>	2	1
	Genetic mapping	<i>P. edulis</i>	1	1
	Development, characterization and selection of markers	<i>P. alata</i> , <i>P. cincinnata</i> , <i>Passiflora contracta</i> , <i>P. edulis</i> , <i>P. setacea</i>	5	7
	Cross-amplification	<i>P. caerulea</i> , <i>P. cincinnata</i> , <i>P. edulis</i> , <i>P. foetida</i> , <i>P. gibertii</i> , <i>P. ligularis</i> , <i>P. maliformis</i> , <i>P. mucronata</i> , <i>P. rubra</i> , <i>P. setacea</i> , <i>P. suberosa</i>	11	2
	Other	<i>P. alata</i>	1	1
RGA	Estimation of diversity (germplasm)	<i>P. caerulea</i> , <i>P. coccinea</i> , <i>P. edulis</i> , <i>P. gibertii</i> , <i>P. nitida</i> , <i>P. odontophylla</i> , <i>P. serratodigitata</i> , <i>P. setacea</i>	8	1
	Genetic mapping	<i>P. alata</i>	1	1
ISSR	Estimation of diversity (germplasm)	<i>P. alata</i> , <i>P. edulis</i>	2	2
SNP	Genetic mapping	<i>P. alata</i>	1	1
Other markers	Estimation of diversity (germplasm)	<i>P. edulis</i>	1	1
	Genetic mapping	<i>P. alata</i>	1	1

RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; SSR, simple sequence repeat; RGA, resistance gene analogs; ISSR, inter-simple sequence repeat; SNP, single-nucleotide polymorphism; The data presented were obtained from queries of the Scopus [69] and Web of Science [70] databases. Detailed information on the references used to construct this table is available in the supplementary material.

Studies on *Passiflora* using co-dominant markers began in 2005 and were mainly limited to the development of microsatellite markers for the species *P. edulis* [85–87], *P. alata* [88,89], *P. cincinnata* [87,90], *P. setacea* [87], and *P. contracta* [91]. Studies on other species of this genus addressed the evaluation of cross-amplification [15,92–94] and hybrid confirmation [45]. Most of these studies were restricted to characterizations in agar gels or polyacrylamide gels and require additional evaluation, including profiles of amplifications and polymorphic loci. The identification and characterization of microsatellite loci in the genus *Passiflora* indicated low levels of polymorphism in these genomic regions, at least for the species evaluated (Table 3). The average number of alleles per characterized microsatellite locus was low in studies of wild and commercial species of *Passiflora*, ranging from 2.8–5 alleles per locus for the wild species *P. cincinnata*, *P. contracta*, and *P. setacea* [87,90,91] and from 3.1–7.6 alleles per locus for the species of greatest commercial interest, namely, *P. edulis* and *P. alata* [85,88].

Regarding the estimates observed for microsatellite loci from passion fruit (Table 3), it is also important to note that the low values of observed heterozygosity, compared with the values of expected heterozygosity, suggest low allelic diversity and tendency toward the fixation of alleles, at least for the germplasm evaluated. The percentage of polymorphic microsatellite loci observed in characterizations of passion fruit species can also be considered low, with an average of approximately 28% of microsatellite loci polymorphic across wild and commercial species; in the wild species *P. cincinnata* and *P. setacea*, the individual values are 21% and 29%, respectively [86,89], and in the commercial species *P. alata* and *P. edulis*, the individual values are 25% and 35%, respectively [86,89].

Table 3. Average values observed for the number of alleles (N_a) and observed (H_o) and expected (H_e) heterozygosity in the characterization of microsatellite loci from passion fruit species (*Passiflora* spp.).

Species	Characteristics of Microsatellite Loci			References
	N_a	H_o	H_e	
<i>P. alata</i>	3.1	0.26	0.53	Pádua <i>et al.</i> [82]
<i>P. cincinnata</i>	5	0.52	0.52	Cerqueira-Silva <i>et al.</i> [84]
<i>P. cincinnata</i>	3.3	0.26	0.36	Cerqueira-Silva <i>et al.</i> [81]
<i>P. contracta</i>	4.9	0.53	0.61	Cazé <i>et al.</i> [85]
<i>P. edulis</i>	7.6	0.58	0.62	Oliveira <i>et al.</i> [79]
<i>P. edulis</i>	3.4	0.31	0.36	Cerqueira-Silva <i>et al.</i> [81]
<i>P. setacea</i>	2.8	0.34	0.41	Cerqueira-Silva <i>et al.</i> [81]

Research that characterizes genetic diversity in wild populations by microsatellite markers remains unpublished, although reports on evaluations of germplasm materials in Colombia [95] and Brazil [94–98] have been published. The microsatellite marker sets used by Ortiz *et al.* [95] in a study with *P. edulis* and by Amorim *et al.* [96] in a study with *P. capsularis* and *P. rubra* have not shown polymorphisms, whereas other studies performed by Reis *et al.* [97,98] have exclusively evaluated genotypes from cycles of recurring selection in *P. edulis* progenies. The hypothesis that microsatellite loci have low levels of polymorphism, which has been based on identification and characterization studies of the microsatellites [85–90], is supported by the absence of polymorphism observed in a study of genetic variability in Colombia that analyzed 70 passion fruit plants (*P. edulis*) collected in 60 commercial

plantations [95]. Ten microsatellite loci reported in *P. edulis* [85] and seven in *P. alata* [88] were used in that study.

The first successful estimates of genetic diversity in *Passiflora* accessions were obtained with SSR markers in 2013 [92,94]. However, those studies were limited in their estimates of intraspecific diversity because of the small number of plants that were evaluated (fewer than six plants for most species). In addition, even for interspecific studies, such as that performed by Oliveira *et al.* [92], where 11 passion fruit species were considered, the average number of alleles was not more than five (ranging from 1–13 alleles per locus).

The variation in the genetic diversity estimated in published papers, primarily those analyzing commercial species, is due to not only the specific characteristics of the genotypes evaluated but also the effects of the different statistical methodologies employed by the authors. Methodological influences on the results of diversity estimates have been reported for a variety of species, including *Mangifera indica* [99], *Olea europaea* [100], *Zea mays* L. [101], and *Solanum lycopersicum* [102]. Differences in methodologies have also been discussed for studies of morphological characteristics and of genetic diversity that used molecular markers to study passion fruit [103]. In the latter work involving passion fruit, comparisons were made among seven distance measures, 14 similarity coefficients, and five grouping methods. The authors concluded that the diversity estimate is significantly influenced by the method employed. Considering the different methodological strategies that have been employed for data analysis in the various studies of diversity among passion fruit accessions, the authors suggest that the choice of the methodology to be used and the comparison of results obtained using different measurements and coefficients should be performed carefully.

Research projects conducted as part of a joint venture between the Universidade Estadual de São Paulo (ESALQ/USP, Piracicaba, Brazil) and Centre National de Ressources Génomiques Végétales (CNRGV/INRA, Toulouse, France) during the past five years have introduced the first SNP identifications in commercial passion fruit species (*P. alata*) [84]. However, the use of these data remains limited to the construction and saturation of genetic maps, as is the case for most microsatellites available for commercial *Passiflora* species.

2.3. Application and Perspectives of Genetic Engineering for Passion Fruit Culture

Considering the diversity of species and intra- and interspecific genetic variability available for use in breeding programs of passion fruit, genetic engineering as a methodological strategy to increase favorable characteristics for passion fruit culture has been specifically employed to search for disease resistance [104]. With regard to diseases such as passion fruit woodiness (PWD) (caused in Brazil by *Cowpea aphid-borne mosaic virus*) and the bacterial spot disease (caused by *Xanthomonas axonopodis* pv. *passiflorae*), for the which chemical control measures are not effective and resistant cultivars are not available, a growing number of advances in the use of genetic engineering have been observed in recent decades [105–108].

Studies attempting to produce transgenic passion fruit have been conducted since the late 1990s and early 2000s at the Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil, and Universidade de São Paulo (USP), São Paulo, Brazil; the first results were published in 2005 [105] and 2006 [106]. In these two studies, plants of yellow passion fruit (*P. edulis*) were transformed from fragments of

genes obtained from viral isolates known to cause PWD. These studies were initiated based on a pathogen-derived resistance (PDR) approach that likely relied on the induction of post-transcriptional gene silencing (PTGS).

An untranslatable construct consisting of two-thirds of the *NIb* gene and the 5' region of the CP gene derived from a viral isolate of CABMV was used by Alfenas *et al.* [105]. In turn, a construct consisting primarily of the CP gene was used by Trevisan *et al.* [106]. In both studies, transgenic plants resistant to PWD were obtained. However, the effective resistance observed in the transformed plants by Alfenas [105] was restricted to the viral isolate from which the fragments of the *NIb* and CP genes were isolated. It is probable that the high variability in the nucleotide sequence of the *NIb* gene was the main factor contributing to this result. Subsequently, the transgenic plants (R_0) were self-crossed to generate homozygous lines (R_1), and a plant of this line was resistant to seven viral isolates [109]. The authors defend the hypothesis that the dose used, due to the presence of homozygous plants for the transgene in the R_1 lineage, contributes substantially to the spectrum of resistance observed [105,109].

In addition, one of the transgenic plants produced by Trevisan *et al.* [106] was immune to all the three isolates considered in your search, but the potential resistance of these transgenic lines to other isolates found in Brazil is not known. Studies performed by Monteiro-Hara [107] identified new transgenic lines of passion fruit and generated transgenic lines R_1 and R_2 , both from transgenic plants characterized by Trevisan *et al.* [106], as well as from the new lines constructed. Homozygous lines did not show symptoms of the viral disease after either mechanical inoculation or inoculation by aphids [107]. New crosses were made between R_2 plants selected by the authors for future studies.

Although in smaller proportions or in less advanced stages, studies devoted to obtaining transgenic plants were also conducted with *P. edulis* for resistance to diseases and herbicides through the introduction of a baculovirus anti-apoptotic gene (*p35* gene) [108] and the resistance to bacterial spot disease by introducing the sequence of the attacin A gene (*attA*) [110]. Plants of *P. edulis* transformed with *p35* gene sequences showed no resistance to CABMV but showed increased tolerance to bacterial spot disease and glufosinate herbicide when compared to non-transgenic plants [108]. Ten plants of *P. edulis* transformed with the *attA* sequence of the gene showed no leaf lesions after inoculation with *X. axonopodis* pv. *Passiflorae*, indicating possible resistance to the pathogen. Initial tests attempting to obtain transgenic plants were conducted with *P. alata* [111]. These experiments demonstrated the possibility of obtaining transgenic plants for *P. alata*. Similar to the work conducted with *P. edulis*, the transformations achieved in *P. alata* were conducted using *Agrobacterium tumefaciens* and a fragment of the CP gene CABMV.

In conjunction, the progress observed with the studies dedicated to the production of transgenic passion fruits indicates that, at least for PWD, it is possible that resistant plants will soon become available for cultivation and/or for use in breeding programs dedicated to the production of resistant cultivars with higher productivity.

2.4. Contributions and Perspectives of Molecular Biology in Pre- and Post-Breeding Programs

The contributions of molecular biology, and especially the use of the molecular markers, in (pre-) breeding programs may be didactically separated into at least six research modes: (i) the direction of convergent and divergent crossings leading the initial stages of breeding; (ii) the confirmation of intra- and interspecies hybrids with a reduction in the occurrence of escapes; (iii) the contribution of retro-crossings with a decrease in the time necessary for the re-composition of the genome; (iv) the genetic mapping and identification of quantitative trait loci (QTLs) that are useful for the subsequent introgression of genes into commercial species and cultivated varieties; (v) the prospection and characterization of resistance genes, both by mapping strategies and through the use of analog markers of resistance genes; and (vi) the identification and protection of hybrids and cultivars. Coupled with the effective use of markers, these joint activities contribute to the establishment of an assisted selection program because all of the information provided enables the efficient use of the available germplasm.

In addition to their contribution to understanding the diversity of populations or groups of regional accessions, estimates of passion fruit genetic diversity have enabled the identification of converging and diverging crosses that can contribute to different stages of a (pre-) breeding program. For commercial species, such as *P. edulis* [80], as well as wild species, such as *P. setacea* [63] and *P. cincinnata* [62], preferred crossings were suggested after the characterization of RAPD markers to generate progenies with greater or lower potential for segregating characteristics. The identification of genetically divergent accessions between yellow and purple passion fruit that has been achieved based on molecular markers [8,58] can also be used to target crosses that aim to broaden the genetic base of breeding programs for *P. edulis*.

The absence of agronomic characterizations for most accessions in the aforementioned studies is the greatest challenge to introducing information from genetic diversity estimates into current breeding programs. Difficulties in the joint use of data from phenotype and genotype characterizations (particularly when related to neutral markers) have been reported for different cultures and are an aspect of the challenge inherent in studies of natural populations [112]. For cultivar development, the pre-breeding programs that completely exploit the use of molecular techniques and field characterizations in germplasm are indicated as bonding strategies between natural variability and the cultivars employed for the maintenance and growth of cultures [67].

Different molecular markers, such as RAPD and microsatellites, are highly efficient and user-friendly techniques that can be used to confirm intra- and interspecies hybrids. The first study to employ molecular markers to confirm hybrids from 17 interspecies crosses of 14 *Passiflora* species of agronomic interest associated with fruit production was published in 2008 [72] and was based on the profile of DNA fragments generated using 12 RAPD primers. Similar molecular markers have since been utilized to confirm and characterize interspecific ornamental hybrids [47,113] based on the profile of DNA fragments generated using seven RAPD primers and one SSR primer. The confirmation of hybrids using molecular markers may decrease the occurrence of escapes and reduce costs via the restricted maintenance of seedlings to be used in sequential stages of a (pre-) breeding program.

Molecular markers also contribute to the characterization and selection of specimens with a higher genomic contribution from the recurrent parent [76,114]. The characterization of parents and segregant

genetic diversity, which are associated with evaluations of the specific phenotype, inform crossings and reduce the number of backcrossing cycles required to obtain the hybrid of interest at the start of breeding. This strategy has improved the introgression of disease resistance found in the wild species *P. setacea* into the cultivars GA-2, AR-1, and EC2-O of *P. edulis* [114]. An experiment performed at EMBRAPA Cerrados re-established a 92% average of the commercial ancestor's genome (*P. edulis*) in hybrids while maintaining the resistance of the wild ancestor (*P. setacea*) [114].

One of the most thorough passion fruit studies, at least with regard to molecular markers within the context of breeding, consists of the construction of genetic maps and identification of regions associated with characteristics of interest (QTLs or, more specifically, quantitative resistance loci, QRLs). Early genetic mapping of passion fruit species began in 2000, and the first maps were published in 2002 [115]. These maps were based on the characterization of a segregant population of *P. edulis* using RAPD markers. The population used to construct the first map helped to identify the first QRLs linked to the genus *Passiflora* using AFLP markers [116]. The quantitative resistance locus detected by Lopes *et al.* [116] explained approximately 16% of the phenotypic variation related to symptoms from bacterial spot disease caused by *X. axonopodis* pv. *passiflorae*.

The use of map integration strategies to construct a single map (rather than two maps representing ascendants) [86] was a landmark in genetic mapping studies and the identification of QTLs in passion fruit. Another important feature of the *Passiflora* map was the use of microsatellite markers for the map construction in addition to AFLP [86]. An important advantage of the integrated construction of a single representative map of a segregant population is an increase in map saturation and length. The extension of research to construct maps and the identification of QTLs in passion fruit plants have occurred only recently for another species of this genus: the first integrated genetic map for *P. alata* was published in 2013 [104]. The use of variable AFLP and SSR techniques and the first characterization of SNPs in the passion fruit plant were included in this map [104].

Research dedicated to the construction of physical maps has also been conducted [42]. Physical maps were obtained from putative genes identified from a passion fruit genomic library inserted into BACs [42,43]. This research is a new development in genetic and genomic studies of passion fruit; it characterizes the species' genomes, identifies evolutionary relationships, and develops new molecular markers, including SSR markers, expressed sequence tags (ESTs), and SNPs.

The advances in the genetic mapping of passion fruit associated with the reduction of costs of next-generation sequencing (NGS) should enable genetic characterizations based on genotyping-by-sequencing (GBS), at least for commercial species, in both the short and medium term. In this context, alternative methodologies that reduce costs and enable GBS, such as the use of restriction enzymes to reduce genome complexity [117], should be considered in future genetic studies of passion fruit; these methodologies can contribute to both population studies and procedures of genome-wide selection in collections and germplasm banks.

Molecular markers are also used in different stages: after cultivars are obtained and distributed (generically termed post-breeding), when paternity is determined (and thus the culture is protected), and when monitoring cultivar genetic purity, regardless of whether the cultivars are clonal or seminal [64]. When legislation to protect cultivars is in effect (Act 9456, Brazil), the growth, development, and use of marker groups for post-breeding will be increasingly necessary for passion fruit cultivation,

particularly because of the increase in the number of varieties that will be developed and made available to producers.

3. Conclusions, Perspectives, and Challenges

Considering the challenges of breeding passion fruit, as well as the available knowledge and conservation of the genetic variability of this genus, we believe that the following research activities should be prioritized: (i) the identification of new accessions to increase the representative breadth of germplasm banks, especially for wild species; (ii) the measurement of population genetics estimates that enable a true understanding of the genetic diversity and structure of passion fruit; (iii) phenotypic characterizations that contribute to both the useful evaluations of the germplasm for interspecific crosses and studies of the association between phenotypic and molecular data; (iv) research efforts devoted to identifying genomic regions associated with resistance to diseases; and (v) the potential use of transgenic strains previously evaluated in crosses targeted in breeding programs.

The recent research on the diversity of wild passion fruit species and the increasing use of molecular markers, particularly co-dominant markers, have made knowledge from population studies available and are promising for further genetic research on passion fruit. Although highly relevant for conservation and breeding activities, population studies of *Passiflora* are only in the initial stages. Coupled with a large gap in group population genetic research, the increasing availability of microsatellite markers for *Passiflora* species and the use of cross-amplification strategies to popularize marker use should increase the number of research studies. Further research should be performed to identify and characterize microsatellite loci because the current results indicate low allelic diversity among SSR loci. In this context, the use of strategies for sequencing and genotyping on a large scale will be important.

In parallel with the population estimates that must be performed for the genus *Passiflora* via molecular markers, strategies for the exploration and conservation of germplasm collections are also necessary to enable morpho-agronomic characterizations to be performed efficiently to increase the understanding of both neutral biological diversity and diversity that is subject to natural selection. In this way, the interactions involving the accessions present in banks, germplasm collections, and elite materials used in breeding programs will be enhanced.

Similar to the expected growth in microsatellite marker usage in population studies, recent genomic investigations of commercial passion fruit species have developed and characterized SNP markers. These studies should be promoted because they will encourage different types of research, as has occurred with the saturation of genetic maps, the identification of QTLs, and the exploitation of mapping by association. Collections, germplasms, or elite accessions can be employed in such a strategy, and thus, a detailed map of the genomic regions of interest can be achieved. Moreover, the development and use of large-scale genotyping should allow for the use of strategies for genome-wide selection (or simply genomic selection), enhancing the association of genetic diversity data with characteristics of agronomic interest. In addition, the use of GBS strategies should help to reduce the costs and time required for germplasm characterization and the selection of passion fruit genotypes.

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Supplementary Information

Table S1. Descriptive and quantitative presentation of papers using molecular markers in the genus *Passiflora*.

Molecular Markers	General Aim of the Article	No. of Article	References
Isoenzyme	Estimation of diversity (germplasm)	3	[118–120]
RFLP	Estimation of diversity (germplasm)	1	[121]
RAPD	Estimation of diversity (germplasm)	15	[7,8,58,62,63,80,81,122–129]
	Characterization and confirmation of hybrids	3	[47,72,113]
	Genetic mapping	1	[115]
	Others	2	[114,130]
AFLP	Estimation of diversity (germplasm)	3	[79,95,131]
	Genetic mapping	2	[84,86]
SSR	Estimation of diversity (germplasm)	4	[95–98]
	Characterization and confirmation of hybrids	1	[47]
	Genetic mapping	1	[86]
	Development, characterization and selection of markers	7	[85–91]
	Cross-amplification	2	[87,92]
	Others	1	[130]
RGA	Estimation of diversity (germplasm)	1	[82]
	Genetic mapping	1	[84]
ISSR	Estimation of diversity (germplasm)	2	[132,133]
SNP	Genetic mapping	1	[84]
Other markers	Estimation of diversity (germplasm)	1	[134]
	Genetic mapping	1	[84]

RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; SSR, simple sequence repeat; RGA, resistance gene analogs; ISSR, inter-simple sequence repeat; SNP, single-nucleotide polymorphism.

Conflicts of Interest

The authors declare no conflict of interest.

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MINI-REVIEWS

A history of passion fruit woodiness disease with emphasis on the current situation in Brazil and prospects for Brazilian passion fruit cultivation

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Abstract Plants of the *Passiflora* genus are widely cultivated for the production of fruit, cosmetics and pharmacological products and for floriculture. Here, we review the history of the identification of viral pathogens involved in passion fruit woodiness disease, which can affect different species of *Passiflora*. Additionally, we discuss prospects for research and development related to this pathosystem and applications to plant breeding. We discuss the different potyviruses identified as potential causal agents of passion fruit woodiness in Africa, Asia, Australia and

Brazil. We focus on the status of the cultivation of different *Passiflora* species in Brazil and place this problem in a global context. Both classical and molecular strategies for obtaining virus-resistant *Passiflora* plants are discussed in this work, thus providing insight into this pathosystem and useful information for breeders of *Passiflora* species.

Keywords Biotechnology · *Passiflora* · Potyvirus · Resistance to disease

Abbreviations

BCMV	Bean common mosaic virus
CAMBV	Cowpea aphid-borne mosaic virus
CTV	Citrus tristeza virus
EAPV	East Asian <i>Passiflora</i> virus
GLDI	Global leaf disease index
PDR	Pathogen-derived resistance
PTGS	Post-transcriptional gene silencing
PaVY	<i>Passiflora</i> virus Y
PWD	Passion fruit woodiness disease
PWV	<i>Passion fruit woodiness virus</i>
UPV	Ugandan <i>Passiflora</i> virus
SAPV	<i>South African Passiflora virus</i>

Introduction

Passiflora is the most commonly encountered genus of the family *Passifloraceae* and is characterized by wide morphological diversity, as it is composed of approximately 530 species and 400 artificial hybrids (Ulmer

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and Mac Dougal 2004). Of these 530 species, at least 140 are native to Brazil (Cervi 2006), and 70 of these native species produce edible fruits and are consequently of potential economic importance for fruit production (Cunha and Barbosa 2002). Brazil stands out globally as the world's largest producer of passion fruit, with a reported annual production of 923,035 t (IBGE 2011). The species *Passiflora edulis* Sims (including both yellow and purple fruits) has particularly high economic value, as it accounts for approximately 95 % of the existing *Passiflora* orchards in Brazil (Meletti 2011).

In addition to being consumed as a food, the passion fruit is used in the pharmaceutical and cosmetic industries and is cultivated as an ornamental plant. The sedative properties of the passion fruit are widely known (Oliveira et al. 2002), and hypoglycemic activity of flour produced from the skin of the fruit has recently been described (Braga et al. 2010).

Wild species of *Passiflora* are suitable for use in breeding programs that aim to produce ornamental plants with decorative attributes, such as lush foliage and a large number of attractive, colourful and exotic flowers (Abreu et al. 2009). Variable genotypes can be generated via interspecific hybridization and backcrossing. Ornamental *Passiflora* hybrids are currently marketed and used to decorate greenhouses in the U.S. and Europe (Ulmer and Mac Dougal 2004). However, in Brazil, which is the origin of approximately one quarter of the members of genus *Passiflora*, the potential of *Passiflora* species remains underexplored. The use of wild relatives as a source of new, interesting traits for commercial *Passiflora* cultivars is therefore of interest.

Within a regional context, species such as *P. setacea* (sleep passion fruit), *P. cincinnata* (wild passion fruit) and *P. alata* (sweet passion fruit) are also of interest with regard to the marketing of their fruits and other derived products (Araújo et al. 2008; Cerqueira-Silva et al. 2010; Cardoso-Silva et al. 2007; Meletti et al. 2003). In particular, *P. alata* has attracted increasing interest in both national and international markets, and according to Maciel et al. (2009), this species accounts for 3 % of the *Passiflora* cultivation area in Brazil.

Because Brazil has high fruit diversity and high production levels of *Passiflora* species, it is not surprising that the expansion of passion fruit cultivation has been accompanied by the appearance of phytosanitary issues, including various pest problems (caused by

caterpillars, fruit flies and various other insects in addition to nematodes) and diseases (caused by fungi, bacteria, viruses and phytoplasmas) (Sampaio et al. 2006; Meletti et al. 2005; Junqueira et al. 2005a). Certain weather conditions may be favourable for the occurrence and development of biotic agents, thus resulting in a reduction of the lifespan of the passion fruit plant from 6 years to 1 year (Melo et al. 2001; Ruggiero et al. 1996). Diseases affecting the cultivation of passion fruit species therefore require immediate control measures; however, much work needs to be done to reduce the losses caused by these diseases. The major diseases that affect the roots and shoots of these plants are blight (*Xanthomonas axonopodis* pv. *passiflorae*), fusarium (*Fusarium oxysporum* f. sp. *passiflorae*), anthracnose (*Colletotrichum gloeosporioides*) (Junqueira et al. 2005b) and nematode-associated galls (*Meloidogyne* spp.) (Garcia et al. 2008). Efforts to generate disease-resistant varieties have involved the application of techniques such as inter- and intra-specific hybridization (Junqueira et al. 2005b) and the use of wild species as resistant rootstocks (Faleiro et al. 2005).

For various reasons (to be discussed throughout this review), the potyvirus-induced woodiness of *Passiflora* fruits (passion fruit woodiness disease; PWD) is considered one of the most important diseases affecting Brazilian passion fruit cultivation, and it is considered a limiting factor for passion fruit production in other regions of the world. The main characteristic symptoms of PWD are reduced size and deformation of the plants because of shortening of the internodes; different levels of wrinkling and mosaic formation, with the possible occurrence of bubbles or blisters on the leaf surface; formation of mosaics on leaves; and woodiness and deformation of the fruits (Rezende 2006; Nascimento et al. 2006). The simultaneous occurrence of these symptoms considerably shortens the life of the plant, and the onslaught of opportunistic pathogens may cause death in susceptible plants.

PWD occurs in various *Passiflora* species, and its symptoms and pathogenesis have been described in several countries, including Australia, Brazil, Nigeria, South Africa, Taiwan and Uganda (Nascimento et al. 2006; Taylor and Greber 1973; Brand et al. 1993; McKern et al. 1994; Iwai et al. 2006; Ochwo-Ssemakula et al. 2012). Thus, PWD is a global disease, though it arguably causes the most serious problems in Brazil, and it can potentially affect the conservation and use of wild species of *Passiflora*.

The development of effective strategies for controlling PWD is still in the initial (or experimental) phase, although, as discussed throughout this review, the number of studies related to this disease has increased greatly in recent decades. The factors that make this virus difficult to address effectively include problems related to controlling its vectors and eliminating hosts that act as reservoirs (Sampaio et al. 2006; Maciel et al. 2009).

Passion fruit woodiness disease

Origin and history of the occurrence of viral woodiness disease

Historically, the first occurrence of the virus that causes PWD was recorded in the early twentieth century in Australian orchards (Cobb 1901). At that time, the symptoms described for PWD were widespread mosaics and ringspots on the leaves, rough and distorted leaves, deformed fruits and woodiness of the pericarp, which are more or less the same symptoms currently considered to be characteristic of the disease. The causative agent of PWD was established in Australia as a virus and designated *Passion fruit woodiness virus* (PWV) (Taylor and Greber 1973). Originally, PWV was considered to occur only in Australia.

In Brazil, the first occurrence of PWD was recorded in the late 1970s in yellow and sweet passion fruit plants grown in the municipality of Feira de Santana in Bahia (Yamashiro and Chagas 1979; Chagas et al. 1981). Within a few years, the disease had spread to other Brazilian states such as Pernambuco in 1983 (Loreto and Vital 1983), Sergipe and Ceará, in 1986 (Kitajima et al. 1986) and São Paulo (Chagas et al. 1992) and Minas Gerais in 1994 (São José et al. 1994). Currently, the disease can be found throughout the passion fruit production fields in the country (Rezende 2006).

Diagnosis of PWD and characterization of the causative agent

Until the mid-1990s, the causative agent of PWD was identified by observing symptoms in plants, performing serological tests and monitoring symptoms in test plants (host range determination). Using these methods, the disease-causing agent in Brazil was identified as PWV. For quite some time, PWV was considered the sole

agent capable of causing passion fruit woodiness syndrome in Brazil.

In the 1990s, the molecular characterization (based on gene sequencing) of viral isolates obtained from passion fruit plants with typical symptoms of PWD resulted in the re-classification of the causative agent. From South Africa, a virus distinct from PWV, and called South African Passiflora virus (SAPV) was described (Brand et al. 1993). This virus was later shown to be synonymous with Cowpea aphid-borne mosaic virus (CABMV; McKern et al. 1994), and also reported from passionfruit in Brazil (Santana et al. 1999; Nascimento et al. 2004; Barros 2007; Cerqueira-Silva et al. 2008; Moreira 2008). In Asia and Uganda, *East Asian Passiflora virus* (EAPV) (Iwai et al. 2006) and *Ugandan Passiflora virus* (UPV) (Ochwo-Ssemakula et al. 2012), respectively, have been designated as other putative etiological agents for the disease.

All of the virus species identified as possible causative agents of PWD exhibit similar biological and serological properties but are genetically distinct (Barros 2007). All of these viruses are members of the genus *Potyvirus* (family *Potyviridae*) and share the following common characteristics: elongated and flexuous viral particles ranging from approximately 680 to 900 nm in length and from 11 to 13 nm in diameter, a single positive-sense RNA genome consisting of approximately 10,000 nucleotides, a virion capsid protein with a molecular mass of approximately 34 KDa, and transmission in a non-persistent manner by aphids (Barros 2007; Gibbs and Ohshima 2010).

Molecular characterization of the causative agents of PWD

In Brazil, the classification of PWV as the sole etiological agent of PWD was questioned when the first partial genome sequences from virus isolates from infected plants became available. These viral isolates came from the main passion fruit-producing states in Brazil (São Paulo, Minas Gerais, Rio de Janeiro, Espírito Santo, Bahia, Pernambuco, Pará and the Federal District) (Braz et al. 1998; Santana et al. 1999).

When Braz et al. (1998) and Santana et al. (1999) compared the amino acid sequences of the coat proteins (CPs) of several Brazilian isolates of potyviruses associated with PWD, they observed high sequence identity of these isolates with CABMV and low sequence identity with PWV isolates.

Subsequently, other researchers expanded the initial characterizations of Braz et al. (1998) and Santana et al. (1999) by obtaining partial CP sequences of isolates from different Brazilian states, including Paraíba, Pernambuco and Sergipe (Nascimento et al. 2004), Espírito Santo, Minas Gerais, Paraíba, Pernambuco, Sergipe, São Paulo and the Federal District (Nascimento et al. 2006), and Bahia (Cerqueira-Silva et al. 2008; Moreira 2008). The high levels of identity with CABMV observed in the Brazilian isolates suggested that most PWD cases reported in Brazilian passion fruit were caused by CABMV, not PWV, thus implicating CABMV as the real cause of the disease and consequent economic losses in Brazil. This situation appears to be different from that in other global regions. For example, recent studies performed in Australia confirmed that the causative agent of PWD in Australia is indeed PWV, as originally described. In particular, the complete genome has been obtained in Australia for *Passion fruit woodiness virus* isolate Gld-1 (Fukumoto et al. 2013).

The combined results of phylogenetic studies, host tests, serological evaluations and molecular evaluations have confirmed that different viral species can cause PWD (Wylie and Jones. 2011; Nascimento et al. 2006; Santana et al. 1999; Braz et al. 1998; McKern et al. 1994). Historically, most occurrences of PWD were justifiably thought to be associated with PWV because the first report of PWD occurred in an area where PWV was endemic. However, later studies conducted in different parts of the world where symptoms of PWD were recorded attested to the existence of different viral species that cause the disease. As a result, the adoption of adequate control measures and comparison of results demands a careful evaluation of viral species identity for each case.

Growing practices for the prevention of PWD

To reduce the loss of productivity in yellow passion fruit genotypes caused by CABMV infection, certain recommendations should be adopted. Although these recommendations may not be completely effective and their effects may not be permanent, their adoption could help prolong the useful life of an orchard and reduce the damage potentially caused by PWD. These recommendations include the use of healthy seedlings obtained from certified nurseries; the complete elimination of old and/or infected plants before new planting is performed;

the adoption of careful practices for pruning and thinning operations to prevent mechanical transmission of the virus; the systematic elimination of plants with mosaic symptoms over the first 7 months after transplantation to the field; increasing the planting density; the use of drip irrigation; and finally, whenever possible, planting in isolated locations that are sufficiently far from known foci of the virus.

In general, these recommendations proposed by different authors (Oliveira 2006; Rezende 2006; Gioria et al. 2000) are aimed at avoiding, or at least reducing, the possibility of early infection of cultivated passion fruit plants, as there is a correlation between losses in production and early infection of these plants (Gioria et al. 2000). The application of these recommendations based on the annual cycle of production, followed by a case study, showed that maintenance of the crop is economically viable, at least under the evaluated conditions, and that passion fruit crops can tolerate the presence of PWD (Sampaio et al. 2008).

Classical strategies for achieving resistance to PWD

Considering the large number of wild species of passion fruit in existence, genetic breeding through the selection of resistant genotypes is a rational method for reducing losses caused by pathogens. In this context, the initial stages of pre-breeding (exploration, characterization and conservation) are very important, especially: (i) the evaluation of agronomic data through the use of uni- and multivariate statistics, and (ii) the characterization of the reactions of various genotypes to diseases based on descriptive keys and disease rates. In general, the reactions of plants to disease is evaluated using descriptive keys (rating scale) and phytopatometric disease rates (McKinney 1923; Silva 1969; Czermainski 1999).

Many studies are currently engaged in the search for passion fruit plants that are resistant to PWD, although an effective solution has yet to be reported. These studies can be summarized as falling into the following categories: (i) pre-immunization strategies; (ii) characterization of range of reactions from commercial passion fruit to PWD; (iii) evaluation of the potential use of wild species as sources of resistance to the virus; and (iv) generation and characterization of resistant hybrids with potential commercial use.

The technique of cross-protection, also referred to as pre-immunization, consists of the use of mild (or attenuated) strains of a given virus to prepare a plant for a

future attack by a severe strain. This technique has been employed to combat other diseases caused by viruses in plants. Examples include the control of the *Citrus tristeza virus* (CTV) pathosystem in Brazil (Muller 2001) and the control of PWD in Australia (Simmonds 1959). However, cross-protection has not yet yielded positive results for control of PWD caused by CABMV in Brazil (Novaes and Rezende 2003). Their studies indicated that the lack of protection seemed to result from the irregular distribution of the mild strains within the tissues of the plants that were initially inoculated, thus providing putative virus-free infection sites that could subsequently be attacked by a severe strain.

In addition to the arguments presented by Novaes and Rezende (2003), other factors, such as delays in obtaining, selecting and testing mild isolates; the possible occurrence of a reversal of a mild isolate into a strong/more severe isolate through mutation; the loss of resistance induced by a mild isolate; and potential synergistic effects between mild isolates and other viral species (Fulton 1986), appear to be limiting factors for the application of cross-protection. It can therefore be concluded that this approach is inadequate for controlling PWD, at least in Brazil.

To characterize the different reactions of passion fruit plants to CABMV in Brazil, we observed that the following were required: (i) examination of standard symptoms using a descriptive key (rating scales) (Junqueira et al. 2003; Nascimento et al. 2006; Novaes and Rezende 1999); (ii) use of these rating scales in association with serological tests (Novaes and Rezende 1999); (iii) assessment of the percentage of leaves and, or fruit infected (Leão et al. 2006; Oliveira et al. 2013); and (iv) evaluation of leaf damage in correlation with agronomic production data from the field (Cerqueira-Silva et al. 2008).

Preliminary evaluations have been made of the resistance of passion fruit plants to PWD, but the findings attest to genetic variability in passion fruit species that must be further characterized. In this context, progeny of the yellow passion fruit plant that show significant differences in PWD resistance, selected in a greenhouse by Leão et al. (2006) are of great interest. Similarly, Cerqueira-Silva et al. (2008) and Oliveira et al. (2013) described the selection of genotypes exhibiting different reactions to CABMV in the first studies on this pathosystem that sought to link leaf symptoms and disease rates with agronomic data on fruit production.

Progress in the characterization and selection of useful germplasms for improving resistance to PWD in commercial accessions of passion fruit will require the examination of disease rates to more effectively gauge symptoms, in addition to multivariate analyses considering production rates and the quality of fruits produced. Cerqueira-Silva et al. (2012) evaluated the efficiency of using rating scales and disease rates to characterize the reaction of passion fruit plants to PWD and concluded that the examination of a disease rate known as the global leaf disease index (GLDI), which considers the overall foliar symptoms presented by infected plants, was most effective.

Studies have also been conducted to identify sources of resistance to PWD in the genetically diverse wild species for incorporation into breeding programs for commercial species (Menezes et al. 1994; Meletti et al. 2005; Oliveira et al. 1994). To this end, Maciel et al. (2009) evaluated the resistance of 16 species of *Passiflora* from different regions of Brazil to CABMV isolates and identified variations in the resistance gradient for all of the isolates tested, especially in the case of *P. suberosa*.

To generate resistant and potentially useful commercial hybrids, studies have aimed to combine the resistance found in wild species (such as *P. setacea* and *P. suberosa*) with the productivity and fruit quality characteristics of commercial species (especially *P. edulis* and *P. alata*). This strategy also allows expansion of the genetic basis of resistance and of the genotypes used in breeding programs. However, to date, only one study has generated partially successful results (Fonseca et al. 2009). This work characterized populations derived from backcrossing fourth and fifth generation plants (RC4 and RC5), in which the successful transfer of resistance genes from a wild species (*P. setacea*) to a commercial species (*P. edulis*, the recurrent parent) was achieved through interspecific hybridization, which was followed by a backcrossing program assisted by the use of molecular markers. A hybrid resistant to CABMV (from crossing between genotypes of *P. edulis* and *P. setacea*) was also recently obtained by Santos (2013), and this finding should assist in the establishment of a breeding program to obtain plants resistant to PWD in the North Fluminense region of Rio de Janeiro in Brazil.

Biotechnological strategies for achieving PWD resistance

In parallel with the “classical strategies” mentioned above, the use of molecular strategies, such as the generation of transgenic plants resistant to CABMV, has been attempted. Studies conducted in the late 1990s and early 2000s at the Universidade Federal de Viçosa (Braz 1999; Alfenas et al. 2005) and Universidade de São Paulo (Trevisan et al. 2006, 2007) were the first to report the application of this research strategy. These studies were initiated based on a pathogen-derived resistance (PDR) approach that likely relied on the induction of post-transcriptional gene silencing (PTGS) (Baulcombe 2000; Mitter et al. 2001).

These experiments made use of two gene sequences: an untranslatable construct consisting of two thirds of the NIb gene and the 5' region of the CP gene derived from a viral isolate known to cause PWD (Braz 1999; Alfenas et al. 2005) and a construct consisting primarily of the CP gene (Trevisan et al. 2006, 2007), both obtained from isolates demonstrated to cause PWD.

Although Braz (1999) and Alfenas et al. (2005) provided evidence of the occurrence of PTGS, they also showed that effective resistance in the transformed plants was restricted to the viral isolate from which the fragments of the NIb and CP genes were isolated. It was therefore concluded that the high variability in the nucleotide sequence of the NIb gene was the main factor contributing to this result.

Using CP gene constructs, Trevisan et al. (2006, 2007) produced a number of transgenic lines resistant to three viral isolates capable of producing PWD symptoms in untransformed plants. However, the potential resistance of these transgenic lines to other isolates found in Brazil is not known. The authors indicated that it would be useful to determine the potential resistance of the strains to infections caused by aphids, which are the natural vectors of PWD transmission in the field, and that the results of experimental inoculations can differ substantially from those of natural vector-mediated virus inoculation. It is thought that the use of strains homozygous for the construct used in the generation of the transgenic line will result in a stronger resistance response in transformed plants that will not be easily overcome by different viral isolates.

Introduction of the p35 gene (an inducer of apoptosis) in a *Baculovirus* genome into yellow passion fruit plants causes these plants to become more tolerant to herbicides

and to *Xanthomonas passiflorae* axonopodis pv *passiflorae*. However, the p35 gene was not effective in generating resistance or tolerance to CABMV. Although there have been reports of increased resistance to viruses in tomatoes transfected with apoptosis inhibitor genes, no such results have been observed in the case of *Passiflora* (Freitas et al. 2007). This situation indicates the need for more information on the molecular mechanisms underlying the interaction of *Passiflora* with potyviruses to enable the development of transgenic resistant cultivars.

Grafting and micropropagation represent another potential strategy for producing virus-free varieties. In the case of *Passiflora*, it has been shown that micrografting is a feasible means of eliminating the clonal CABMV virus and thus cleansing sour passion fruit (Ribeiro et al. 2008).

Another strategy for combating PWD based on biotechnology tools is somatic hybridization via protoplast fusion, which is a possible alternative to gene transfer from related or non-related species. The fusion of somatic cells can potentially be applied in genetic breeding in cases of high sexual incompatibility or when reproductive barriers exist between unrelated species (Liu et al. 2005). Otoni et al. (1996) identified the wild species *P. suberosa* as a source of resistance to PWD and were also able to isolate protoplasts from the mesophyll of *P. suberosa*. Thus, it may be possible to generate resistant plants that can potentially be used as rootstock via somatic hybridization (Monteiro et al. 2000).

Analogies with other pathosystems involving potyviruses

Potyviruses affect different wild and cultivated species of passion fruit and Leguminosae in Australia. In recent studies, five different potyviruses were detected in nine different plant species in Australia. These viruses were used in cross-inoculations of 15 different plant species, including species of *Passiflora*, and it was found that isolates of PWV and *Passiflora virus Y* (PaVY) from *P. foetida* were able to infect *P. edulis* and *P. caerulea*, whereas they only occasionally infected legumes. In contrast, a PaVY isolate from *P. foetida* did not infect plants of *P. edulis* but did infect several legumes that could serve as reservoirs of the virus. These results, combined with data from the partial sequencing of these viruses, allowed the drawing of inferences regarding the potential invasive ability of potyviruses in new hosts found at the interface

of natural vegetation and crop acreage. The potential ability of indigenous viruses to infect introduced plants was also previously discussed (Coutts et al. 2011).

The causal agents of PWD identified in Brazil and South Africa (CABMV), East Asia (EAPV) Uganda (UPV) and Australia (PWV) are all viruses belonging to the bean common mosaic virus (BCMV) group, which includes at least 37 potyviruses, approximately two thirds of which can infect *Passiflora*. Recent reviews have included phylogenetic analyses and hypotheses to explain the spread of potyviruses in various plant species. In general, it appears that certain lineages of plants are especially susceptible to natural infection by a particular lineage of potyviruses (Gibbs et al. 2008; Gibbs and Ohshima 2010). In the case of *Passiflora*, it appears that different potyviruses affect endemic species in different parts of the world. However, the process of domestication and the recent distribution of these plants (and their pathogens) around the world over the past 500 years have likely also led to the dissemination of potyviruses in different plant species. This hypothesis is supported by the occurrence of CABMV in both the passion fruit and cowpea in Africa and in Brazil. Finally, Gibbs and Ohshima (2010) emphasized that the great diversity and plasticity of potyviruses and the speed with which they spread in crops imply that these pathogens can serve as excellent models for the study of plant-virus interactions.

Different species of *Passiflora* have demonstrated interactions with different potyviruses in studies conducted in various regions. It is possible that each species of potyvirus could exhibit great genetic diversity because recombination events are common in potyviruses (Chare and Holmes 2006). EAPV was initially isolated from *Passiflora edulis* fruits presenting symptoms of PWD. However, this pathogen was not able to infect approximately 15 different species of plants tested. This potyvirus multiplies and systematically spreads in different cultivars of *Phaseolus vulgaris* following artificial inoculation, causing mild symptoms. In various species of *Passiflora* (*P. maliformis*, *P. edulis*, *P. quadrangularis*, *P. incarnata* and *P. suberosa*), this virus causes mosaics and shriveled leaves 10 to 15 days after inoculation (Iwai et al. 2006). A group of 14 CABMV isolates obtained from *P. edulis* in different regions of Brazil was found to be able to infect *N. benthamiana*, *N. clevelandii*, *P. vulgaris* and *Vigna unguiculata*, in addition to *P. edulis* (Nascimento et al. 2006). In contrast, among 16 different species of *Passiflora* inoculated with four CABMV isolates, only one was not infected, thus demonstrating the susceptibility

of most of the *Passiflora* species evaluated. Therefore, biological studies using isolates of different potyvirus (CABMV and EAPV) from *Passiflora* show that these potyviruses exhibit high genetic diversity and can infect a wide range of *Passiflora*.

Current scenario and perspectives for the pathosystem

For the “passion fruit plant vs. woodiness disease” pathosystem, advances have been made regarding: (i) measures for managing and “living with” the disease; (ii) identifying the etiology of the actual causative agents of the disease in Brazil; (iii) increasing the resistance of commercial and wild passion fruit plants to different viral isolates; and (iv) increasing the response and potential use of resistant transgenic plants. However, the control of this disease still presents a major challenge for Brazilian passion fruit cultivation.

The results drawn from advances in recent genetic breeding programs for passion fruit and from biotechnology research will lead to the production of plants that show greater resistance and desirable agronomic traits for the maintenance and growth of Brazilian passion fruit crops.

Future research involving this pathosystem, at least in the context of the culture of passion fruit in Brazil, should involve efforts dedicated to increasing the number of wild species of *Passiflora* evaluated for CABMV resistance; obtaining and evaluating interspecific hybrids of *Passiflora edulis* and wild species for the development of CABMV resistant cultivars; and generating genetic maps of genes involved in CABMV resistance.

Together, these efforts will contribute to the implementation of a program of marker-assisted selection (MAS) for passion fruit, especially to attain varietal and inter- and intraspecific resistance to CABMV. Alternative approaches for achieving genetic control of this virus are being examined in studies addressing the molecular mechanisms involved in the interaction between passion fruit and infective pathogens.

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CAPÍTULO II

Desenvolvimento e caracterização de marcadores microsatélites

ARTIGO 1.

C.B.M. Cerqueira-Silva; E.S.L. Santos; A.M. Souza; G.M. Mori; E.J. Oliveira; R.X. Corrêa; A.P. Souza (2012). **Development and characterization of microsatellite markers for the wild South American *Passiflora cincinnata* (Passifloraceae).** *American Journal of Botany*. doi:10.3732/ajb.1100477

ARTIGO 2.

C.B.M. Cerqueira-Silva; E.S.L. Santos; J.G.P. Vieira; G.M. Mori; O.J. Nunes; R.X. Corrêa; A.P. Souza (2014). **New microsatellite markers for wild and commercial species of *Passiflora* (Passifloraceae) and cross-amplification.** *Applications in Plant Sciences*. doi.org/10.3732/apps.1300061

CAPÍTULO II

Desenvolvimento e caracterização de marcadores microsatélites

Neste segundo capítulo o objetivo central foi o desenvolvimento de marcadores microsatélites para três espécies de maracujazeiro, a saber: *Passiflora edulis* Sims., espécie comercial que ocupa mais de 90% das áreas agrícolas destinadas ao cultivo do maracujazeiro no Brasil, e as espécies silvestres *P. cincinnata* Mast. e *P. setacea* DC., ambas de comercialização regional e com grande interesse para programas de melhoramento genético, visto que apresentam resistência a fatores bióticos e abióticos. Em síntese, são apresentados os primeiros marcadores microsatélites desenvolvidos para as duas espécies silvestres, bem como um novo set de marcadores para a espécie comercial. As atividades desenvolvidas possibilitaram o fortalecimento das parcerias existentes entre diferentes instituições de pesquisa, destacando-se a interação entre a Universidade Estadual de Campinas - UNICAMP, Universidade Estadual do Sudoeste da Bahia - UESB (*campus* de Itapetinga), Universidade Estadual de Santa Cruz - UESC e Empresa Brasileira de Pesquisa e Agropecuária - EMBRAPA (unidade mandioca e fruticultura).

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR THE WILD SOUTH AMERICAN *PASSIFLORA* *CINCINNATA* (PASSIFLORACEAE)¹

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- **Premise of the study:** We developed the first set of microsatellite markers for the passion fruit, *Passiflora cincinnata*, to provide tools for further study of its genetic diversity and to support current conservation and genetic studies.
- **Methods and Results:** We used a microsatellite-enriched library approach to isolate and characterize 25 new molecular markers. Seven primer pairs successfully amplified polymorphic microsatellite regions, with an average of five alleles per locus. The mean values of expected and observed levels of heterozygosity were 0.516 and 0.525, respectively.
- **Conclusions:** The microsatellite markers identified in this study may be valuable tools for population genetic studies, and this set of markers also may be useful in the design of a genetic conservation strategy, mating system, and hybridization studies.

Key words: genetic diversity; genomic microsatellite-enriched library; molecular markers; *Passiflora cincinnata*; Passifloraceae; wild passion fruit.

The family Passifloraceae originated in tropical America and comprises approximately 580 species and 18 genera (Bernacci, 2003). The genus *Passiflora* L. consists of approximately 400 species, and Brazil is considered a center of diversity, presenting nearly one-third of the *Passiflora* species (approximately 129 species) (Cervi et al., 2010). *Passiflora cincinnata* Mast. is a widely distributed South American passion fruit that has been found from northeastern Brazil to eastern Paraguay and western Bolivia. This species is locally cultivated, and its fruits are sold commercially in small cities in Brazil. The aerial parts of this plant are traditionally used for ethnopharmacological and ornamental purposes. Although this species is not seriously threatened by human activities within its habitat, there are current conservation and breeding endeavors focused on this species. Molecular genetic diversity studies of *P. cincinnata* are limited to the work of Cerqueira-Silva et al. (2010) using dominant markers. The development of microsatellite markers for the genetic studies of *Passiflora* was first reported for *P. edulis* Sims

(Oliveira et al., 2005) and *P. alata* Curtis (Pádua et al., 2005). However, these loci were not successfully cross-amplified or were not found to be polymorphic in *P. cincinnata*. Thus, to address this problem and to evaluate the molecular diversity that is present in *P. cincinnata* populations, we isolated and characterized a set of microsatellite markers from this species.

METHODS AND RESULTS

A microsatellite-enriched library was developed using DNA from the *P. cincinnata* (Pc UESB-01) accession collected in the Active Collection of *Passiflora* Work Germplasm (CAGT-*Passiflora*) of the Universidade Estadual do Sudoeste da Bahia (UESB) in Vitória da Conquista, Bahia, Brazil. Genomic DNA was isolated from fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) method, and the library construction followed the methodology of Billote et al. (1999). The DNA samples (5 µg) were digested with *AfaI* and ligated into the double-stranded adapters, 5'-CTCTTGCTTACGCGTGGACTA-3' and 5'-TAGTCCACGCGTAAGCAAGAGCACA-3'. The enrichment procedure was performed using a hybridization-based capture with (GT)₈ and (CT)₈ biotin-linked probes and streptavidin-coated magnetic beads (Streptavidin MagneSphere Paramagnetic Particles, Promega, Madison, Wisconsin, USA). The selected fragments were cloned into a pGEM-T Easy (Promega) vector and used to transform *Escherichia coli* XL1-Blue competent cells (Stratagene, La Jolla, California, USA). The recombinant colonies were selected using white/blue screening. We randomly selected a total of 192 positive clones, which were then double-sequenced using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). Every sequence was aligned and edited using SeqMan software (DNASTAR, Madison, Wisconsin, USA) analysis. MICROSAT software (A. M. Risterucci, Centre de Coopération Internationale en Recherche Agronomique pour le Développement [CIRAD], personal communication) was used to eliminate the adapters and restriction sites from the sequences. The Simple Sequence Repeat Identification Tool

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TABLE 1. Characteristics of the 25 microsatellite markers that were developed for *Passiflora cincinnata*.

Marker	Primer sequences (5'–3')	Allele size (bp) ^a	Repeat motif	AC ^b	GenBank accession no.
mPc-UNICAMP01	F: AGACAGACATTTCAGTCCGGTC R: TCAAGAAGCTCCTCCAACCTGA	300–310	(AG) ₄	TD65–55	JF815377
mPc-UNICAMP02	F: CATTTCGAAGCGTGGTTCT R: AAGCGCCATGGTTTACAG	180–184	(TCC) ₄	TD65–55	JF815378
mPc-UNICAMP03	F: CTAGCCATATCCCGCATCAT R: GGGGATTACATTACCCATCG	281–284	(CT) ₅	TD65–55	JF815379
mPc-UNICAMP04	F: ATGACAACGTGGTTAGGGAA R: CACGCGTGTGTGGAATAC	270–340	(AC) ₁₆ (AT) ₄	TD65–55	JF815380
mPc-UNICAMP05	F: TAAGCTTCCTCCAACCAA R: AGGAAGGAGGAAGCAGGAAG	178–182	(AC) ₄	TD65–55	JF815381
mPc-UNICAMP06	F: AAGCAGGTAGCAGACTCAAC R: TTCTCAGGTGACAGGGGAAC	230–252	(TG) ₈ (TG) ₆ (TG) ₇	TD65–55	JF815382
mPc-UNICAMP07	F: GCGACTAATGTGCCACTGTCT R: ATTGTGGTGTGCGTAGAGC	260–330	(CAT) ₅	TD65–55	JF815383
mPc-UNICAMP08	F: CACAGCAACGAAAACGAGA R: ACGTGTGACTGTTGGCACTT	103	(AT) ₄	TD65–55	JF815384
mPc-UNICAMP09	F: GAGAAAGAGTTTCACCGGCTA R: TGCACCCATACTGCTCTTTG	203	(GAGC) ₃	TD65–55	JF815385
mPc-UNICAMP10	F: GGCGATCAACAGCACTAC R: CCTGAGAAACCGGATGAAG	230	(GAG) ₆	TD65–55	JF815386
mPc-UNICAMP11	F: TGCCACACAACGTACCTCAT R: CGAGACGCCCAGAGAAGTAG	194	(TACA) ₃	TD65–55	JF815387
mPc-UNICAMP12	F: AGTCCCGACCTCCAACCTTT R: TTCCTGAGACTGGAGAGCAAC	113	(GAT) ₆	TD65–55	JF815388
mPc-UNICAMP13	F: GCACAGTCTTACGCCAGTGTT R: ATGCTCCTCCTCTGGGGTAT	231	(CCAG) ₃	TD65–55	JF815389
mPc-UNICAMP14	F: GCGATCAACGAGCACTATCA R: GTCGCTCTGCGAAGTAATCC	129	(GAG) ₅	TD65–55	JF815390
mPc-UNICAMP15	F: GCACGTCAGTGGGTTTACA R: AGTCTGTGCTGGGCAGTT	161	(AC) ₄	TD65–55	JF815391
mPc-UNICAMP16	F: TAAGCTTCCTCCAACCAA R: AGGAAGGAGGAAGCAGGAAG	253	(AC) ₄	TD65–55	JF815392
mPc-UNICAMP17	F: GTCCACCATATCCTGGCATC R: CTGAAGGAGGCTAGACAGCTT	176	(CT) ₄	TD65–55	JF815393
mPc-UNICAMP18	F: CAAAACCATACAAGGCGTCA R: TATGGCACTTCTGTATCAGCAT	209	(AT) ₄	TD65–55	JF815394
mPc-UNICAMP19	F: ACGAGACAGAATTTACCCGG R: AAGCTCAGATCTCCATGGACA	109	(GAGC) ₃	TD65–55	JF815395
mPc-UNICAMP20	F: CGAGAAGATTCTGTAAGCAGGC R: GCATTATCTCGGTGTGATGGT	141	(TG) ₄	TD65–55	JF815396
mPc-UNICAMP21	F: CCGTATTCCTGCCTTTTAAG R: GTGGCTTCGTCTGATGTGAA	151	(TA) ₅ (TG) ₁₂	TD65–55	JF815397
mPc-UNICAMP22	F: TCTCCTCCTCCTCCTCCTTC R: TTTTGAGCTAGCCCGTGAGT	189	(TTC) ₄ (TC) ₃	TD65–55	JF815398
mPc-UNICAMP23	F: CGCCAACAACATGAAAGC R: CCCCAGAGAGTGCCCAAGA	105	(TGG) ₄	TD58–48	JF815399
mPc-UNICAMP24	F: GTTTCAGCCCAACGACTT R: ACATATTGAACAGTCCGAACAT	225	(TTAA) ₃ (TAAT) ₃	TD65–55	JF815400
mPc-UNICAMP25	F: CCTTTATATCTCGCAACCGC R: GGAGTCGGTAGGCTCAAGAC	139	(CGCT) ₃	TD58–48	JF815401

^aFragment size ranges for polymorphic loci and expected product sizes based on the clone fragment for monomorphic markers.^bOptimal PCR amplification conditions (AC). TD65–55 and TD58–48 indicate touchdown PCR programs with temperatures ranging from 65°C to 55°C and 58°C to 48°C, respectively.

(SSRIT) (Temnykh et al., 2001) was used to identify the microsatellites. Of the 62 microsatellites found, dinucleotide motifs were the most abundant (60%), followed by trinucleotide and tetranucleotide motifs (22% and 18%, respectively). The number of repetitions of dinucleotide microsatellites varied from four to 33 (average: 6.4). Primer pairs were designed using PrimerSelect (DNASTAR) and Primer3Plus (Untergasser et al., 2007). The main criteria adopted for the design of the primers were as follows: annealing temperature 45–65°C (with a maximum difference of 3°C between the forward and reverse primers), GC content 40–70%, and amplified product sizes that vary from 100 and 300 bp for accurate genotyping using vertical electrophoresis in 1× TBE buffer and 6% polyacrylamide gels.

PCR was conducted using a final volume of 15 µL containing 15 ng of template DNA, 1× PCR buffer (20 mM Tris HCl [pH 8.4] and 50 mM KCl),

1.5 mM MgCl₂, 0.2 µM of each dNTP, 0.5 µM of each primer, and 1 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, California, USA). All of the primers were evaluated using three PCR amplification protocols with an annealing temperature of 60°C, as follows: 94°C for 5 min, 34 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min, and two different touchdown PCR protocols, TD 65–55°C and TD 58–48°C. The touchdown protocols consisted of the following five steps: (1) initial denaturation (94°C for 5 min); (2) 10 cycles of denaturation (94°C for 1 min), annealing (65°C – 1°C per cycle for 1 min for TD65–55 or 58°C – 1°C per cycle for 1 min for TD58–48), and extension (72°C for 1 min); (3) step 2 was repeated; (4) 14 cycles of denaturation (94°C for 1 min), annealing (55°C or 48°C for 1 min), and extension (72°C for 1 min); and (5) a final extension (72°C for 10 min). Superior results were obtained using the touchdown programs. The amplification products

TABLE 2. Results of the initial screening of polymorphic microsatellite markers in one population of *Passiflora cincinnata* (N = 31).

Marker	A	H _o	H _e	PIC	HWE
mPc-UNICAMP01	2	0.428	0.342	0.28	0.30
mPc-UNICAMP02	3	0.620	0.472	0.411	0.15
mPc-UNICAMP03	2	0.000	0.063	0.061	0.02 [†]
mPc-UNICAMP04	9	0.576	0.838	0.803	0.001* [†]
mPc-UNICAMP05	3	0.880	0.522	0.397	0.001*
mPc-UNICAMP06	8	0.535	0.567	0.537	0.35
mPc-UNICAMP07	8	0.636	0.813	0.769	0.01

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; PIC = polymorphism information content.

*Departs significantly from HWE after Bonferroni correction ($P < 0.007$).

[†]Significant values for the occurrence of null alleles.

were visualized on ethidium bromide-stained agarose gels (3%) in 0.5× TBE before vertical electrophoresis on 6% denaturing polyacrylamide gels run in 1× TBE and staining with silver nitrate. The product sizes were determined using a 10-bp DNA ladder (Invitrogen). Statistical analyses were performed using GENEPOP (Raymond and Rousset, 1995), PIC Calculator (Kemp, 2002), and MICRO-CHECKER (van Oosterhout et al., 2004) software. We designed 33 primer pairs that matched the predefined criteria, and 25 generated consistent patterns of amplification that matched the expected band sizes based on the size of the sequenced fragment; these were used for further characterization (Table 1). The remaining eight loci were discarded due to amplification failure in the preliminary tests.

For the polymorphism analysis, 31 *P. cincinnata* accessions from the germplasm collection of the Brazilian Agricultural Research Corporation (EMBRAPA—Mandioca e Fruticultura Tropical) were analyzed. Among the 25 microsatellite markers that were developed, seven markers were polymorphic, with varying degrees of polymorphism (Table 2). The mean number of alleles was five (with a range from two to nine alleles). The mean values of expected and observed levels of heterozygosity were 0.516 (0.063–0.838) and 0.525 (0–0.88), respectively, and the polymorphism information content varied from 0.061 to 0.803. A small number of polymorphic microsatellites have been reported in the literature for *Passiflora* species, and the low number of polymorphic loci seems to be characteristic of the genus. Of the microsatellites developed for *P. cincinnata*, 21% were polymorphic, which is similar to the percentage of polymorphic microsatellites reported for *P. edulis* (20%; Oliveira et al., 2008) and for *P. alata* (26%; Pereira, 2010).

The analysis revealed significant deviations from Hardy–Weinberg equilibrium at only two microsatellite loci (mPc-UNICAMP03 and mPc-UNICAMP04) ($P < 0.007$ after Bonferroni correction). These results might be due to the probable occurrence of null alleles or the linkage disequilibrium (LD) that is observed for the mPc-UNICAMP04 locus (Table 1). With the exception of one pair of markers, mPc-UNICAMP04 and mPc-UNICAMP07, none of the other 21 marker pairs showed a significant LD. Furthermore, none of the microsatellite loci exhibited a significant probability of stuttering or large allele dropout (Table 2). Two of the loci (mPc-UNICAMP03 and mPc-UNICAMP04) showed significant values for the occurrence of null alleles using Bonferroni correction. Eighteen microsatellite markers were monomorphic for the evaluated sample (Table 2). However, these markers might be polymorphic among other *P. cincinnata* populations or other *Passiflora* species.

CONCLUSION

The microsatellite markers characterized in this study are the first to be developed for *P. cincinnata* and are potentially useful for genetic studies on other wild populations of this species.

Considering the low frequency of polymorphic loci previously reported and the great difficulty associated with obtaining informative microsatellites for *Passiflora* species, the markers developed in this study might be important tools to assess the genetic diversity of other species from this genus.

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PRIMER NOTE

NEW MICROSATELLITE MARKERS FOR WILD AND COMMERCIAL SPECIES OF *PASSIFLORA* (PASSIFLORACEAE) AND CROSS-AMPLIFICATION¹

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- **Premise of the study:** We developed the first microsatellites for *Passiflora setacea* and characterized new sets of markers for *P. edulis* and *P. cincinnata*, enabling further genetic diversity studies to support the conservation and breeding of passion fruit species.
- **Methods and Results:** We developed 69 microsatellite markers and, in conjunction with assessments of cross-amplification using primers available from the literature, present 43 new polymorphic microsatellite loci for three species of *Passiflora*. The mean number of alleles per locus was 3.1, and the mean values of the expected and observed levels of heterozygosity were 0.406 and 0.322, respectively.
- **Conclusions:** These microsatellite markers will be valuable tools for investigating the genetic diversity and population structure of wild and commercial species of passion fruit (*Passiflora* spp.) and may be useful for developing conservation and improvement strategies by contributing to the understanding of the mating system and hybridization within the genus.

Key words: genetic diversity; genomic microsatellite-enriched library; molecular markers; *Passiflora*; simple sequence repeats; wild passion fruit.

The genus *Passiflora* L. (Passifloraceae) comprises approximately 400 species, of which at least 30% are distributed within Brazilian forests (Cervi et al., 2010). Species such as *P. edulis* Sims are important because of the economic value of their fruit (Faleiro et al., 2005). Certain wild species, including *P. setacea* DC. and *P. cincinnata* Mast., are of interest because of their potential use in genetic breeding. However, the limited number of molecular genetic diversity studies of this genus (Faleiro et al., 2005; Cerqueira-Silva et al., 2012) attests to the need for and relevance of novel molecular tools for studies of its populations and mating system.

Although diversity studies of passion fruit began in the late 1990s, efforts to use microsatellites only began in 2005 (Oliveira et al., 2005; Pádua et al., 2005), and studies related to the development of microsatellites have been published for *P. cincinnata*

(Cerqueira-Silva et al., 2012) and *P. contracta* Vitta (Cazé et al., 2012) only recently. The markers available are still insufficient for performing consistent genetic studies of most *Passiflora* species because the evaluated populations exhibit low variability and percentages of polymorphic loci (between 0% and 26%) (Pereira, 2010; Ortiz et al., 2012; Cerqueira-Silva et al., 2012). Thus, considering the difficulty in obtaining informative microsatellites for *Passiflora* and to enhance the genetic investigation of both wild and commercial populations, we isolated, characterized, and evaluated the cross-amplifications of microsatellites for *P. edulis*, *P. setacea*, and *P. cincinnata*.

METHODS AND RESULTS

Two microsatellite-enriched genomic libraries were developed using genotypes from the germplasm collection of *P. edulis* (Pe-UESB01) and *P. setacea* (Ps-UESB01) from the Universidade Estadual do Sudoeste da Bahia (UESB; Itapetinga, Bahia, Brazil). Genomic DNA was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method, and libraries were constructed following Billote et al. (1999). DNA samples (5 µg) were digested with *AfaI* and ligated to the double-stranded adapters 5'-CTCTTGCTTA-CGCGTGGACTA-3' and 5'-TAGTCCACGCGTAAGCAAGAGCACA-3'. Enrichment was performed using a hybridization-based capture with (GT)₈ and (CT)₈ biotin-linked probes and streptavidin-coated magnetic beads (Streptavidin Magnosphere Paramagnetic Particles; Promega Corporation, Madison, Wisconsin, USA). The selected fragments were cloned into a pGEM-T Easy Vector (Promega Corporation) and used to transform *Escherichia coli* x1-blue competent

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TABLE 1. Characteristics of the 69 new microsatellite markers developed for passion fruit species (17 markers for *Passiflora edulis* and 52 markers for *P. setacea*) and cross-amplification assays.

Locus	Primer sequences (5'–3')	Allele size (bp)	Repeat motif	PCR amplification conditions ^a	GenBank accession no.	Cross-amplification		
						Pe	Ps	Pc
mPe-UNICAMP01	F: CCTGTCGGAAAGACTTCTGC R: GGATCGTTGTGGAGTGTGGT	230–232	(AC) ₄	TD58	KF142650		232	232
mPe-UNICAMP02	F: TCGAGTGAGATTGGCAGTG R: TTGGCTTCGAGGAGAAGAA	165–171	(GT) ₈	TD58	KF142651		161–163	163
mPe-UNICAMP03	F: ATAGGCATTTCACACAGCAC R: AAGCATCCGTGAGACAGGT	261	(AC) ₈	TD58	KF142652		261	261
mPe-UNICAMP04	F: GCTAACAAAGCCCAATCAAC R: CAGACCATGAGACGGCAGTA	296	(CA) ₅	TD65	KF142653		296	296
mPe-UNICAMP05	F: CGGGGTTATGCAAGTAACA R: ACTGGGTGGACTAGGAAACG	121	(TG) ₈	TD65	KF142654		—	—
mPe-UNICAMP06	F: GTTCGAACCTTGGTTCTCTTG R: AATCCTCTCCCGGTATCCAC	292	(TG) ₄	TD65	KF142655		—	290–320
mPe-UNICAMP07	F: GGAACCGTGTGATGGGATAC R: ACCGATTGACAGCTCTGCC	255	(AG) ₈	TD65	KF142656		255	255
mPe-UNICAMP08	F: GCTGAGAACCCCGTGACTTA R: CGAGTATGGCACATCCCTG	196	(CA) ₄	TD65	KF142657		196	196
mPe-UNICAMP09	F: TGCCTCTCGGATATTACAGC R: CGCATGTCCCATACGAC	212	(AC) ₅	TD58	KF142658		212	248–261
mPe-UNICAMP10	F: GTCACTGCAGCCTGGTATAGTT R: GAACATATTTCGGCAGATGGA	251	(CT) ₅	TD58	KF142659		251	251
mPe-UNICAMP11	F: GCAGCAATCAATGCAATCAG R: GCCATTCTCCTCTCACCCTA	180	(CA) ₉ (AT) ₅	TD58	KF142660		172	176
mPe-UNICAMP12	F: CACACAAGGCGTTTCTTACG R: TGATATGAACGATACGGTAGGC	214	(CA) ₇	TD65	KF142661		—	—
mPe-UNICAMP13	F: TTCGTGCATTGTTTCATTACC R: GCCTTCTTTGTCATGTTGGA	202	(TC) ₅	TD58	KF142662		202	166–168
mPe-UNICAMP14	F: GACTTCGTATGACGCCAGGT R: TGCAAGAATCCGAAGACTCA	263	(CA) ₈	TD65	KF142663		263	260
mPe-UNICAMP15	F: CATTCCCTCACCTCACGAA R: TGGTTGTGTGGTTTGTGCTT	253	(AC) ₅	TD58	KF142664		253	253
mPe-UNICAMP16	F: CGTGGGTGAGTGTGAATGAG R: TGATGTGAGCATGGTTGGTT	195	(AT) ₄ (TG) ₁₁	TD65	KF142665		—	—
mPe-UNICAMP17	F: GCCACGTGCAATGTCTCAGT R: CGTGCTGTGACCAAGGAG	300	(AC) ₉	TD65	KF142666		—	—
mPs-UNICAMP01	F: TAGCTTAACACAATGCAACAGA R: CAACGGAGAACGATGTCAG	153–154	(TG) ₅ (TG) ₅	TD58	KF171014	158–168		154
mPs-UNICAMP02	F: TAGCTTAACACAATGCAACAGA R: CAACGGAGAACGATGTCAG	154–156	(TG) ₅ (TG) ₅	TD58	KF171015	160–170		156
mPs-UNICAMP03	F: GTAGCGTCTCGGCAGGTC R: ACTCTAAGTCGGCCACTCTTG	176–177	(CT) ₄	TD65	KF171016	176		176
mPs-UNICAMP04	F: CAACAGGAGGTGAGGTGTGA R: GACAGTGCAACTTTAGGCGAC	156–157	(TG) ₄	TD65	KF171017	156		156
mPs-UNICAMP05	F: TCGGTCTTCGTATTCAACTCTG R: GAGGAACCTGGCATCGCAT	194–218	(CT) ₈	61.5°C	KF171018	210–220		213–216
mPs-UNICAMP06	F: GTTGGATCAAAGGGTCACA R: CAACTACTGGATCGAACTGGTA	218–224	(CGTG) ₃ (ATGA) ₃	TD65	KF171019	194–224		215
mPs-UNICAMP07	F: ACAGGGGTGAGGCACATTC R: TCTGTTATTATCATCGGCAGGA	143–145	(CA) ₄	TD58	KF171020	—		—
mPs-UNICAMP08	F: AGTGCCAGTGGCTTCGTATT R: GATCGTCATGGCTGTTGCTA	207–211	(TGCAA) ₃	TD65	KF171021	174		176
mPs-UNICAMP09	F: GGGCCGTTGTCAAAGTAGT R: GAGGTTAAGGCAAGCACTG	250–268	(AC) ₄	61.5°C	KF171022	258–260		260
mPs-UNICAMP10	F: ACTCTACCTCAATCGACC R: AATTGTTACTCCGTTTCTCTGA	256–260	(AG) ₄ (GT) ₅ (GT) ₄	60°C	KF171023	264–268		260–268
mPs-UNICAMP11	F: CAGACGTTGTGTTTGGTAAT R: TCAGGTTAGGAAGCTGCATC	232–270	(CA) ₄ (CA) ₄ (AT) ₄	60°C	KF171024	262		—
mPs-UNICAMP12	F: ACAGGGGTGAGGCACATACA R: GTAGTGCCTGGCTTGGGTAG	201–204	(CA) ₄	TD65	KF171025	208		208
mPs-UNICAMP13	F: CCTATACCTGCCAGTCAGC R: ACTTAAGCACCCCAATCGTT	146–148	(CA) ₄	TD65	KF171026	144		—
mPs-UNICAMP14	F: CGTTTCATAAGTGAATCAGTCAA R: GGATCGACAAACAAGGTAGA	112–116	(CA) ₄	TD65	KF171027	114		114
mPs-UNICAMP15	F: TATGGAGTTGCGAGGCTTTAG R: CGGGCAACGAACACTTTATT	145–148	(GT) ₄	60°C	KF171028	143–145		146
mPs-UNICAMP16	F: GAGAAAGCGAGTCAGCGAGA R: GACTCCAATATCGGCACTTCA	157–165	(GAG) ₆ (CAA) ₄	TD65	KF171029	163–167		159–170

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TABLE 1. Continued.

Locus	Primer sequences (5'-3')	Allele size (bp)	Repeat motif	PCR amplification conditions ^a	GenBank accession no.	Cross-amplification		
						Pe	Ps	Pc
mPs-UNICAMP17	F: CATCCAACCTCCGAACCTTA R: TACCCAGTCCGGTCCATTAG	142–148	(AC) ₃	60°C	KF171030	147		146
mPs-UNICAMP18	F: GGGGTTCTTCACTCATCCAC R: TGACGACTAGGGGATTCCAGG	262–278	(CA) ₁₀ (AT) ₆	TD65	KF171031	—		—
mPs-UNICAMP19	F: CTGTGGCAAGTGGCTAACAA R: CCACCTACTCGACCACTC	290–294	(TG) ₄	60°C	KF171032	290		290
mPs-UNICAMP20	F: GCTGGCTCTAGCTCAACTCG R: GCCAGCATAGGATGTCAGGT	200	(CT) ₅	TD65	KF171033	200		200
mPs-UNICAMP21	F: CCCAATCGCTGAGAGGAGT R: CGGTAGGCTCATTCGTGTCA	228	(TG) ₄	TD58	KF171034	—		—
mPs-UNICAMP22	F: AGGCATGCCCCATCAATG R: CACTAAACCTGCAAGCGAA	131	(GT) ₃ (GT) ₄	TD58	KF171035	—		—
mPs-UNICAMP23	F: GAGCAGCTAAAAGAACCTAC R: TAGAGGTTGTGCTGGAGTC	298	(AC) ₃ (CA) ₄	TD58	KF171036	298		298
mPs-UNICAMP24	F: GAGGTCCACCACTGTCAGT R: CTAGCGTCACCTCCAGAAG	254	(AG) ₄	TD58	KF171037	254		258–260
mPs-UNICAMP25	F: GTGTTTGTGGCGATGTGATTA R: GACAAACGTTGTTCCGCTC	162	(AAG) ₅	TD58	KF171038	162		162
mPs-UNICAMP26	F: TGTGGCATGTGTATGACTTGAT R: CATAGATATGGGATGAGCGACA	166	(TG) ₄	TD58	KF171039	166		174
mPs-UNICAMP27	F: AGATGGAACAGGTGGGTGAG R: TAGGCTTGTCTGGCTCTGG	151	(CCA) ₅	TD58	KF171040	151		151
mPs-UNICAMP28	F: AATTGTCATCGGTAACCTGC R: TGCCATTGCGAGTGAATAAG	274	(AC) ₆	TD58	KF171041	274		274
mPs-UNICAMP29	F: GAGAAATCTCAGCACACGCA R: CGGTTCTTGGTTTGTGGAT	204	(CA) ₅	TD58	KF171042	—		—
mPs-UNICAMP30	F: CGGCTGAAGGAGGAGGTAG R: TGAAAAACAAGTCAGCCAACA	118	(GT) ₆	TD58	KF171043	—		—
mPs-UNICAMP31	F: GGTGTGGTAGCCTGTTTGTC R: CCGCATCTCTTACATCGTTA	211	(TG) ₄ (GT) ₅	TD65	KF171044	215		215–219
mPs-UNICAMP32	F: CAGACGTTGCATCTTGGTAAT R: CATCGGAGGAGTTTACACATT	172	(CA) ₄ (AC) ₉ (AT) ₆	TD65	KF171045	172		172
mPs-UNICAMP33	F: GCAGCAATCAATGCAATCAG R: GCCATTCTCCTCACCCTGA	184	(AT) ₄ (CA) ₁₀ (AT) ₆	TD65	KF171046	184		184
mPs-UNICAMP34	F: GGCAGGATATGCTTTGGTT R: GCTGTGCGACACATGGAC	162	(TC) ₁₀	TD65	KF171047	160		158–161
mPs-UNICAMP35	F: TCGAGAGTTGCGTGTGTTTC R: CATTCTCCTGCCACCTGAGT	183	(TG) ₄	TD65	KF171048	183		183
mPs-UNICAMP36	F: GGGAGTCGGGTTGAGTTA R: AGTCGAGGACCAAGTCAAAG	228	(TG) ₄ (TG) ₇	TD65	KF171049	228		228
mPs-UNICAMP37	F: TTGTTTGGGTTAGCGTGTGAG R: CCCTGCCACCTGAGTAATCA	172	(TG) ₆	TD65	KF171050	172		172
mPs-UNICAMP38	F: CCTGACCTCTGGCACTACC R: GAGGCGTATCAGGCTTTGA	112	(TGC) ₆	TD65	KF171051	112		112
mPs-UNICAMP39	F: GGAGGGTTGTTGTGTGAGTG R: CTCCTGTGCGAAAGACTTCTG	230	(GT) ₄	TD65	KF171052	230		—
mPs-UNICAMP40	F: GAATCAATGGAACACAAGCA R: CCAGCCCACTAGACCACCT	224	(AC) ₅	TD65	KF171053	234		230
mPs-UNICAMP41	F: CTTCACTGACAGCTTCCAT R: ATACCGATACTCGCCTTGATAG	168	(GT) ₄	60°C	KF171054	168		170
mPs-UNICAMP42	F: AGTGCCAGTGGCTTCGTATT R: GATCGTCATGGCTGTTGCTA	174	(TGCAA) ₃	61.5°C	KF171055	174		174
mPs-UNICAMP43	F: CTCAGTGAGGAATAAGCAATCA R: ATTTGGCATGCTGTTACGC	192	(CA) ₄	61.5°C	KF171056	198		198
mPs-UNICAMP44	F: AGTCGTGCTTGTGTTGTGAG R: CCACTGTTGAGGTCCAGATG	275	(GATT) ₃	TD65	KF171057	280		275
mPs-UNICAMP45	F: CCTATACCTGCCCCAGTCAG R: GTATGTGTGTGCCGTGGATT	110	(AT) ₄ (CA) ₄	TD65	KF171058	110		110
mPs-UNICAMP46	F: TGGCTGTTGTCCACCAT R: GACTGAGCGGACTCACATCA	138	(CT) ₈	TD65	KF171059	138		138–139
mPs-UNICAMP47	F: AAATTCGGCATGGTTTATG R: CCGAGATCGTTGGAGCTTA	298	(AC) ₃ (CA) ₄	60°C	KF171060	294		298
mPs-UNICAMP48	F: AGCTTACCGGCTCACTCTTG R: GACAGGCTTGGAAGTGAAT	144	(AC) ₆	60°C	KF171061	143		142
mPs-UNICAMP49	F: TGTATGAGTGAGAAAGAGCCCA R: CAATCAACATGAGACAAGCGG	118	(TA) ₄	TD65	KF171062	126		126
mPs-UNICAMP50	F: TTCTGCGAAACTGGTGAGTG R: CGCCCGTATTTTGTGATGA	202	(TA) ₆	60°C	KF171063	202		202

TABLE 1. Continued.

Locus	Primer sequences (5'–3')	Allele size (bp)	Repeat motif	PCR amplification conditions ^a	GenBank accession no.	Cross-amplification		
						Pe	Ps	Pc
mPs-UNICAMP51	F: CTTGCACACTCAGGCTAAA R: CAACCTACTGGATCGAAGTAA	152	(GT) ₅	60°C	KF171064	152		150–152
mPs-UNICAMP52	F: GTCGGTTGAGAACCCGTA R: ACCAATCGTTGAGAGTTCGTG	118	(AT) ₅	60°C	KF171065	118		—

Note: — = unsuccessful amplification; Pe = *Passiflora cincinnata*; Ps = *Passiflora edulis*; Pc = *Passiflora setacea*.

^aTD65 and TD58 indicate touchdown PCR programs with temperatures ranging from 65°C to 55°C and 58°C to 48°C, respectively.

cells (Stratagene, La Jolla, California, USA). The recombinant colonies were selected using blue/white screening. In total, 480 positive clones (192 for *P. edulis* and 288 for *P. setacea*) were randomly selected and double-sequenced using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). Every sequence was aligned and edited using SeqMan software (DNASTAR, Madison, Wisconsin, USA). We used the MICROSAT software developed by A. M. Risterucci at the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD, France; unpublished) to identify and eliminate the adapters and restriction sites from the edited sequences.

Sequences containing microsatellites (134 for *P. edulis* and 114 for *P. setacea*) were identified using the SSR Identification Tool (SSRIT; Temnykh et al., 2001). Approximately 85% of the microsatellite motifs observed for both of the species were dinucleotides. We designed a total of 30 (*P. edulis*) and 75 (*P. setacea*) primer pairs using PrimerSelect (DNASTAR) and Primer3Plus

(Untergasser et al., 2007). The 105 primer pairs exhibited the following characteristics: annealing temperatures ranging from 45°C to 65°C (with a maximum difference of 3°C between the forward and reverse primers), CG concentrations ranging from 40% to 70%, and amplified product sizes varying from 100 to 300 bp. We used 16 genotypes of passion fruit (eight for each species) for the amplification tests. PCRs were conducted using a final volume of 15 µL (containing 15 ng of template DNA) with the reagents and concentrations described by Cerqueira-Silva et al. (2012). Every marker was evaluated by PCR amplification as follows: 94°C for 5 min; 34 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The loci that showed unsatisfactory amplification with an annealing temperature of 60°C were subjected to two different touchdown PCR protocols (TD 65–55°C and TD 58–48°C) as follows: an initial denaturation (94°C for 5 min); 10 cycles of 94°C for 1 min and an annealing temperature decreasing by 1°C from 65–55°C or 58–48°C every cycle for 1 min; 14 cycles of 94°C for 1 min, 55°C or 48°C

TABLE 2. Results of the initial screening of polymorphic microsatellite markers in populations of *Passiflora edulis*, *P. setacea*, and *P. cincinnata*.

Locus	<i>P. edulis</i> (N = 42)				<i>P. setacea</i> (N = 42)				<i>P. cincinnata</i> (N = 31)			
	A	H _o	H _e	PIC	A	H _o	H _e	PIC	A	H _o	H _e	PIC
mPe-UNICAMP01	2	0.051	0.047	0.476	1	—	—	—	1	—	—	—
mPe-UNICAMP02	4	0.651	0.515	0.404	2	0.261	0.497	0.371 ^a	1	—	—	—
mPe-UNICAMP06	1	—	—	—	0	—	—	—	2	0.083	0.079	0.077
mPe-UNICAMP09	1	—	—	—	1	—	—	—	6	0.458	0.679	0.628 ^a
mPe-UNICAMP13	1	—	—	—	1	—	—	—	3	0.055	0.205	0.191 ^{ab}
mPs-UNICAMP01	5	0.578	0.723	0.642 ^a	2	0.333	0.512	0.393 ^a	1	—	—	—
mPs-UNICAMP02	5	0.631	0.768	0.704	2	0.333	0.511	0.389 ^a	1	—	—	—
mPs-UNICAMP03	1	—	—	—	2	0.311	0.266	0.225	1	—	—	—
mPs-UNICAMP04	1	—	—	—	2	0.142	0.133	0.123	1	—	—	—
mPs-UNICAMP05	4	0.381	0.471	0.476 ^b	4	0.261	0.593	0.468 ^{a,b}	3	0.401	0.513	0.392
mPs-UNICAMP06	4	0.191	0.176	0.157	2	0.424	0.401	0.322	1	—	—	—
mPs-UNICAMP07	0	—	—	—	2	0.251	0.221	0.194	0	—	—	—
mPs-UNICAMP08	1	—	—	—	2	0.102	0.097	0.093	1	—	—	—
mPs-UNICAMP09	2	0.024	0.024	0.023	4	0.761	0.614	0.551	1	—	—	—
mPs-UNICAMP10	3	0.119	0.197	0.186 ^a	3	0.357	0.583	0.493 ^{ab}	4	0.448	0.637	0.577 ^a
mPs-UNICAMP11	1	—	—	—	3	0.166	0.157	0.149	0	—	—	—
mPs-UNICAMP12	1	—	—	—	2	0.208	0.187	0.371	1	—	—	—
mPs-UNICAMP13	1	—	—	—	2	0.282	0.456	0.351 ^a	0	—	—	—
mPs-UNICAMP14	1	—	—	—	4	0.589	0.674	0.678 ^b	1	—	—	—
mPs-UNICAMP15	2	0.024	0.024	0.023	3	0.101	0.531	0.411 ^{ab}	1	—	—	—
mPs-UNICAMP16	3	0.476	0.585	0.499	3	0.391	0.485	0.395	4	0.561	0.541	0.464
mPs-UNICAMP17	1	—	—	—	4	0.833	0.714	0.656 ^b	1	—	—	—
mPs-UNICAMP18	0	—	—	—	4	0.524	0.454	0.412	0	—	—	—
mPs-UNICAMP19	1	—	—	—	2	0.189	0.173	0.566	1	—	—	—
mPs-UNICAMP24	1	—	—	—	1	—	—	—	2	0.125	0.187	0.169
mPs-UNICAMP31	1	—	—	—	1	—	—	—	3	0.217	0.326	0.282
mPs-UNICAMP34	0	—	—	—	1	—	—	—	5	0.401	0.671	0.592 ^{ab}
mPs-UNICAMP46	1	—	—	—	1	—	—	—	2	0.041	0.041	0.041
mPs-UNICAMP51	1	—	—	—	1	—	—	—	2	0.033	0.033	0.038
mPe-UNICAMP11 ^c	4	0.237	0.447	0.424 ^b	0	—	—	—	1	—	—	—
mPe-UNICAMP19 ^c	0	—	—	—	4	0.418	0.411	0.367	1	—	—	—

Note: — = information not available; A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; PIC = polymorphism information content.

^aMarkers with the probability of null allele occurrence after a Bonferroni correction.

^bMarkers deviating from Hardy-Weinberg equilibrium after a Bonferroni correction ($P < 0.004$ [*P. edulis* and *P. cincinnata*]; $P < 0.002$ [*P. setacea*]).

^cMicrosatellite markers published by Cerqueira-Silva et al. (2012).

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Cerqueira-Silva et al.—*Passiflora* microsatellites

for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. For markers that showed inconsistent amplification after the touchdown protocols, we tested reactions with an annealing temperature gradient ranging from 65°C to 50°C. The products were visualized using vertical electrophoresis on 6% denaturing polyacrylamide gels run in 1× TBE and stained with silver nitrate. The product sizes were determined using a 10-bp DNA ladder (Invitrogen, Carlsbad, California, USA). In total, 17 and 52 markers generated consistent patterns of amplification that matched the expected sizes based on the sequenced fragments from *P. edulis* and *P. setacea*, respectively (Table 1). Cross-amplification assays were performed according to previously described protocols, with all 69 primer pairs showing a high percentage of amplification (88% [*P. edulis*], 70% [*P. setacea*], and 80% [*P. cincinnata*]) (Table 1). Cross-amplification assays were also performed with the 25 loci previously characterized for *P. cincinnata* (Cerqueira-Silva et al., 2012), presenting a percentage of amplification of 48% in *P. edulis* (mPc-UNICAMP02, -04, -06, -10, -11, -14, -15, -17, -18, -20, -21, and -24) and 28% in *P. setacea* (mPc-UNICAMP02, -04, -06, -10, -15, -19, and -20).

To characterize all the loci, we used genotypes from the germplasm collection of the Embrapa Mandioca Fruticultura Center (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA]), Cruz das Almas, Bahia, Brazil, and of the UESB, Itapetinga, Bahia, totaling 114 genotypes. For each species, 42, 42, and 30 genotypes from *P. edulis* (all from EMBRAPA), *P. setacea* (30 from EMBRAPA and 12 from UESB), and *P. cincinnata* (all from EMBRAPA), respectively, were used (Appendix S1). We performed a descriptive statistical analysis for all the polymorphic loci using GENEPOP software (Raymond and Rousset, 1995; Table 2). The polymorphism information content was calculated using PIC Calculator software (Kemp, 2002), and the probability of null alleles was estimated using MICRO-CHECKER software (van Oosterhout et al., 2004), with significant probabilities between two and six loci observed for the three species evaluated (Table 2).

The percentage of polymorphic microsatellites observed was 15% in *P. edulis*, 29% in *P. setacea*, and 20% in *P. cincinnata*, totaling 11, 21, and 11 polymorphic loci, respectively (Table 2). This low number of polymorphic loci was expected because low variability appears to be a characteristic of the genus *Passiflora*, as suggested by Cerqueira-Silva et al. (2012). The number of alleles per locus ranged from two to six, with a mean of 3.1 for the three species evaluated; overall, the observed heterozygosity was lower than expected heterozygosity. Of the 31 polymorphic microsatellites, only one (*P. edulis*), six (*P. setacea*), and two (*P. cincinnata*) showed significant deviation from Hardy-Weinberg equilibrium (HWE) after a Bonferroni correction. Deviations from HWE can be explained by linkage disequilibrium (LD) or the occurrence of null alleles. Among the 320 possible pairs of microsatellites, we observed significant LD for two pairs (in *P. edulis*; $P < 0.004$), 49 pairs (in *P. setacea*; $P < 0.002$), and one pair (in *P. cincinnata*; $P < 0.004$) after a Bonferroni correction. However, with no additional information, LD should not be attributed solely to physical linkages among loci because of the possibility of population processes such as nonrandom mating (Hedrick, 2005).

CONCLUSIONS

We present the first set of microsatellites developed for *P. setacea* and characterize new markers for *P. edulis* and *P. cincinnata*, thereby increasing the number of available markers for these species. This effort potentiates the use of microsatellites in genetic studies of wild and commercial populations of *Passiflora* species, enabling the development of more efficient conservation and genetic breeding strategies.

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APPENDIX S1. Identification of the genotypes used in this study.^a

Species	Genotype identification code	Collection
<i>Passiflora edulis</i> Sims	BGP007-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP009-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP017-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP020-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP022-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP023-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP025-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP028-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP029-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP031-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP033-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP034-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP037-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP038-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP041-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP043-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP044-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP048-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP049-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP051-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP061-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP064-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP071-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP078-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP079-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP089-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP092-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP094-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP104-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP116-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP121-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP123-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP129-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP158-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP164-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP168-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP169-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP176-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP177-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP179-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP180-P1	EMBRAPA
<i>Passiflora edulis</i> Sims	BGP181-P1	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP237-P1	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP237-P2	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP237-P3	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP237-P4	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP237-P5	EMBRAPA

<i>Passiflora setacea</i> DC.	BGP237-P6	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP238-P1	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP238-P2	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP238-P3	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP238-P4	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP238-P5	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P1	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P2	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P3	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P4	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P5	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P6	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P7	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP242-P8	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP245-P1	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP245-P2	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP245-P3	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP245-P4	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P1	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P2	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P3	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P4	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P5	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P6	EMBRAPA
<i>Passiflora setacea</i> DC.	BGP272-P7	EMBRAPA
<i>Passiflora setacea</i> DC.	Ps-UESB01	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB02	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB03	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB04	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB05	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB06	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB07	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB08	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB09	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB10	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB11	UESB
<i>Passiflora setacea</i> DC.	Ps-UESB12	UESB
<i>Passiflora cincinnata</i> Mast.	BGP002-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP002-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP200-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP200-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP215-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP215-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP215-P3	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP232-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP268-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP268-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP268-P3	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP274-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP275-P1	EMBRAPA

<i>Passiflora cincinnata</i> Mast.	BGP275-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP275-P3	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP280-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP280-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP290-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP290-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP290-P3	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP292-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP292-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP299-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP299-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP312-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP312-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP312-P3	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP322-P1	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP322-P2	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP322-P3	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP322-P4	EMBRAPA
<i>Passiflora cincinnata</i> Mast.	BGP322-P5	EMBRAPA

Note: EMBRAPA = Empresa Brasileira de Pesquisa Agropecuária; UESB = Universidade Estadual do Sudoeste da Bahia.

^a All material is available upon request. Contact information for the researchers responsible for the genotypes: onildo.nunes@embrapa.br (EMBRAPA) and csilva@uesb.edu.br (UESB).

CAPÍTULO III

Caracterização de diversidade presente em acessos de banco de germoplasma

ARTIGO 1.

C.B.M. Cerqueira-Silva; E.S.L. Santos; O.N. Jesus; J.G.P. Vieira; G.M. Mori; R.X. Corrêa; A.P. Souza (2014). **Molecular Genetic Variability of Commercial and Wild Accessions of Passion Fruit (*Passiflora* spp.) Targeting *Ex Situ* Conservation and Genetic Breeding.** *International Journal Molecular Science*. Artigo Submitido.

ARTIGO 2.

C.B.M. Cerqueira-Silva; O.N. Jesus; E.J. Oliveira; R.X. Corrêa; A.P. Souza (2014). **Characterization and selection of passion fruit (yellow and purple) accessions based on molecular markers and disease reactions for use in breeding programs.** *Euphytica*. doi: 10.1007/s10681-014-1235-9

CAPÍTULO III

Caracterização de diversidade presente em acessos de banco de germoplasma

Neste capítulo os principais objetivos foram (i) avaliar a estratégia de amplificação cruzada de 109 locos microssatélites entre 14 espécies de maracujazeiros, como forma de otimizar e potencializar o uso destes marcadores em estudos genéticos do gênero *Passiflora*; caracterizar (ii) a diversidade genética molecular em acessos das espécies *P. cincinnata*, *P. edulis* e *P. setacea*, e; (iii) a reação à doenças (virose do endurecimento dos frutos, verrugose e antracnose) em acessos de maracujazeiro ‘amarelo’ e ‘roxo’ (*P. edulis*) mantidos no banco ativo de germoplasma da Embrapa Mandioca e Fruticultura. A estratégia de amplificação cruzada mostrou-se eficiente, sendo observado um percentual de amplificação médio superior a 70% entre os locos microssatélites testados nas 14 espécies. Para as três espécies que tiveram sua diversidade caracteizada a partir dos acessos mantidos no banco de germoplasma da Embrapa, foram observados a formação de dois a cinco *pool* gênicos, sendo menor diversidade e maior estruturação genética verificada para as espécies silvestres, especialmente para *P. setacea*. Para as caracterizações de reação a doenças, foi observado gradiente de resistência/suscetibilidade para todas as três doenças avaliadas. Destaca-se também que em todo o capítulo são apresentadas e discutidas possíveis combinações de acessos a serem priorizados em cruzamentos divergentes e convergentes, passíveis de serem realizadas em diferentes etapas nos programas melhoramento genético do maracujazeiro.

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Article

Molecular Genetic Variability of Commercial and Wild Accessions of Passion Fruit (*Passiflora* spp.) Targeting *Ex Situ* Conservation and Genetic Breeding

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Abstract: *Passiflora* species are widely distributed throughout Latin America, with Brazil and Colombia being centers of diversity for this genus. We performed cross-species amplification to evaluate 109 microsatellite loci in 14 *Passiflora* species, and we estimated the diversity and structure present among species. A total of 127 accessions were examined (85 accessions of *P. edulis* and 42 accessions of 13 species). Cross-amplification strategy was effective for obtaining microsatellite loci (average cross-amplification of 70%). The average number of alleles per locus (5) was relatively low, and the average diversity ranged from 0.52 in *P. cincinnata* to 0.32 in *P. setacea*. Estimates of structure via Bayesian analyses indicated that the accessions were distributed into two groups for *P. cincinnata* and *P. setacea* and five groups for *P. edulis*. Private alleles were identified and suggestions

for core collections are presented. The data indicate the need for further collection and the information generated may be useful for breeding programs and conservation.

Keywords: core collection; germplasm; genetic diversity; molecular markers; microsatellites.

1. Introduction

The terms “passion fruit” and “passion flower” are generally associated with species of the Passifloraceae family, especially those belonging to the genus *Passiflora*. Although the taxonomic classification of some species is unclear, and therefore, the total number of species is unknown, the genus *Passiflora* is nevertheless recognized as the richest genus in terms of the number of species within the family, with approximately 520 species [1]. The vast majority of *Passiflora* species are distributed throughout the Americas, which contain approximately 96% of the known species, although species are also found in India, China, Southeast Asia, Australia, and Oceania and the surrounding regions [2]. Brazil and Colombia are prominent centers of diversity for this genus; approximately 30% of *Passiflora* species (~150 species in Brazil and ~170 species in Colombia) are found in these regions, and 89 species are endemic to Brazil [3,4,5,6]. In general, passion fruit species are considered allogamous plants and exhibit self-incompatibility, which prevents self-fertilization and the crossing of different plants that contain the same incompatibility alleles [7].

Taxonomic and phylogenetic studies, aided by molecular biology techniques, have shown that the organization of the genus *Passiflora* tends toward the formation of four subgenera (*Astrophaea*, *Decaloba*, *Deidamiodes* and *Passiflora*) [1,8,9,10], contradicting the previous hypotheses of 22 [11] and 23 [12] subgenera. A recent study based on analyses of genomic sequences and plastidial and mitochondrial regions indicates that, historically, *Passiflora* ancestors arrived in Central America and diversified quickly from there, with numerous dispersion events [9]. These authors also argue that the divergence among the *Passiflora* subgenera occurred approximately ~32 to ~38 Mya, which indicates that this diversification is quite ancient. Cytogenetic data on *Passiflora* spp. are still modest, and studies are restricted to less than 20% of species in the genus *Passiflora* [13]. Most *Passiflora* species are diploid, with $2n = 12$, 18 or 20 [14,15]. Four basic chromosome numbers are considered for passion fruit species, and these are of according to the division of the genus into four subgenera: $x = 6$ for *Decaloba*; $x = 9$ for *Passiflora*, with some instances of $x = 10$ or 11; $x = 10$ for *Astrophaea*; and $x = 12$ for *Deidamiodes* [1,14].

Passion fruit species are used as a natural resource for the pharmaceutical [16,17,18], cosmetic [19,20] and juice industries [21,22]; they are also used as ornamental plants [23,24] and are consumed as a fresh food item [21,22]. Brazil is prominent on the international stage as the largest producer and consumer of passion fruit [21,25]. Brazilian passion fruit production was estimated at 676,000 tons annually for the last 10 years, with approximately 776,000 tons in 2012; thus, Brazil produces approximately 70% of the global supply of passion fruit [21,25,26]. Brazil's position as the largest producer of passion fruit is primarily explained by an increase in the agricultural area dedicated to growing passion fruit, as crop productivity is considered low [27,28]. Currently, the average Brazilian

productivity is estimated to be 14 t ha⁻¹ [26], with the potential for producing 50 t ha⁻¹ year⁻¹ [27,28]. Therefore, efforts to increase crop production, such as the popularization and implementation of appropriate cultivation techniques and management, coupled with the genetic breeding of passion fruit, are clearly needed.

The passion fruit genetic breeding program conducted by the Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Bahia, Brazil, has developed more productive hybrids with good physicochemical fruit characteristic; however, no reported varieties exhibit resistance to major pests and diseases. Wild species are potential sources of biotic and abiotic stress resistance [29,30,31] and extensive phenotypic variation in morpho-agronomic traits [32,33,34,35,36]. Molecular markers are important for germplasm characterization because they contribute to different steps in pre-breeding and breeding programs and reduce the time required for the release of cultivars. For most *Passiflora* species, no studies concerning heritable variation have been conducted, and molecular genetics efforts have been restricted to approximately 15% of the species within the genus, with the majority of studies using only a few accessions per species, along with dominant markers [37].

Since 1998, when the first effort to estimate the variability in passion fruit using molecular markers random amplified polymorphic DNA (RAPD) was published [5], different markers have been used. In recent years, the use of DNA-based techniques, such as those based on *random amplified polymorphic DNA* (RAPD) [38,39,40,41,42], amplified fragment length polymorphism (AFLP) [43], inter-simple sequence repeat (ISSR) [44], resistance gene analog (RGA) [45], simple sequence repeat (SSR) [43,46,47,48,49] and single nucleotide polymorphism (SNP) [50] markers, have been used to examine genetic variation. However, considering the broad time range, most studies were conducted using dominant markers, and SSR and SNP markers have been used in a limited number of studies or were restricted to the development and characterization of new loci.

Considering that genetic characterizations provide useful information for understanding, conserving and harnessing genetic diversity, thereby allowing for greater interaction between pre-breeding and breeding programs [51,52], the aim of the present study was to measure and describe the genetic variation within the *Passiflora* genus to promote interactions between *Passiflora ex situ* conservation and pre-breeding efforts [51, 52]. We evaluated the cross-species amplification of 109 microsatellite loci in 14 wild and commercial passion fruit species, and we estimated the intraspecific genetic variability of 116 accessions associated with 364 plants belonging to three species of *Passiflora* that are recognized to be of interest to genetic breeding programs. We then investigated the use of microsatellite markers and genetic diversity in breeding programs and *ex situ* conservation actions. Finally, we identified preferred crosses among accessions and the minimum acceptable sample size for the core collection of each species.

2. Results

2.1. Tests for cross-amplification

Cross-amplification was shown to be an efficient strategy for obtaining microsatellite loci in passion fruit species, with an average cross-amplification success rate of approximately 70% for the 14 *Passiflora* species examined in this study (Table 1). The highest percentages of cross-amplification (>

80%, corresponding to more than 90 SSR loci) were found for *P. alata*, *P. galbana*, *P. malacophylla* and *P. watsoniana*. In contrast, the lowest percentages of cross-amplification (< 60%, corresponding to fewer than 65 SSR loci) were observed for *P. laurifolia*, *P. morifolia*, *P. rubra*, *P. setacea* and *P. suberosa*. At least 12 new species of passion fruit now have a substantial number of SSR markers, with an average of 75 new loci characterized for each species (Figure 1).

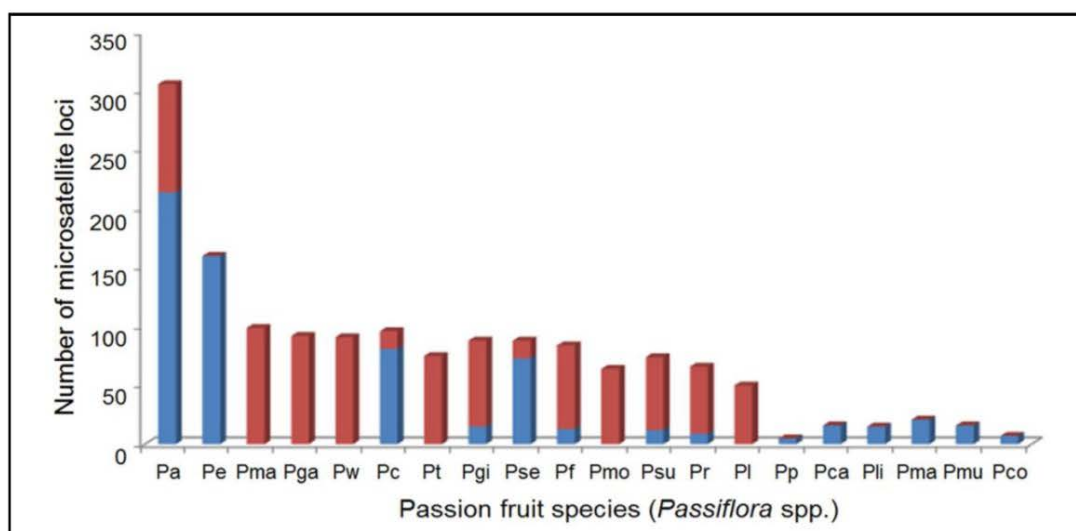
We observed a slightly higher average percentage of successful amplifications in loci derived from *P. edulis* and *P. setacea* ($\geq 70\%$) compared with the loci derived from *P. cincinnata* (< 60%) in the different species studied (Table 1). Moreover, the cross-species amplification of the 109 SSR loci revealed wide variation, occurring specific loco amplification in the species for which a given microsatellite marker was developed (three SSR loci, i.e., mPe-Unicamp11 and 17 and mPs-Unicamp18). At other loci, 100% cross-amplification was identified between the 14 passion fruit species (23 SSR loci, i.e., Unicamp06 and mPe-15; mPe-Unicamp09 and 13; mPs-Unicamp03, 05, 08, 09, 12, 15, 24, 31, 36, 41, 42, 43, 45, 46, 47, 48 and 49; and PE-07 and 18) (Supplementary Material – SM 1). The average estimated distance between the 14 species, based on the complement of the simple coincidence coefficient, was 0.24 and ranged from 0.07 (between *P. alata* and *P. morifolia* and between *P. alata* and *P. watsoniana*) to 0.42 (between *P. laurifolia* and *P. setacea* and between *P. laurifolia* and *P. edulis*) (Supplementary Material – SM 2).

Table 1. Cross-amplification observed using a set of 109 primer pairs developed for *P. cincinnata* (25 loci), *P. edulis* (32) and *P. setacea* (52) in 14 passion fruit species from the germplasm bank of Embrapa Cassava & Fruits.

<i>Passiflora</i> species	<i>P. cincinnata</i>		<i>P. edulis</i>		<i>P. setacea</i>		Total	
	Nº loci	%	Nº loci	%	Nº loci	%	Nº loci	%
<i>P. alata</i> Curtis	19	76	29	91	44	85	92	84
<i>P. cincinnata</i> Mast	----	----	24	75	42	81	66	76
<i>P. edulis</i> Sims	12	48	----	----	46	88	58	75
<i>P. foetida</i> L.	14	56	22	69	35	67	71	65
<i>P. galbana</i> Mast	19	76	28	88	45	87	92	84
<i>P. gibertii</i> N.E.Br	16	64	21	66	36	69	73	67
<i>P. laurifolia</i> L.	12	48	12	38	26	50	50	46
<i>P. malacophylla</i> Mast	22	88	30	94	47	90	99	91
<i>P. morifolia</i> Mast	12	48	16	50	36	69	64	59
<i>P. rubra</i> L.	13	52	16	50	28	54	57	52
<i>P. setacea</i> DC	6	24	21	66	----	----	27	47
<i>P. suberosa</i> L.	13	52	19	59	30	58	62	57
<i>P. tenuifolia</i> Killip	15	60	22	69	38	73	75	69
<i>P. watsoniana</i> Mast	18	72	30	94	43	83	91	83
Total	15	59	22	70	38	73	70	68

^aMicrosatellite markers previously developed by Oliveira [53] and Cerqueira-Silva et al. [46,49].

Figure 1. Descriptive histogram of the passion fruit species, including the microsatellite markers characterized in this study (red columns) or published previously (blue columns). Note: *Passiflora alata* (Pa), *P. edulis* (Pe), *P. malacophylla* (Pma), *P. galbana* (Pga), *P. watsoniana* (Pw), *P. cincinnata* (Pc), *P. tenuifolia* (Pt), *P. gibertii* (Pgi), *P. setacea* (Pse), *P. foetida* (Pf), *P. morifolia* (Pmo), *P. suberosa* (Psu), *P. rubra* (Pr), *P. laurifolia* (Pl), *P. pohlii* (Pop), *P. caerulea* (Pca), *P. ligularis* (Pli), *P. maliformis* (Pma), *P. mucronata* (Pmu) and *P. contracta* (Pco).



2.2. Polymorphism analysis of microsatellite loci

The results of the descriptive statistical analyses of all the SSR loci used for the genetic characterization of *P. cincinnata*, *P. edulis* and *P. setacea* are summarized in Table 2, and details are available in the supplementary material (Supplementary Material – SM 3, 4 and 5). We identified a total of 355 alleles for the three species evaluated, with 137 alleles for *P. cincinnata* (average of 6 alleles for 22 SSR loci used), 141 alleles for *P. edulis* (average of 6 alleles for 23 SSR loci used) and 77 alleles for *P. setacea* (average of 3 alleles for 22 SSR loci used).

The H_O values were generally lower than the H_E values for most (70%) of the SSR loci in the three species of passion fruit. The average values of H_O and H_E were 0.42 (ranging from 0.01 to 0.92) and 0.52 (ranging from 0.01 to 0.90), respectively, for *P. cincinnata*; 0.43 (ranging from 0.01 to 0.77) and 0.50 (ranging from 0.01 to 0.86), respectively, for *P. edulis*; and 0.26 (ranging from 0.02 to 0.82) and 0.36 (ranging from 0.02 to 0.69), respectively, for *P. setacea*. Finally, the mean F values in the three species were 0.17 for *P. cincinnata*, 0.13 for *P. edulis* and 0.28 for *P. setacea*, and the estimated t values were 0.74, 0.91 and 0.85.

Table 2. Descriptive results of the values observed in the characterization of accessions of *P. cincinnata* (24 accessions and 22 microsatellite loci), *P. edulis* (85 accessions and 23 microsatellite loci) and *P. setacea* (7 accessions and 25 microsatellite loci).

<i>Passiflora</i> species	Values	Na	H _O	H _E	F	F _{is}
<i>P. cincinnata</i>	Minimum	2	0.00	0.01	-0.41	-0.36
	Maximum	16	0.92	0.89	1.00	0.66
	Average	6	0.42	0.52	0.17	0.15
	Standard deviation	±4.7	±0.2	±0.3	±0.3	±0.07
<i>P. edulis</i>	Minimum	2	0.01	0.01	-0.16	-0.27
	Maximum	18	0.77	0.86	0.38	0.31
	Average	6	0.43	0.50	0.13	0.05
	Standard deviation	±3.7	±0.2	±0.3	±0.1	±0.03
<i>P. setacea</i>	Minimum	2	0.02	0.02	-0.65	-0.86
	Maximum	6	0.82	0.69	0.87	0.68
	Average	3	0.25	0.36	0.28	0.08
	Standard deviation	±1.4	±0.2	±0.2	±0.4	±0.08

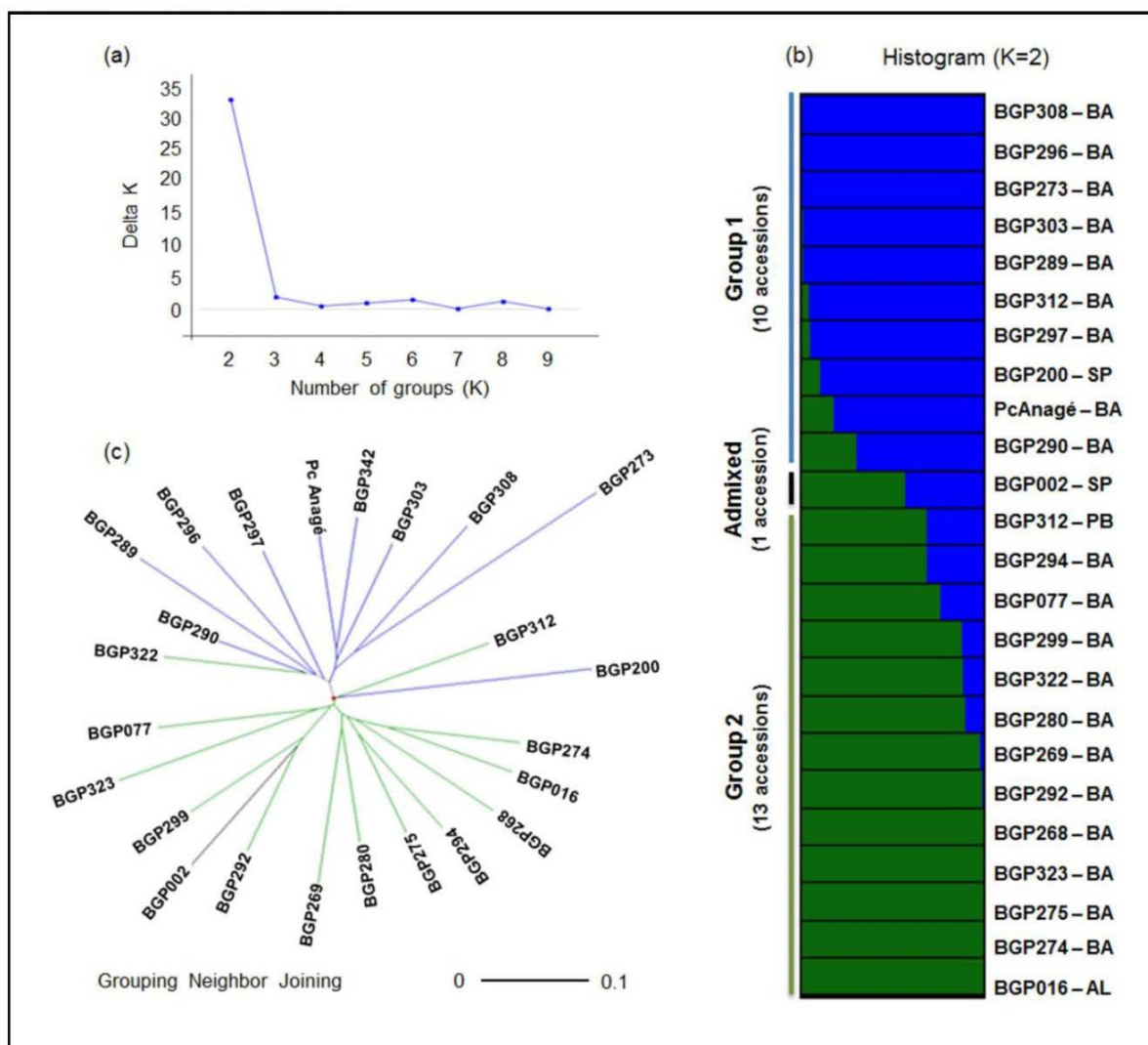
Note: The microsatellite loci were previously developed by Oliveira [53] and Cerqueira-Silva et al. [46, 49]. Na = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; F = fixation index; and F_{is} = inbreeding coefficient.

2.3. Genetic structure and diversity among passion fruit accessions

Bayesian clustering indicated the existence of two genetic groups (K = 2) for the 24 accessions of *P. cincinnata*, which were evident for 10 and 13 accessions in groups 1 and 2, respectively (Figure 2a, b). Only the BGP002 accession was identified as admixed, with estimated membership q values of ≤ 0.60 (approximately 0.43 and 0.57) for each genetic group. The distribution of the accessions in the two suggested groups corresponded to an intermediate level of genetic structure, with a G_{ST} value of 0.08. Furthermore, AMOVA indicated that approximately 7% of the observed variation arose from differentiation between these groups (P < 0.01).

The average proportion of polymorphic loci (97.5%) did not differ between the groups. However, a large proportion of the identified alleles (41%) were not shared between the two groups, and most of these alleles occurred at a low frequency (below 5%); therefore, these alleles may be considered private and rare alleles (Table 3). The estimated F values (0.20 and 0.09 for groups 1 and 2, respectively) revealed an uneven distribution of the alleles in each group and a more substantial reduction in H_O compared with H_E in group 2. The mean modified Rogers' distance [54] among the 24 accessions was 0.47 (± 0.08), with extreme values recorded for two pairs of accessions: BGP290 and BGP342 (0.28) and BGP273 and BGP323 (0.69). This distance clustering based on the neighbor-joining method supported the distribution of accessions into two groups by the Bayesian method (Figure 2c).

Figure 2. Intraspecific genetic structure of *P. cinnamomata*. Inferred clusters based on Bayesian analyses considering the most probable number of groups (K) estimated with the method described by Evanno et al. [55] (a). Each column (histogram) (b) and line (unrooted neighbor-joining) (c) represents the genotyping data from three plants. The tree was constructed using Rogers' genetic distance, as modified by Wright [54], among 24 passion fruit accessions. The colors used in the histogram and tree represent the most likely ancestry of the cluster from which the accessions were derived.



For the 85 *P. edulis* accessions, the Bayesian assignment method revealed five and three clusters as the most probable K [55], and these groups were partially concordant with the dendrogram based on the neighbor-joining method (Figure 3c).

The distribution of the accessions into five groups revealed that the distribution of the number of accessions among the groups was not homogeneous, and the numbers of accessions in the five groups ranged from three to 22. Seventeen accessions were classified as admixed and did not have membership $q \geq 0.6$ for a single group. The geographic origins of the accessions were not decisive for the formation of the groups, as indicated by the Bayesian approach (Figure 3b). Accessions of passion fruit from Bahia and São Paulo were distributed in all the groups. Accessions from Minas Gerais were included in groups 5 and 6. Moreover, accessions from Pará and Santa Catarina were included in group 4. Finally, the accessions from Paraná were distributed into groups 4 and 6. However, the hybrids and cultivars clustered into group 1, indicating that these hybrids might have a genetic background in this group. The genetic structure estimated with the formation of the five groups could be considered intermediate ($G_{ST} = 0.15$), and the diversity inherent to the groups corresponded to 10% of the total variance, according to the AMOVA ($P < 0.01$).

The number of polymorphic loci differed among the groups, with percentages varying from 78% (group 3) to 96% (groups 4 and 5). The estimated F values ranged from -0.025 (group 1) to 0.145 (group 5). Thirty-one private alleles were identified among the groups estimated using the Bayesian method, and approximately 60% of these alleles were rare alleles with a relative frequency lower than 5% (Table 3). The mean estimated modified Rogers' distance [54] among 85 accessions was 0.41 (± 0.07), with extreme values recorded for the following pairs of accessions: BGP009 and BGP034, BGP009 and BGP051, BGP007 and BGP222 (with an average distance of 0.21) and BGP071 and BGP234 (with a distance of 0.67).

Considering the seven accessions of *P. setacea*, we observed two likely scenarios based on the Bayesian method, with $K = 2$ and $K = 3$. Both scenarios were completely compatible with the dendrogram based on the neighbor-joining method (Figure 4). For $K = 2$, all six accessions from Embrapa were grouped together, and only the UESB accession was separated into a distinct group. For $K = 3$, the accessions from Embrapa were divided into two groups, and the UESB accession was retained as a separate group. In the two-cluster scenario, we observed a G_{ST} value of 0.36, and the between-group variation corresponded to 36% of the total diversity ($P < 0.01$), whereas when $K = 3$, there was a slightly higher G_{ST} value (0.38), but a lower portion of the total variance (29%) was due to differentiation between the inferred groups ($P < 0.01$).

Figure 3. Intraspecific genetic structure of *P. edulis*. Inferred clusters based on Bayesian analyses considering the most probable number of groups (K) estimated with the method described by Evanno et al. [55] (a). Each column (histogram) (b) and line (unrooted neighbor-joining) (c) represents the genotyping data from three plants. The tree was constructed using Rogers' genetic distance, as modified by Wright [54], among 85 passion fruit accessions. The colors used in the histogram and tree represent the most likely ancestry of the cluster from which the accessions were derived. Accessions with fruits of yellow and purple peel are indicated with * and #, respectively.

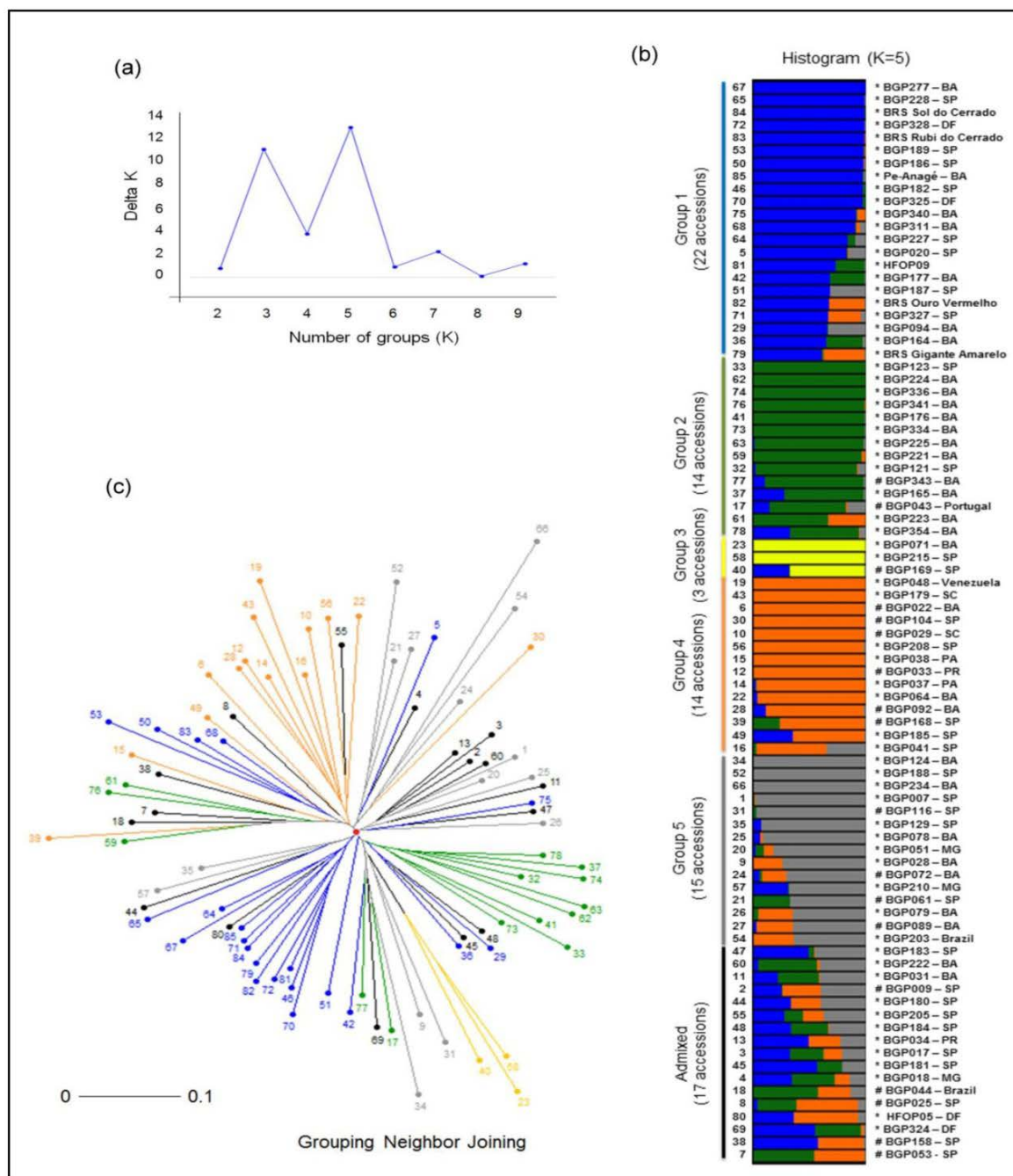
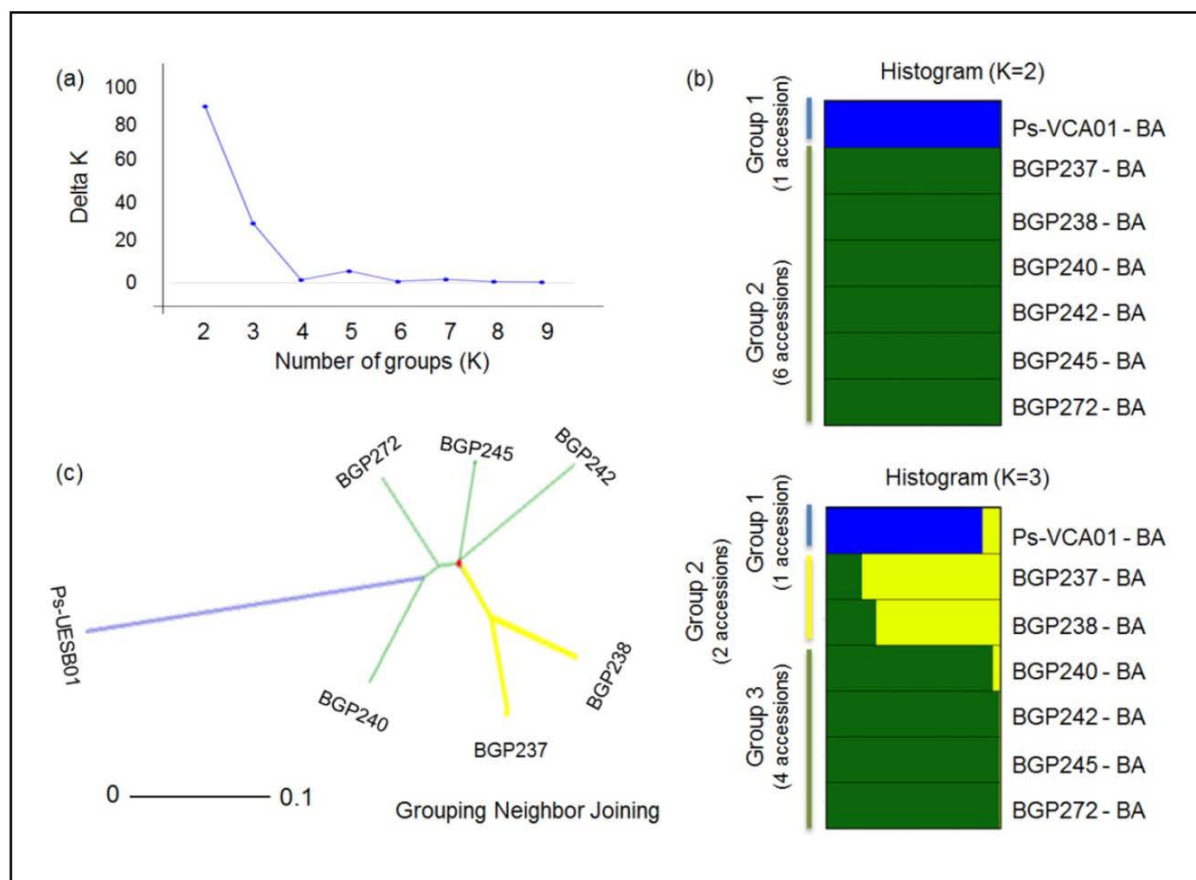


Figure 4. Intraspecific genetic structure of *P. setacea*. Inferred clusters based on Bayesian analyses considering the most probable number of groups (K) estimated with the method described by Evanno et al. [55] (a). Each column (histogram) (b) and line (unrooted neighbor-joining) (c) represents the genotyping data from three to 12 plants. The tree was constructed using Rogers' genetic distance, as modified by Wright [54], among seven passion fruit accessions. The colors used in the histogram and tree represent the most likely ancestry of the cluster from which the accessions were derived.



The percentage of polymorphic loci differed between the groups and varied from 92% (group 1) to 64% (group 2) for K = 2 and 92% (group 1), 52% (group 2) and 60% (group 3) for K = 3. The fixation index estimates also differed between the groups and ranged from 0.008 (group 2) to 0.231 (group 1). Thirty alleles might be classified as private and rare alleles, with the vast majority of these alleles restricted to group 1 (Table 3). The estimated mean genetic distance among the seven *P. setacea* accessions was 0.32 ($\pm$ 0.12), with extreme values recorded for the following pairs of accessions: BGP245 and UESB-VCA, BGP272 and UESB-VCA (both with distances of 0.19) and BGP237 and BGP242 (with a distance of 0.54).

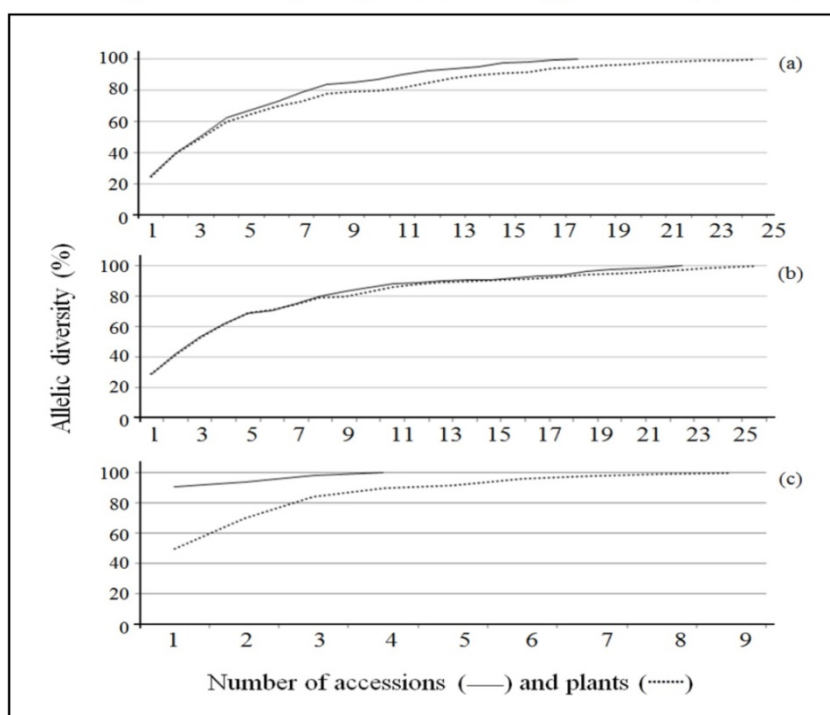
Table 3. Number of microsatellite loci with private alleles (NI), the total number of these alleles (Na) and the percentage of rare alleles (Na_R) identified in each genetic group, as estimated based on Bayesian analyses of three passion fruit species.

Groups	<i>P. cincinnata</i>			<i>P. edulis</i>			<i>P. setacea</i>		
	NI	Na	Na _R	NI	Na	Na _R	NI	Na	Na _R
Group 1	10	27	70%	2	2	100%	17	24	25%
Group 2	13	29	69%	1	1	100%	2	2	100%
Group 3	---	---	---	8	13	31%	3	4	50%
Group 4	---	---	---	6	8	75%	---	---	---
Group 5	---	---	---	6	7	75%	---	---	---
Total	16	56	70%	16	31	61%	22	30	27%

2.4. Estimates for the formation of core collections

Regarding the minimum number of accessions necessary to represent the diversity present in the germplasm evaluated for the three passion fruit species, only five, six and one accession were sufficient to represent at least 70% of the allelic diversity observed in *P. cincinnata*, *P. edulis* and *P. setacea*, respectively. Moreover, 18 (25 plants), 23 (26 plants) and 4 (9 plants) accessions were required for all of the alleles identified from the SSR loci to be retained in a core collection for *P. cincinnata*, *P. edulis* and *P. setacea*, respectively (Figure 5 and Supplementary Material – SM 6).

Figure 5. Allelic diversity as a function of the numbers of plants included in the core collections of three passion fruit species, *P. cincinnata* (a), *P. edulis* (b) and *P. setacea* (c).



3. Discussion

The primary actions associated with the conservation of wild *Passiflora* species are related to the pre-breeding of *Passiflora*, which highlights the importance of characterizing the genetic diversity of *Passiflora* species and the construction and maintenance of active germplasm banks. However, the numbers of accessions/species maintained in collections and germplasm banks are considered unrepresentative of the genus *Passiflora* [21,56]. In addition, reports have indicated reductions in the numbers of accessions and collections maintained by Brazilian institutions [19]. Despite this reality, the availability of representative and well-characterized germplasm banks is critical to the advancement of breeding programs because intra- and interspecific hybridization is indispensable.

In addition to the technical and financial difficulties associated with prospecting and maintaining accessions in germplasm banks, the lack of biological and agronomic information makes it extremely difficult to effectively use the material stored in the banks in breeding programs [57]. Therefore, the effective use of germplasm banks at different stages of breeding is dependent on the quality of the work performed during the steps that comprise pre-breeding programs (prospecting, characterizing and conserving germplasm) [19,51,58]. In this context, the use of molecular techniques associated with agronomic characterization tends to maximize the volume and quality of information generated during the stages of pre-breeding and reduce the costs and time required for the stages of germplasm characterization [57,59].

Although the assessment of the available genetic variability in germplasm banks is a first step toward the rational conservation and efficient use of genetic resources for plant breeding, this work is the first study to investigate the molecular genetic diversity and structure of a large number of commercial and wild accessions of passion fruit, including different species of the genus *Passiflora*. In addition, estimates of genetic diversity performed using microsatellite markers for passion fruit have been developed only recently and are restricted to studies that monitor diversity in full-sib progenies associated with recurrent selection programs [60,61] or studies of variability using the cross-amplification of microsatellite loci in a few accessions of passion fruit [48,62].

In summary, our results not only facilitate the use of microsatellite markers for genetic studies of the genus *Passiflora* but also elucidate the structure and diversity of accessions stored in one of the most robust passion fruit germplasm banks (maintained by Embrapa at Cruz das Almas, Bahia, Brazil). Our findings will enable the discussion of strategies related to gathering expeditions and directed crosses.

3.1. Tests for cross-amplification

Considering that assessments of microsatellite loci are based on primer pair hybridization to specific regions of the genome, different species are expected to contain some of these conserved regions, albeit in different proportions. The success rate of cross-amplification among the 14 *Passiflora* species evaluated (an average of 70%, ranging between 47 and 91%) (Table 1) can be considered high compared with the results presented by Paiva [62] (an average of 45%, ranging from 12.5 to 64%) and is more readily comparable to the results reported by Oliveira et al. [48] (an average of 67%, ranging from 43 to 100%). According to Barbará et al. [63], success rates for the cross-

amplification of SSR loci were highly variable among plant species and were related, leading to the variation observed in the success rates for cross-amplifying microsatellite loci.

The first cross-amplification assays in *Passiflora* species were performed by Pádúa [10] and Cerqueira-Silva [64]. Together, these authors presented information on the cross-amplification of approximately 100 species. Although considerable, the data reported by Pádúa [10] and Cerqueira-Silva [64] are limited in the sense that they provide information on a limited number of SSR loci (approximately 10) for each species evaluated. In addition, those tests were restricted to agarose gel electrophoresis, which limits the evaluation of the amplification profiles of the loci. Recently, more robust studies of cross-amplification were conducted by Oliveira et al. [48], using 11 passion fruit species and 21 SSR loci; by Paiva [62], using 10 species and 28 SSR loci; and by Cerqueira-Silva et al. [49], using three species and 94 SSR loci. Together, the information provided by evaluating cross-amplification in the genus *Passiflora* attests to the effectiveness of this strategy as a means of providing microsatellite markers for different species of this genus at a reduced cost.

With the results obtained using cross-amplification (Figure 1), the 14 passion fruit species analyzed in this study now have a substantial number of SSR loci (ranging from 50 to 99 SSR loci) that are potentially useful for different genetic approaches. Given that studies of germplasm genetic diversity and population genetics using microsatellites rely on 10 to 30 polymorphic loci, the results presented herein facilitate studies of this nature focusing on wild and commercial species of passion fruit. However, because the number of characterized and available SSR markers for the genus *Passiflora* was less than the approximately 415 loci and because these markers were developed from the genomic DNA of only six species (*P. alata*, *P. edulis*, *P. cincinnata*, *P. contracta*, *P. pohlii* and *P. setacea*) [46, 47, 49, 65, 67, 68, 69], both cross-amplification and the development of new markers are required to advance microsatellite-based genetic studies of the more than 500 species of *Passiflora*.

The identification of loci using cross-amplification among species not only enhances intraspecific genetic studies but also aids studies of interspecific diversity. Barbará et al. [63] considered SSR markers one of the most popular and useful markers for ecological studies, with the cross-species amplification of positive characteristics critical to the success of these studies. In this context, the different subsets of SSR loci conserved among the *Passiflora* species (Supplementary Material – SM 1) could promote the first studies of population genetics of the genus *Passiflora*, as well as diversity characterizations of wild and commercial accessions found in germplasm banks. Notably, of the loci we studied, the 23 SSR loci that showed 100% cross-amplification between 14 *Passiflora* species represent a significant number of loci, which should be considered in interspecific characterizations. Moreover, the identification of sets of SSR loci with different amplification profiles among species (for example, the loci mPs-Unicamp18, mPe-Unicamp11 and 17, which were amplified only in the species of origin) (Supplementary Material – SM 1) is also of great importance both for population genetics efforts related to the species for which they were developed and for the confirmation of interspecific hybrids in pre-breeding programs and natural hybridization in sympatry zones.

The use of cross-amplification-derived SSR loci has contributed to the pre-breeding of passion fruit, although the effective use of this approach remains limited due to its recent development. Microsatellite cross-amplification to confirm ornamental hybrids derived from crosses among wild species of passion fruit has become a regular component of the activities undertaken in the breeding program developed at the Universidade Estadual de Santa Cruz, Bahia, Brazil [41]. Moreover,

characterizations of variability among accessions from germplasm banks using cross-amplification of SSR loci were also initiated in recent years. Interspecific diversity studies were conducted by the research group at the Universidade Estadual do Norte Fluminense, Rio de Janeiro, Brazil, where a total of 56 plants, representing 10 species, were evaluated using 28 SSR loci [62]. Additionally, in searches conducted as part of the Embrapa breeding program, genetic distances for a total of 61 plants, representing 11 species, were estimated using 21 SSR loci [48]. With the increasing number of SSR markers available for species of passion fruit, which arise from cross-amplification tests and the identification of new loci, an increase in the number of studies devoted to characterizing and understanding the diversity of the genus *Passiflora* is also expected.

The dendrogram created using these data (Supplementary Material – SM 2) illustrates the grouping of species according the number of shared SSR loci; together with the information available for the percentage of cross-amplification presented in the Supplementary Material (SM 1), the dendrogram indicates the amount of effort required for interspecific characterizations based on SSR loci. Although the grouping shown in Supplementary Material (SM 2) enables the clustering of a subset composed of two species of the subgenus *Decaloba* (i.e., *P. rubra* and *P. suberosa*), it does not represent the systematic distribution of the *Passiflora* species. According to Oliveira et al. [48], the high mutation rates associated with microsatellite loci may contribute to the problem of not being able to faithfully reproduce taxonomic classifications (such as genera and subgenera) with the same efficiency that these loci show for intraspecific approaches.

3.2. Polymorphism analysis of microsatellite loci

The average number of six alleles per locus observed for *P. cincinnata* and *P. edulis*, as well as the average of three alleles per locus observed in *P. setacea*, conforms to the pattern of the results previously found in characterizations of SSR loci in different species of *Passiflora*. In commercial species of passion fruit, such as *P. alata* and *P. edulis*, the average number of alleles per locus observed by Pádua et al. [67] and Oliveira et al. [68, 69] ranged from 3.5 to 5.3. For wild species of passion fruit, such as *P. contracta*, *P. cincinnata* and *P. setacea*, the average number of alleles per locus observed ranged from 2.8 to 4.9 [46, 49, 65].

In addition to the relatively low numbers of alleles per locus, the percentages of polymorphic SSR loci observed in studies of the development and characterization of SSR primers for passion fruit did not exceed 35% [46,47, 49,50, 69,70], with average polymorphic loci of less than 22% in these reports. As for SSR loci characterized as polymorphic, identifying polymorphisms in unrelated germplasms is difficult. For example, Ortiz et al. [43] did not observe polymorphism when evaluating the germplasm of wild and commercial passion fruit, distributed throughout the Colombian territory, using 17 SSR primers previously characterized by Oliveira et al. [68] and Pádua et al. [67]. In this context, Cerqueira-Silva et al. [46] reported that low polymorphism in SSR loci seemed to be a pattern among species of the genus *Passiflora*.

The absence of a correlation between the high diversity observed for agronomic traits of passion fruit and the low estimates of genetic diversity was discussed by Viana et al. [6]. These researchers observed low levels of polymorphism among genotypes of passion fruit that were characterized using

RAPD markers, positing that environmental influences were largely responsible for the morphological changes observed in commercial plantings of passion fruit.

The lower H_O values, compared with the H_E values (Table 3), indicate a tendency toward inbreeding in the evaluated germplasm. Similar results were observed by Oliveira et al. [68] for *P. edulis* (average values of $H_O = 0.58$ and $H_E = 0.62$), by Pádua et al. [67] for *P. alata* (average values of $H_O = 0.26$ and $H_E = 0.53$), by Cerqueira-Silva et al. [46, 49] for *P. cincinnata* (average values of $H_O = 0.39$ and $H_E = 0.44$), by Cazé et al. [65] for *P. contracta* (average values of $H_O = 0.53$ and $H_E = 0.61$) and by Cerqueira-Silva et al. [49] for *P. setacea* (average values of $H_O = 0.34$ and $H_E = 0.41$).

The mean values of F in all three species (0.17, 0.13 and 0.28 for *P. cincinnata*, *P. edulis* and *P. setacea*, respectively) reinforce the idea of the loss of heterozygotes, as suggested by the values of H_O and H_E , which is more evident for *P. setacea*. These results may be partly explained by the origins of the accessions present in germplasm banks, with most accessions originating from the same geographical region. All of the *P. setacea* accessions, and approximately 79% and 36% of the *P. edulis* and *P. cincinnata* accessions, respectively, were from Bahia (Figure 6 and Supplementary Material SM 7). As expected, the outcrossing rate estimated for these three species, calculated from the values of F_{IS} , was high ($t = 0.84$). Nevertheless, because these are cross-pollinated species [7].

3.3. Genetic diversity and structure among passion fruit accessions

Given that the accessions examined in this study do not represent the entire natural geographic distribution of *P. edulis*, *P. cincinnata* and *P. setacea* and that most of the accessions (approximately 70%) were from only two Brazilian states (Bahia and São Paulo), the genetic groups defined by the STRUCTURE program could not be correlated with geographic regions. Thus, for population studies of the genetic diversity of passion fruit, new samples must be acquired from natural populations, especially for *P. cincinnata* and *P. setacea*, which were less represented in germplasm banks. Studies of diversity among passion fruit accessions based on AFLP [66] and ISSR [44] markers found no association between estimated genetic diversity and geographic origin. In these studies, the authors considered the high flow of biological material among producers as a likely factor contributing to a lack of association between the genetic diversity and geographic origin of the accessions. The flow of biological material and the self-incompatibility of passion fruit, which both favor the exchange of alleles among passion fruits, were possible causes of the lack of definition of groups by the structure program for seventeen accessions of *P. edulis* (Figure 3b). These accessions likely have multiple ancestries compared with other accessions.

Considering the formation of two groups using the Bayesian clustering method for *P. cincinnata*, stands out beyond the distribution of accessions from the Bahia state, the meeting of the accessions from the Alagoas and Pernambuco states (both from northeastern Brazil) in the same group (Figure 2). The tree constructed using the neighbor-joining method corroborated the Bayesian results, but it indicated a union between the accessions from São Paulo state and the accessions from Alagoas and Pernambuco states. The distribution of *P. setacea* accessions in two or three groups suggested by STRUCTURE and corroborated by the neighbor-joining tree reinforces the hypothesis that a low number of accessions are represented in relation to the diversity of natural species. We highlight the fact that six of the seven accessions (BGP237, 238, 240, 242, 245 and 272, totaling 38 plants)

presented low values of genetic diversity (68% of polymorphic loci) and few private alleles (six, representing only 20% of the private alleles identified) compared with the values observed for the Ps-UESB01 accession (accounting for 12 plants), which showed 92% polymorphic loci and 24 private alleles (representing 80% of the private alleles identified). High genetic variability among *P. setacea* accessions from fragments of native forest in the Southwest region of Bahia (fragments collected in the Forest of Liana, 14° 53 'S and 40° 47' W) was also observed by Cerqueira-Silva et al. [42].

Together, the results reveal that both *P. cincinnata* and *P. setacea* were undersampled in the evaluated germplasm and that diversity has maintained high levels of genetic structure, indicating the potential occurrence of inbreeding in this material. Future efforts should be directed toward increasing the representation of these two species in collections, both because of their importance for genetic breeding [27,31] and because of the importance of collections and germplasm banks for ex situ conservation [51, 52,64]. In relation to the conservation of *P. cincinnata* and *P. setacea*, it is noteworthy that in studies of accessions from fragments of degraded native forest, low variability was observed among accessions of *P. cincinnata* [38], and although a similar study identified significant variability in *P. setacea* [42], it should be noted that the latter species is endemic to the Brazilian territory [71]; therefore, its diversity is potentially more vulnerable to the effects of genetic drift.

The distribution of *P. edulis* accessions in the groups estimated using the Bayesian approach and corroborated by the neighbor-joining method led to the significant finding of five of the six hybrids and cultivars evaluated in the same group (Figure 3b). These data provide evidence of the homogeneity of this material, indicating that these hybrids could have a genetic background in this group. In agreement with this evidence, we highlight the fact that the HFOP09 hybrid has the BGP165 accession and the "BRS Gigante Amarelo" variety as parents. In addition, low genetic diversity in commercial accessions of this species was also suggested by different authors, such as the estimates performed using RAPD [6], as well as AFLP and SSR [43]. Low genetic diversity was observed among commercial varieties of *P. edulis*, as evaluated by Faleiro et al. [58]. These results corroborate the hypothesis of a narrowing in the genetic base of commercial cultivars and accessions that are available for yellow passion fruit [6, 39,58]. The low diversity observed in *P. edulis*, especially when associated with commercial accessions, could be due to germplasm exchange within Brazil because in most cases, passion fruit producers generate their own seedlings, either from seeds collected in their neighborhood or from fresh fruits purchased at the market [44].

Therefore, we highlight the need to increase the genetic variability in collections composed of commercial accessions with the addition of wild accessions that, according to Faleiro et al. [58] and Viana et al. [6], will improve breeding programs. However, it is not sufficient to increase the number of accessions stored in germplasm banks because, as discussed by Freitas et al. [72], the dearth of information due to the lack of characterization is one of the major obstacles to the effective use of new accessions in breeding programs. These same authors argued that as a consequence of this lack of information, breeders are encouraged to continue exploring small collections, which consequently limits the use of germplasm and leads to a narrowing of the genetic base of cultivated passion fruit. The low genetic diversity available and characterized in active collections can undermine the advancement of breeding programs and the effective ex situ conservation of the diversity available in natural populations. In this context, studies by Junqueira et al. [30] suggest that the low genetic variability present in passion fruit cultivars contributes to the susceptibility of this material to the most

important diseases affecting the crop. Considering the potential use of wild accessions as a source of genetic variability and the limited representation of accessions from different geographic regions in the evaluated germplasm relative to the natural distribution of passion fruit species (Table 4 and Figure 6), new collecting expeditions are required.

Regarding diversity studies conducted in *P. edulis*, Bellon et al. [25] observed greater genetic distance between accessions of fruits with yellow and purple peels. From the germplasm evaluated in this study, 18 of 85 accessions had fruits with purple peels and were represented in four of the five groups inferred using the Bayesian analysis (Figure 3b). Although there was no clear distinction between groupings of accessions with yellow and purple fruits, we observed the presence of 37 private alleles in accessions of yellow passion fruit (approximately 26% of the alleles identified in the germplasm). Although these accessions are recognized as the same species [73], these results reinforce the need, as suggested by Bellon et al. [25], to distinguish accessions of *P. edulis* by the color of their fruit. This variation may be understood as a phenotypic marker of more robust genetic variability. Those microsatellite loci with the most private alleles identified among accessions of fruits with purple and yellow peels (mPe-UNICAMP02, Pe-19, Pe-20, Pe-23, Pe-24, Pe-42, Pe-58, mPc-UNICAMP11, mPs-UNICAMP09 and mPs-UNICAMP16) could be prioritized in future studies to characterize and monitor variability among progeny derived from contrasting accessions for fruit traits. Considering that *P. edulis* has at least 150 characterized SSR loci, it would be possible to build and characterize a mapping population to elucidate the genetic architecture that contributes to this feature and to understand the genetic basis of this phenotype. Thus, marker-assisted breeding, at least for *P. edulis* and *P. alata*, is fairly plausible in the medium and long term, both because of the commercial interest in these species and because of the large numbers of available SSR markers.

Estimates of genetic distance also contribute to breeders' decisions regarding the selection and crosses of divergent and convergent parents [59,66]. In this context, molecular markers could help produce direct or indirect estimates (depending on the type of molecular marker used) of the difference in alleles present in a population, as well as in germplasm banks, and can enhance the genetic gains derived from crosses that explore the maximum heterosis effect. The indication of molecular markers in this context is discussed and advocated for breeding programs with passion fruit [61].

In the present study, we identified various possibilities for intraspecific crosses (convergent and divergent) that increase (BGP071 and BGP234, BGP273 and BGP323, BGP237 and BGP242) or reduce (BGP009 and BGP034, BGP009 and BGP051, BGP007 and BGP222, BGP290 and BGP342, BGP245 and UESB-VCA, BGP272 and UESB-VCA) the available genetic variability in the evaluated accessions. Obviously, information on the agronomic traits of interest should also be considered during the selection of preferred crosses, as well as after the crosses, when selecting segregating progeny. Although such crosses are limited by a lack of agronomic information for most of the genotyped accessions, as considered by Freitas et al. [72], we recommend potential genotypes for crosses to generate segregating progeny with the potential for use in the fresh fruit trade and in the production of juice.

The BGP185 and BGP051 accessions, indicated by Freitas et al. [72] as promising for the fresh fruit market, were positioned in different groups for the STRUCTURE program and were shown to be divergent in the grouping performed by the neighbor-joining method (Figure 3b, c). Therefore, in addition to possessing desirable agronomic characteristics, crosses between these accessions may

produce progeny with desirable variability for future stages of selection and/or present positive effects of heterosis. Moreover, considering the accessions indicated by Freitas et al. [72] as promising for use in the production of juice in combination with the diversity estimated using microsatellite markers (Figure 3b, c), it is possible to identify both divergent (BGP034 and 181 or BGP0123, BGP0079 and BGP123 or 181) and convergent crosses (BGP034 and BGP079, BGP123 and BGP181). We believe that information derived from future agronomic evaluations (both for the physical-chemical characteristics of fruits and for disease resistance) and the molecular genetic diversity data generated in this study will enable a more conscious use of the variability available in germplasm banks.

Finally, considering that no direct correlations between geographical and genetic distances were observed and that we observed high values of inbreeding (relative to the fixation index) for the three species considered in this study (Table 2), we understand that expeditions to obtain new accessions must consider the different biomes present in the political units (Brazilian states). We understand that the collection of a larger number of plants should be prioritized at the expense of the number of fruits/seeds per plant. In addition, the actions of different pollinators of *P. setacea* (pollinated by bats) and *P. cincinnata* and *P. edulis* (pollinated by bees) should be taken into account when establishing collection strategies. Moreover, additional information regarding the ancestry of commercial materials should be prioritized for the addition of new accessions to germplasm banks because the characteristics inherent in the commercial cultivation of the passion fruit crop in Brazil, including seed production being performed largely by the producers and the flow of material between crop regions [44], can lead to redundancy in the genetic information among the accessions that should be maintained in germplasm banks.

3.4. Formation of core collection

The COREFINDER analysis showed that for the three passion fruit species, the entire set of alleles found in this study could be represented by core collections with 75%, 27% and 57% of the accessions from *P. cincinnata*, *P. edulis* and *P. setacea*, respectively (Figure 5). In addition, only 10% of the 116 accessions used to represent the three *Passiflora* species were indicated as sufficient to maintain 70% of the alleles identified in this study. The difference observed between the number of accessions and plants required to retain all of the alleles identified by the SSR loci in core collections of *P. cincinnata* (18 accessions and 25 plants), *P. edulis* (23 accessions and 26 plants) and *P. setacea* (4 accessions and 9 plants) (Figure 5) (Supplementary Material – SM 6) attests to the existence of significant variability among plants of the same accession. Because passion fruit is allogamous, due to its self-incompatibility [7], genetic variability is expected even among plants of the same accession (even if they are the progeny of controlled pollination between related plants). A similar result was observed by Santos et al. [44] when using ISSR markers to characterize genetic diversity in accessions of commercial species (*P. edulis* and *P. alata*).

Due to the potential flow of alleles among passion fruit accessions, special attention should be given to strategies for managing and restoring the active germplasm banks of passion fruit to avoid contamination among accessions and the loss of alleles. In this context, Santos et al. [44] discussed potential actions, such as monitoring the accession variability and studying allelic loss and effective population size.

The core collection indicated for *P. cincinnata* included accessions from all three Brazilian states, whereas the core collection suggested for *P. edulis* included accessions from four of the seven represented Brazilian states (Bahia, São Paulo, Minas Gerais and Pará) and one accession from Portugal. Not included in the collection proposed for *P. edulis* were accessions from the states of Paraná, Santa Catarina or the Federal District, as well as the accession from Venezuela. The non-inclusion of accessions from certain geographical regions does not necessarily follow from the low genetic variability of these regions or the redundancy among accessions because the sampling was very uneven among geographic regions.

Certain factors may have contributed to the different numbers of accessions in the core collections suggested here, including the different numbers of accessions evaluated for each species and the differences in the geographical origins of representative accessions of each species, ranging from one to seven Brazilian states. We think that the prevalence of accessions collected from one or a few geographic regions may, to some extent, compromise the genetic diversity present in the germplasm bank, resulting in a reduced number of individuals compared with what is necessary to fully represent allelic diversity. One possible effect of the sample composition of a germplasm bank leading to a reduced number of representative accessions of a core collection was suggested by Santos-Garcia et al. [74], who used genetic diversity information to establish core collections of important forage legumes from tropical and subtropical regions.

According to the general criteria available in the literature to form a core collection [51,75,76], one should retain 5 to 20% of the available germplasm that represents at least 70% of the total diversity. In this context, the core collection proposed for *P. edulis* is adequate, with minimal maintenance of 27% of the characterized accessions and 100% of the allelic diversity estimated via the SSR loci. The scenario is different for *P. cincinnata* and *P. setacea*, however. Although the suggested core collection is also adequate for the representation of variability criteria, due to the low number of accessions characterized in the base collection and the low representation of these accessions in relation to species distribution, it is advisable to increase the number of accessions in a core collection of these passion fruit species.

In addition, molecular markers arising from SNPs could also contribute to greater coverage of the genome. This information, coupled with phenotypic data for traits of interest, such as high productivity and resistance to both abiotic factors and disease, would help to more effectively identify the accessions that should be maintained in a core collection.

4. Experimental Section

4.1. Germplasm material and genomic DNA extraction

A total of 127 passion fruit accessions were analyzed, including three accessions from the Universidade Estadual do Sudoeste da Bahia (UESB) collection, Itapetinga, Bahia, Brazil, and 124 accessions from the germplasm collection of Embrapa Cassava & Fruits, Cruz das Almas, Bahia, Brazil, which included 85 accessions of *P. edulis* Sims (yellow and purple) and 42 accessions of *Passiflora* spp. (13 species of *Passiflora*). For each accession, an average of three plants was used (ranging from 1 to 12 plants per accession). Table 4 and Figure 6 present general information about the

germplasm used, such as the number and origins of the accessions and the genotypes of each species. Detailed information regarding the record of each accession is available in the supplementary material (Supplementary Material – SM 7).

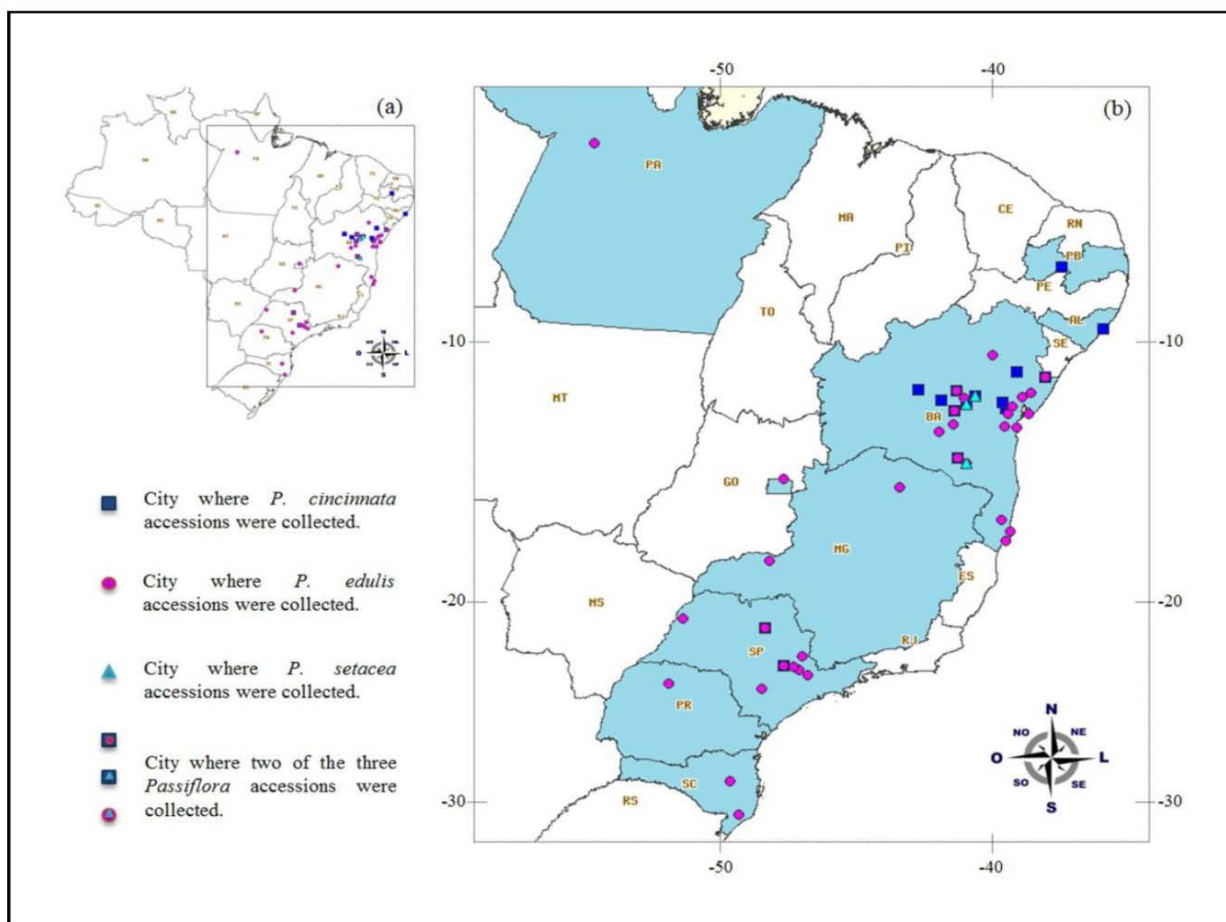
Genomic DNA was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method [77] and was stored in duplicate aliquots in an ultra-cold freezer at the Laboratory of Applied Molecular Genetics of UESB, Itapetinga, Bahia, Brazil, and at the Laboratory of Molecular and Genetic Analysis of Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil. The quality of the DNA samples was assessed via 1% (m/v) agarose gel electrophoresis (90 V electric current for 90 min) followed by ethidium bromide staining. The DNA concentrations (ng μ L⁻¹) were estimated by comparison with known quantities of lambda phage DNA (undigested lambda DNA).

4.2. Analysis of molecular markers

A set of 109 SSR loci was used at different stages in this study, including 15, 25 and 69 loci that were originally identified and characterized by Oliveira [53], Cerqueira-Silva et al. [46] and Cerqueira-Silva et al. [49], respectively. We performed a polymerase chain reaction (PCR) for a cross-amplification test of the SSR loci, and diversity estimates were performed in a final volume of 15 μ L containing 12 ng of template DNA, 0.4 mM of each primer (reverse and forward) and 1.0 U of Taq DNA polymerase (Invitrogen Co., Carlsbad, CA, USA). The other reagents (dNTP concentrations, MgCl₂ and buffers) and the amplification conditions (annealing temperature and PCR cycle characteristics) were exactly as described in the original articles reporting the identification and characterization of these loci.

The amplification products were initially visualized by electrophoresis on 3% (m/v) agarose gels in 0.5 $\times$ TBE buffer, after which the gels were stained with ethidium bromide. To characterize the amplification profiles, we then performed vertical electrophoresis on 6% (m/v) denaturing polyacrylamide gels in 1 $\times$ TBE buffer, followed by silver nitrate staining [78]; a 10-bp DNA ladder (Invitrogen) was used as a reference to determine the product sizes. We evaluated the reproducibility of the electrophoretic profiles by re-genotyping at least of 10% of the samples.

Figure 6. Brazilian geographical areas (a) highlighting the areas sampled for passion fruit accessions (*Passiflora* spp.) (b). Location of accessions plotted on a map using the SpeciesMapper tool, which is available at SpeciesLink [79].



4.3. Tests for cross-amplification

The results of cross-amplification of the 109 SSR loci in 14 *Passiflora* species considered in this study were recorded as binary values, i.e., the presence (1) or absence (0) of bands. Descriptive analyses associated with the cross-amplifications were performed, and we calculated the percentage of cross-amplifications by species and loci and the mean values and deviations.

The genetic dissimilarity among the accessions of 14 *Passiflora* species was estimated using the complement of the simple coincidence method and was graphically represented by generating a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA) clustering algorithm. The efficiency of the cluster matrices was evaluated using a bootstrap analysis (with 1000 resampling values). The statistical analyses were performed using Genes software [80].

Table 4. General information about the 14 passion fruit species characterized in this study.

<i>Passiflora</i> species	Popular name in Brazil	Natural occurrence (Brazilian macro-regions)	Total of accesses ^b	Total of plants	Location of origin
<i>P. cincinnata</i> Mast	maracujá do mato / mochila	North - Northeast - Midwest – Southeast	24	67	Alagoas, Bahia, São Paulo, Paraíba
<i>P. edulis</i> Sims	maracujá azedo / amarelo	North - Northeast - Midwest - Southeast - South	85	247	Bahia, Distrito Federal, Minas Gerais, São Paulo, Santa Catarina, Pará, Paraná, Portugal, Venezuela,
<i>P. setacea</i> DC ^a	maracujá do sono / sururuca	Northeast - Midwest - Southeast	7	50	Bahia
<i>P. alata</i> Curtis ^a	maracujá doce	North - Northeast - Midwest - Southeast - South	1	3	Distrito Federal
<i>P. gibertii</i> N.E.Br	-----	Midwest	1	3	Unavailable
<i>P. laurifolia</i> L.	maracujá laranja / perola	North - Northeast - Midwest	1	3	Unavailable
<i>P. tenuifolia</i> Killip ^a	maracujá de cobra	South - Southeast	1	3	São Paulo
<i>P. morifolia</i> Mast	maracujá da capoeira / peludo	Midwest - Southeast - South	1	3	São Paulo
<i>P. galbana</i> Mast ^a	maracujá luxo	Northeast - Southeast	1	3	Unavailable
<i>P. watsoniana</i> Mast ^a	-----	Northeast - Southeast	1	3	Unavailable
<i>P. rubra</i> L.	maracujá de estalo	Northeast - Southeast	1	3	São Paulo
<i>P. suberosa</i> L.	maracujá cortiça / maracujazinho	Northeast - Midwest - Southeast - South	1	3	São Paulo
<i>P. foetida</i> L.	-----	North - Northeast - Midwest - Southeast - South	1	3	São Paulo
<i>P. malacophylla</i> Mast ^a	-----	Northeast - Southeast	1	3	Unavailable

^a Species endemic to Brazilian territory [71];^b With the exception of two accessions (one accession each of *P. cincinnata* and *P. setacea*, which are present in the collection of the UESB, Itapetinga, Bahia, Brazil), all 125 accessions are maintained in the germplasm bank of the Embrapa Cassava & Fruits/EMBRAPA, Cruz das Almas, Bahia, Brazil.

4.4. Characterization of intraspecific genetic diversity

We studied the organization of the genetic variation in three species that are of great interest to passion fruit breeding programs: *P. cincinnata* (67 plants, representing 24 accessions), *P. edulis* (247 plants, representing 85 accessions) and *P. setacea* (50 plants, representing 7 accessions) (Table 4; Figure 6) (Supplementary Material – SM 7). For this purpose, we performed different estimates to characterize intra-species genetic diversity and structure. For each species, we performed a descriptive statistical analysis for all the SSR loci, and the number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F) were calculated using GenAlEx v. 6.5 [81]. The apparent outcrossing rate (t) was calculated from the inbreeding coefficient (F_{IS}) using the equation $t = (1 - F_{IS}) / (1 + F_{IS})$ [82] (Weir, 1990). Moreover, deviations from Hardy-Weinberg equilibrium and the occurrence of linkage disequilibrium were evaluated using GENEPOP software [83]. The significance level was adjusted using the sequential Bonferroni correction for multiple comparisons.

Analyses of population structure were performed using the Bayesian method that is available in the STRUCTURE program, version 2.3.4 [84,85]. Considering that the present study was conducted using accessions (represented by plants from controlled crosses within each accession) maintained in a germplasm bank, we used a no-admixture model, with independent allele frequencies in each population, and the burn-in time and replication numbers were set to 100,000 and 1,000,000, respectively, for each run. The number of groups (K) was systematically varied from 1 to 10, and 20 simulations were performed to estimate each K. We used the ΔK ad hoc method described by Evanno et al. [55] and implemented in the online tool Structure Harvester [86] to estimate the most likely K in each set of passion fruit species. After estimating the most likely K, we used the greedy algorithm implemented in CLUMPP v.1.1.1 [87] with a random input order and 1,000 permutations to align the runs. The results were visualized using DISTRUCT v.1.1 [88]. Based on the posterior probability of membership (q) of a given accession belonging to a given group compared to the total number of groups (K), we classified individuals with $q > 0.60$ as a member of a given cluster, whereas for clusters with membership $q \leq 0.60$, the accession was classified as admixed.

The modified Rogers' distance [54] was calculated using Tools for Population Genetic Analysis (TFPGA) software [89] (Miller 1997), and the result was used to infer the genetic relationships among accessions using the UPGMA criteria. These relationships were also studied by analyzing genotypic data using the neighbor-joining clustering method in DARwin 5.0 [90] (Perrier and Jacquemoud-Collet 2006). A hierarchical analysis of molecular variance (AMOVA) and Nei's GST among the groups, as defined by the STRUCTURE analysis, were calculated using GenAlEx v.6.5 [81]. This software was also used to identify private and rare alleles present in the estimated clusters for each species.

Finally, we used COREFINDER [91] to assemble a core collection estimated to represent both 70% and 100% of the genetic diversity present within each of the three species.

5. Conclusions

Considering the high percentage of successful observed with the cross-amplification reactions for obtaining microsatellite loci in passion fruit species, the cross-amplification was shown to be an efficient strategy and of low cost for molecular genetic studies in the genus *Passiflora*. The data indicate the need for further collection expeditions, especially to wild *Passiflora* species (*P. cincinnata* and *P. setacea*), as well as the diversity estimates confirm the pattern of low levels of genetic

polymorphism in microsatellite loci characterized in species of the genus *Passiflora*. The information generated by this work may be useful for breeding programs, conservation, preferential crossings based on genetic estimates and agronomic data.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Material

SM 1 Cross-amplification observed for a set of 109 loci developed for *P. cincinnata* (identified as mPc-01 to -25), *P. edulis* (identified as mPe-01 to 17, and Pe-) and *P. setacea* (identified as mPs-01 to -52) in 14 passion fruit species from the germplasm bank of Embrapa Cassava & Fruits.

<i>Loci^a</i>	Passion fruit species (<i>Passiflora</i> spp.) ^b														Total	%
	<i>Pa</i>	<i>Pc</i>	<i>Pe</i>	<i>Pf</i>	<i>Pga</i>	<i>Pgi</i>	<i>Pl</i>	<i>Pma</i>	<i>Pmo</i>	<i>Pr</i>	<i>Pse</i>	<i>Psu</i>	<i>Pt</i>	<i>Pw</i>		
mPc-01	1	1	0	0	1	0	0	1	0	0	0	0	1	1	6	43
mPc-02	1	1	1	1	1	1	1	1	1	1	0	1	1	1	13	93
mPc-03	1	1	0	1	1	1	1	1	1	1	0	1	1	1	12	86
mPc-04	0	1	1	0	0	0	0	0	0	0	1	0	0	0	3	21
mPc-05	1	1	0	1	1	1	1	1	1	1	0	1	1	1	12	86
mPc-06	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPc-07	1	1	0	1	1	1	1	1	1	1	0	1	1	1	12	86
mPc-08	1	1	0	0	1	1	0	1	1	0	0	0	1	1	8	57
mPc-09	1	1	0	0	1	1	0	1	0	0	0	0	0	1	6	43
mPc-10	1	1	1	1	1	1	0	1	0	0	1	0	0	0	8	57
mPc-11	0	1	0	0	0	0	0	1	0	1	1	1	0	0	5	36
mPc-12	1	1	0	0	1	0	0	1	0	1	0	0	0	1	6	43
mPc-13	1	1	0	1	1	1	1	1	1	1	0	1	1	1	12	86
mPc-14	0	1	1	0	0	0	0	1	0	0	0	0	0	0	3	21
mPc-15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPc-16	1	1	0	1	0	1	0	1	1	0	0	1	1	1	9	64
mPc-17	1	1	1	0	1	1	0	1	0	0	0	0	0	1	7	50
mPc-18	0	1	1	0	1	0	0	1	0	0	0	0	0	0	4	29
mPc-19	1	1	1	1	1	1	1	1	1	1	0	1	1	1	13	93
mPc-20	0	1	1	0	0	0	0	0	0	0	1	0	0	0	3	21
mPc-21	1	1	1	1	1	1	1	1	0	1	0	1	1	1	12	86
mPc-22	1	1	0	0	1	0	0	1	0	0	0	0	0	0	4	29
mPc-23	1	1	0	1	1	1	1	1	1	1	0	1	1	1	12	86
mPc-24	1	1	1	1	1	1	1	1	1	0	0	0	1	1	11	79
mPc-25	0	1	0	1	0	0	1	0	0	1	0	1	1	1	7	50
mPe-01	1	1	1	0	1	0	1	1	0	1	1	0	1	1	10	71
mPe-02	1	1	1	1	1	0	1	1	0	0	1	1	1	1	11	79
mPe-03	1	1	1	0	1	1	1	1	1	0	1	0	1	1	11	79
mPe-04	1	1	1	1	1	1	0	1	1	1	1	1	1	1	13	93
mPe-05	1	0	1	1	1	1	1	1	1	1	0	1	1	1	12	86
mPe-06	1	1	1	1	1	1	1	1	1	0	0	0	1	1	11	79
mPe-07	1	1	1	0	1	0	0	1	0	0	1	1	1	1	9	64
mPe-08	1	1	1	0	1	1	0	1	1	0	1	0	1	1	10	71
mPe-09	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPe-10	1	1	1	1	1	1	0	1	1	1	1	1	0	1	12	86
mPe-11	1	1	1	1	1	0	0	1	0	1	1	1	0	1	10	71
mPe-12	1	0	1	1	0	1	0	1	0	0	0	1	0	1	7	50
mPe-13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100

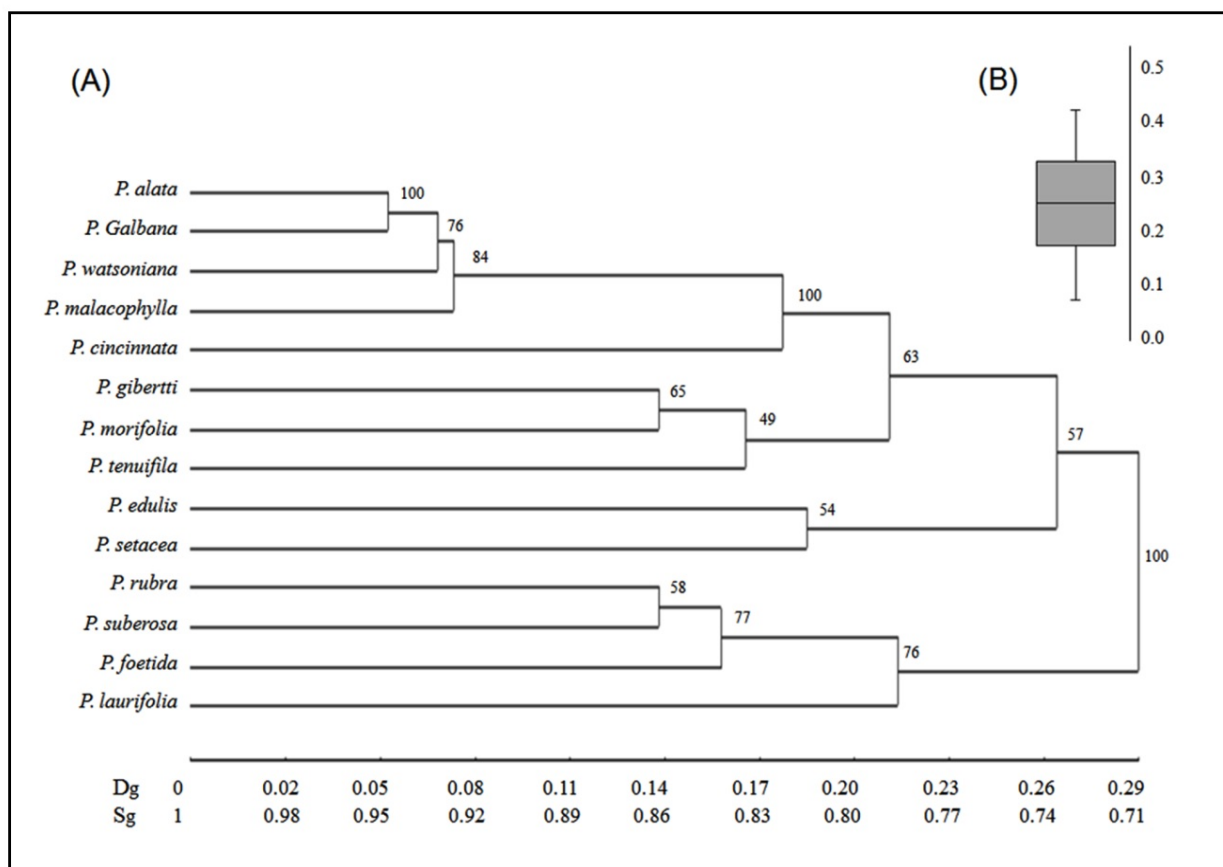
mPe-14	0	1	1	1	0	0	0	1	0	0	1	1	0	1	7	50
mPe-15	1	1	1	1	1	0	1	1	0	0	1	1	1	1	11	79
mPe-16	1	0	1	1	1	1	0	1	1	1	0	1	1	1	11	79
mPe-17	1	0	1	1	1	1	0	1	0	1	0	1	1	1	10	71
Pe-07	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
Pe-08	1	1	1	0	1	0	0	1	0	0	1	0	1	1	8	57
Pe-10	1	1	1	1	1	1	1	1	1	1	0	1	1	1	13	93
Pe-11	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	7
Pe-15	1	1	1	1	1	1	0	1	1	1	1	0	1	1	12	86
Pe-16	1	1	1	1	1	1	0	1	0	1	0	1	0	1	10	71
Pe-17	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	7
Pe-18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
Pe-19	1	1	1	1	1	1	0	1	1	0	1	1	1	1	12	86
Pe-20	1	1	1	1	1	1	0	1	1	1	1	0	1	1	12	86
Pe-23	1	0	1	0	1	0	0	1	0	0	0	0	0	1	5	36
Pe-24	1	1	1	0	1	1	0	1	0	0	1	0	1	1	9	64
Pe-28	1	0	1	0	1	1	0	1	0	0	0	0	1	1	7	50
Pe-42	1	1	1	1	1	1	1	1	1	1	1	1	0	1	13	93
Pe-58	1	1	1	1	1	0	0	1	0	0	1	0	0	1	8	57
mPs-01	1	1	1	1	1	1	0	1	1	1	1	1	1	1	13	93
mPs-02	1	1	1	1	1	1	1	1	0	0	1	0	1	1	11	79
mPs-03	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-04	1	1	1	0	1	1	1	1	1	0	1	0	1	1	11	79
mPs-05	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-06	0	1	1	0	0	0	0	0	0	0	1	0	0	0	3	21
mPs-07	1	0	0	1	1	1	0	1	0	0	1	0	0	1	7	50
mPs-08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-09	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-10	1	1	1	1	1	0	0	1	0	0	1	0	0	0	7	50
mPs-11	1	0	1	0	1	1	0	1	0	0	1	0	1	1	8	57
mPs-12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-13	0	0	1	0	0	0	0	1	0	0	1	1	1	0	5	36
mPs-14	1	1	1	0	0	0	0	1	1	1	1	0	0	1	8	57
mPs-15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-16	1	1	1	0	1	1	1	0	1	0	1	1	1	0	10	71
mPs-17	1	1	1	1	1	1	1	1	1	1	1	1	0	1	13	93
mPs-18	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	7
mPs-19	1	1	1	1	1	1	0	1	1	1	1	0	1	1	12	86
mPs-20	1	1	1	1	1	1	0	1	1	1	1	1	1	1	13	93
mPs-21	0	0	0	0	1	0	0	1	0	0	1	0	0	0	3	21
mPs-22	1	0	0	0	1	1	0	1	1	0	1	0	1	1	8	57
mPs-23	1	1	1	0	1	1	0	1	1	0	1	0	1	1	10	71
mPs-24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-25	1	1	1	1	0	1	0	1	0	0	1	0	1	0	8	57
mPs-26	1	1	1	0	1	1	0	1	1	0	1	0	1	1	10	71
mPs-27	1	1	1	1	1	1	0	1	1	0	1	1	1	1	12	86

mPs-28	1	1	1	0	1	0	0	1	0	0	1	0	0	1	7	50
mPs-29	1	0	0	0	1	0	0	1	0	0	1	0	1	1	6	43
mPs-30	1	0	0	1	1	0	1	1	1	0	1	1	0	1	9	64
mPs-31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-32	1	1	1	1	1	1	0	1	0	1	1	1	0	1	11	79
mPs-33	1	1	1	1	1	1	1	1	1	0	1	0	1	1	12	86
mPs-34	0	1	1	0	0	0	0	0	0	0	1	0	0	0	3	21
mPs-35	1	1	1	1	1	1	0	1	1	1	1	1	1	1	13	93
mPs-36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-37	1	1	1	1	1	1	1	1	1	0	1	1	1	1	13	93
mPs-38	1	1	1	1	1	0	1	1	1	1	1	1	1	1	13	93
mPs-39	0	0	1	0	1	0	0	1	0	1	1	1	0	1	7	50
mPs-40	0	1	1	0	0	0	0	1	0	0	1	0	0	0	4	29
mPs-41	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-42	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-43	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-44	0	1	1	0	1	0	0	0	1	0	1	0	0	1	6	43
mPs-45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-46	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-49	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14	100
mPs-50	1	1	1	1	1	1	0	1	1	1	1	1	1	1	13	93
mPs-51	1	1	1	1	1	0	1	1	1	1	1	0	1	1	12	86
mPs-52	1	0	1	1	1	0	0	1	0	0	1	0	1	1	8	57

^aSSR loci previously characterized by Cerqueira-Silva et al. [46], identified as mPc-01 to -32; Cerqueira-Silva et al. [49], identified as mPe-01 to -17 and mPs-01 to -52; and Oliveira [53], identified as Pe-7, 8, 10, 11, 15, 16, 17, 18, 19, 20, 23, 24, 28, 42, 58.;

^b*Passiflora alata* (Pa), *P. cincinnata* (Pc), *P. edulis* (Pe), *P. foetida* (Pf), *P. galbana* (Pga), *P. gibertii* (Pgi), *P. laurifolia* (Pl), *P. malacophylla* (Pma), *P. morifolia* (Pmo), *P. rubra* (Pr), *P. setacea* (Pse), *P. suberosa* (Psu), *P. tenuiflora* (Pt) and *P. watsoniana* (Pw).

SM 2. Dendrogram constructed with UPGMA method using the distance matrix (A) and descriptive presentation of the average and standard deviations (in box-plot graphic) of the estimated distance to the 14 species from the passion fruit complement coefficient of simple coincidence (B). Presents appropriate values of cophenetic correlation ($CCC = 0.77$), stress ($S = 19$), distortion ($D = 3.9$) and consistency (average of 74%) after bootstrap with 1.000 resampling analysis.



SM 3 Characteristics of the microsatellite markers used in the genotyping of 24 passion fruit (*Passiflora cincinnata* Mast) accessions from the germplasm bank of Embrapa Cassava & Fruits.

Markers ^a	Na	H _o	H _E	F
mPc-Unicamp01	3	0.32	0.50	0.35
mPc-Unicamp02	2	0.01	0.01	-0.01
mPc-Unicamp03	6	0.20	0.26	0.23
mPc-Unicamp04	4	0.48	0.58	0.18
mPc-Unicamp05	5	0.71	0.60	-0.20
mPc-Unicamp06	3	0.21	0.28	0.26
mPc-Unicamp07	3	0.39	0.43	0.10
mPe-Unicamp06	5	0.51	0.56	0.10
mPe-Unicamp09	2	0.13	0.13	-0.07
mPe-Unicamp13	3	0.34	0.29	-0.20
Pe-07	2	0.01	0.06	1.00
Pe-08	3	0.49	0.38	-0.30
Pe-15	2	0.58	0.41	-0.41
Pe-18	15	0.59	0.89	0.34
Pe-19	5	0.25	0.63	0.60
mPs-Unicamp05	11	0.59	0.61	0.03
mPs-Unicamp10	11	0.59	0.87	0.32
mPs-Unicamp16	8	0.52	0.78	0.34
mPs-Unicamp24	4	0.13	0.57	0.78
mPs-Unicamp31	16	0.92	0.90	-0.03
mPs-Unicamp46	16	0.63	0.87	0.28
mPs-Unicamp51	8	0.60	0.72	0.17
Average	6	0.42	0.52	0.18
Confidence interval*	4.5-7.9	0.32-0.49	0.40-0.61	0.04-0.29

^aSSR loci previously characterized by Cerqueira-Silva et al. [46], identified as mPc-UNICAMP; Cerqueira-Silva et al. [49], identified as mPe-UNICAMP and mPs-UNICAMP; and Oliveira [53], identified as Pe-.

*Confidence interval estimated by bootstrap (100,000 simulations).

SM 4 Characteristics of the microsatellite markers used in the genotyping of 85 passion fruit (*Passiflora edulis* Sims) accessions from the germplasm bank of Embrapa Cassava & Fruits and from collection of Universidade Estadual do Sudoeste da Bahia (UESB, Itapetinga).

Markers ^a	Na	H _o	H _E	F
mPe-Unicamp01	7	0.59	0.72	0.17
mPe-Unicamp02	7	0.58	0.72	0.20
Pe-07	4	0.77	0.67	-0.16
Pe-08	2	0.16	0.15	-0.04
Pe-15	3	0.25	0.36	0.31
Pe-16	4	0.48	0.53	0.09
Pe-17	2	0.01	0.01	0.00
Pe-18	2	0.13	0.12	-0.07
Pe-19	3	0.03	0.04	0.18
Pe-20	7	0.43	0.60	0.29
Pe-23	10	0.64	0.71	0.09
Pe-24	7	0.47	0.68	0.31
Pe-42	10	0.49	0.79	0.38
Pe-58	18	0.76	0.86	0.12
mPc-Unicamp11	6	0.52	0.58	0.10
mPs-Unicamp01	5	0.55	0.53	-0.02
mPs-Unicamp02	4	0.40	0.48	0.17
mPs-Unicamp05	5	0.12	0.18	0.31
mPs-Unicamp06	9	0.63	0.80	0.21
mPs-Unicamp09	9	0.68	0.67	-0.01
mPs-Unicamp10	4	0.45	0.44	-0.02
mPs-Unicamp15	3	0.05	0.07	0.21
mPs-Unicamp16	10	0.65	0.71	0.08
Average	6	0.43	0.50	0.13
Confidence interval*	4.7-7.4	0.33-0.51	0.38-0.59	0.07-0.17

^aSSR loci previously characterized by Cerqueira-Silva et al. [46], identified as mPc-UNICAMP; Cerqueira-Silva et al. [49], identified as mPe-UNICAMP and mPs-UNICAMP; and Oliveira [53], identified as Pe-.

*Confidence interval estimated by bootstrap (100,000 simulations).

SM 5 Characteristics of the microsatellite markers used in the genotyping of seven passion fruit (*Passiflora setacea* DC) accessions from the germplasm bank of Embrapa Cassava & the Fruits and from collection of Universidade Estadual do Sudoeste da Bahia (UESB, Itapetinga).

Markers ^a	Na	H _o	H _E	F
mPs-Unicamp01	2	0.15	0.32	0.54
mPs-Unicamp02	2	0.28	0.50	0.44
mPs-Unicamp03	2	0.27	0.49	0.45
mPs-Unicamp04	2	0.14	0.33	0.58
mPs-Unicamp05	2	0.05	0.30	0.82
mPs-Unicamp07	3	0.14	0.32	0.56
mPs-Unicamp08	2	0.06	0.06	-0.03
mPs-Unicamp09	6	0.50	0.59	0.15
mPs-Unicamp10	2	0.82	0.50	-0.65
mPs-Unicamp11	5	0.15	0.48	0.70
mPs-Unicamp12	4	0.16	0.38	0.58
mPs-Unicamp13	3	0.52	0.60	0.13
mPs-Unicamp14	3	0.12	0.28	0.57
mPs-Unicamp15	4	0.76	0.67	-0.13
mPs-Unicamp16	2	0.12	0.11	-0.06
mPs-Unicamp17	2	0.10	0.13	0.23
mPs-Unicamp18	2	0.08	0.08	-0.04
mPs-Unicamp19	3	0.06	0.46	0.87
mPe-Unicamp02	4	0.58	0.60	0.03
Pe-07	2	0.04	0.04	-0.02
Pe-08	2	0.04	0.25	0.83
Pe-15	2	0.02	0.02	-0.01
Pe-18	6	0.48	0.69	0.31
Pe-19	5	0.20	0.28	0.30
mPc-Unicamp03	5	0.55	0.48	-0.15
Average	3	0.26	0.36	0.28
Confidence interval*	2.6-3.5	0.17-0.34	0.28-0.43	0.13-0.40

^aSSR loci previously characterized by Cerqueira-Silva et al. [46], identified as mPc-UNICAMP; Cerqueira-Silva et al. [49], identified as mPe-UNICAMP and mPs-UNICAMP; and Oliveira [53], identified as Pe-.

*Confidence interval estimated by bootstrap (100,000 simulations).

SM 6 Descriptive list of the number of accessions and plants recommended for constructing a core collection for three species of passion fruit

Species	Total Accessions	Total Plants	List of Accessions ^a (Number of Plants)
<i>P. cincinnata</i> Mast	18	25	BGP002, 016, 077, 200, 268, 269, 273, 274 ^b , 280, 289, 290, 296, 297 ^b , 299, 303 ^b , 312 ^b , 323, Pc-Anagé ^b .
<i>P. edulis</i> Sims	23	26	BGP007, 018, 020, 037, 038, 041, 043, 071 ^b , 079, 104, 116, 124, 129, 165, 168, 169, 203, 208, 215 ^b , 223, 234, 341, HFOP09.
<i>P. setacea</i> DC	4	9	BGP238, 245, 272 ^b , Ps-VCA ^b .

^aA complete description of the accessions is presented in SM 7 of the supplementary material;

^bAccessions with more than one plant selected for the construction of the core collection.

SM 7 List of the wild and commercial accessions of passion fruit (*Passiflora* spp.) used for this study.

Code	Species	Variety	City, State	Country	Germplasm Bank ^a
BGP002	<i>P. cincinnata</i> Mast	Does not apply	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP007	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP009	<i>P. edulis</i> Sims	Purple	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP016	<i>P. cincinnata</i> Mast	Does not apply	Maceió, Alagoas	Brazil	EMBRAPA
BGP017	<i>P. edulis</i> Sims	Yellow	Andradina, São Paulo	Brazil	EMBRAPA
BGP018	<i>P. edulis</i> Sims	Yellow	Araguari, Minas Gerais	Brazil	EMBRAPA
BGP020	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP022	<i>P. edulis</i> Sims	Purple	Cruz das Almas, Bahia	Brazil	EMBRAPA
BGP023	<i>P. edulis</i> Sims	Purple	Mogi-Guaçu, São Paulo	Brazil	EMBRAPA
BGP025	<i>P. edulis</i> Sims	Purple	Mogi-Guaçu, São Paulo	Brazil	EMBRAPA
BGP028	<i>P. edulis</i> Sims	Yellow	Nova Soure, Bahia	Brazil	EMBRAPA
BGP029	<i>P. edulis</i> Sims	Purple	Urussanga, Santa Catarina	Brazil	EMBRAPA
BGP031	<i>P. edulis</i> Sims	Yellow	Vitória da Conquista, Bahia	Brazil	EMBRAPA
BGP033	<i>P. edulis</i> Sims	Purple	Maringá, Paraná	Brazil	EMBRAPA
BGP034	<i>P. edulis</i> Sims	Yellow	Maringá, Paraná	Brazil	EMBRAPA
BGP037	<i>P. edulis</i> Sims	Yellow	Santarém, Pará	Brazil	EMBRAPA
BGP038	<i>P. edulis</i> Sims	Yellow	Santarém, Pará	Brazil	EMBRAPA
BGP041	<i>P. edulis</i> Sims	Yellow	Monte Alegre do Alto, São Paulo	Brazil	EMBRAPA
BGP043	<i>P. edulis</i> Sims	Purple	Funchal, Ilha da Madeira	Portugal	EMBRAPA
BGP044	<i>P. edulis</i> Sims	Purple	Unavailable	Brazil	EMBRAPA
BGP048	<i>P. edulis</i> Sims	Yellow	Caracas	Venezuela	EMBRAPA
BGP051	<i>P. edulis</i> Sims	Yellow	Janauba, Minas Gerais	Brazil	EMBRAPA
BGP061	<i>P. edulis</i> Sims	Purple	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP064	<i>P. edulis</i> Sims	Yellow	Conceição do Jacuípe, Bahia	Brazil	EMBRAPA
BGP071	<i>P. edulis</i> Sims	Yellow	Presidente Tancredo Neves, Bahia	Brazil	EMBRAPA
BGP072	<i>P. edulis</i> Sims	Purple	Presidente Tancredo Neves, Bahia	Brazil	EMBRAPA
BGP077	<i>P. cincinnata</i> Mast	Does not apply	Andaraí, Bahia	Brazil	EMBRAPA
BGP078	<i>P. edulis</i> Sims	Yellow	Andaraí, Bahia	Brazil	EMBRAPA
BGP079	<i>P. edulis</i> Sims	Yellow	Andaraí, Bahia	Brazil	EMBRAPA
BGP089	<i>P. edulis</i> Sims	Purple	Ibicoara, Bahia	Brazil	EMBRAPA
BGP092	<i>P. edulis</i> Sims	Purple	Livramento Nossa Senhora, Bahia	Brazil	EMBRAPA
BGP094	<i>P. edulis</i> Sims	Yellow	Livramento Nossa Senhora, Bahia	Brazil	EMBRAPA
BGP104	<i>P. edulis</i> Sims	Purple	São Paulo	Brazil	EMBRAPA
BGP105	<i>P. tenuifolia</i> Killip	Does not apply	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP107	<i>P. morifolia</i> Mast	Does not apply	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP109	<i>P. galbana</i> Mast	Does not apply	Unavailable	Brazil	EMBRAPA
BGP116	<i>P. edulis</i> Sims	Purple	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP117	<i>P. rubra</i> L	Does not apply	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP121	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP123	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP124	<i>P. edulis</i> Sims	Yellow	Cruz das Almas, Bahia	Brazil	EMBRAPA
BGP129	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP143	<i>P. suberosa</i> L	Does not apply	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP153	<i>P. foetida</i> L	Does not apply	Nova Odessa, São Paulo	Brazil	EMBRAPA

BGP158	<i>P. edulis</i> Sims	Purple	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP164	<i>P. edulis</i> Sims	Yellow	Alagoinhas, Bahia	Brazil	EMBRAPA
BGP165	<i>P. edulis</i> Sims	Yellow	Alagoinhas, Bahia	Brazil	EMBRAPA
BGP168	<i>P. edulis</i> Sims	Purple	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP169	<i>P. edulis</i> Sims	Purple	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP170	<i>P. malacophylla</i> Mast	Does not apply	Unavailable	Brazil	EMBRAPA
BGP176	<i>P. edulis</i> Sims	Yellow	Cruz das Almas, Bahia	Brazil	EMBRAPA
BGP177	<i>P. edulis</i> Sims	Yellow	Alcobaça, Bahia	Brazil	EMBRAPA
BGP179	<i>P. edulis</i> Sims	Yellow	Rio do Sul, Santa Catarina	Brazil	EMBRAPA
BGP180	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP181	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP182	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP183	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP184	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP185	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP186	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP187	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP188	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP189	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP198	<i>P. gibertii</i> N.E.Br	Does not apply	Unavailable	Brazil	EMBRAPA
BGP200	<i>P. cincinnata</i> Mast	Does not apply	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP203	<i>P. edulis</i> Sims	Yellow	Unavailable	Brazil	EMBRAPA
BGP205	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP208	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP210	<i>P. edulis</i> Sims	Yellow	Araguari, Minas Gerais	Brazil	EMBRAPA
BGP215	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP221	<i>P. edulis</i> Sims	Yellow	Salvador, Bahia	Brazil	EMBRAPA
BGP222	<i>P. edulis</i> Sims	Yellow	Salvador, Bahia	Brazil	EMBRAPA
BGP223	<i>P. edulis</i> Sims	Yellow	Salvador, Bahia	Brazil	EMBRAPA
BGP224	<i>P. edulis</i> Sims	Yellow	Salvador, Bahia	Brazil	EMBRAPA
BGP225	<i>P. edulis</i> Sims	Yellow	Salvador, Bahia	Brazil	EMBRAPA
BGP227	<i>P. edulis</i> Sims	Yellow	Campinas, São Paulo	Brazil	EMBRAPA
BGP228	<i>P. edulis</i> Sims	Yellow	Campinas, São Paulo	Brazil	EMBRAPA
BGP234	<i>P. edulis</i> Sims	Yellow	Morro de São Paulo / Tinharé, Bahia	Brazil	EMBRAPA
BGP235	<i>P. alata</i> Curtis	Does not apply	Brasília, Distrito Federal	Brazil	EMBRAPA
BGP237	<i>P. setacea</i> DC	Does not apply	Lagedinho, Bahia	Brazil	EMBRAPA
BGP238	<i>P. setacea</i> DC	Does not apply	Lagedinho, Bahia	Brazil	EMBRAPA
BGP240	<i>P. setacea</i> DC	Does not apply	Ibiquera, Bahia	Brazil	EMBRAPA
BGP242	<i>P. setacea</i> DC	Does not apply	Ibiquera, Bahia	Brazil	EMBRAPA
BGP245	<i>P. setacea</i> DC	Does not apply	Lagedinho, Bahia	Brazil	EMBRAPA
BGP268	<i>P. cincinnata</i> Mast	Does not apply	Ruy Barbosa, Bahia	Brazil	EMBRAPA
BGP269	<i>P. cincinnata</i> Mast	Does not apply	Ruy Barbosa, Bahia	Brazil	EMBRAPA
BGP272	<i>P. setacea</i> DC	Does not apply	Ruy Barbosa, Bahia	Brazil	EMBRAPA
BGP273	<i>P. cincinnata</i> Mast	Does not apply	Ruy Barbosa/Utinga, Bahia	Brazil	EMBRAPA
BGP274	<i>P. cincinnata</i> Mast	Does not apply	Ruy Barbosa/Utinga, Bahia	Brazil	EMBRAPA
BGP275	<i>P. cincinnata</i> Mast	Does not apply	Ruy Barbosa/Utinga, Bahia	Brazil	EMBRAPA

BGP277	<i>P. edulis</i> Sims	Yellow	Ruy Barbosa/Utinga, Bahia	Brazil	EMBRAPA
BGP280	<i>P. cincinnata</i> Mast	Does not apply	Rui Barbosa/Utinga, Bahia	Brazil	EMBRAPA
BGP289	<i>P. cincinnata</i> Mast	Does not apply	Seabra/Ibotirama, Bahia	Brazil	EMBRAPA
BGP290	<i>P. cincinnata</i> Mast	Does not apply	Brotas de Macaúbas, Bahia	Brazil	EMBRAPA
BGP292	<i>P. cincinnata</i> Mast	Does not apply	Brotas de Macaúbas, Bahia	Brazil	EMBRAPA
BGP294	<i>P. cincinnata</i> Mast	Does not apply	Brotas de Macaúbas, Bahia	Brazil	EMBRAPA
BGP296	<i>P. cincinnata</i> Mast	Does not apply	Araci, Bahia	Brazil	EMBRAPA
BGP297	<i>P. cincinnata</i> Mast	Does not apply	Bahia	Brazil	EMBRAPA
BGP299	<i>P. cincinnata</i> Mast	Does not apply	Curralinho, Bahia	Brazil	EMBRAPA
BGP303	<i>P. cincinnata</i> Mast	Does not apply	Curralinho, Bahia	Brazil	EMBRAPA
BGP308	<i>P. cincinnata</i> Mast	Does not apply	Ibiquera, Bahia	Brazil	EMBRAPA
BGP311	<i>P. edulis</i> Sims	Yellow	Nova Viçosa, Bahia	Brazil	EMBRAPA
BGP312	<i>P. cincinnata</i> Mast	Does not apply	Teixeira, Bahia	Brazil	EMBRAPA
BGP322	<i>P. cincinnata</i> Mast	Does not apply	Rafael Jambeiro, Bahia	Brazil	EMBRAPA
BGP323	<i>P. cincinnata</i> Mast	Does not apply	Rafael Jambeiro, Bahia	Brazil	EMBRAPA
BGP324	<i>P. edulis</i> Sims	Yellow	Planaltina, Distrito Federal	Brazil	EMBRAPA
BGP325	<i>P. edulis</i> Sims	Yellow	Planaltina, Distrito Federal	Brazil	EMBRAPA
BGP327	<i>P. edulis</i> Sims	Yellow	Jarinu, São Paulo	Brazil	EMBRAPA
BGP328	<i>P. edulis</i> Sims	Yellow	Planaltina, Distrito Federal	Brazil	EMBRAPA
BGP334	<i>P. edulis</i> Sims	Yellow	Santo Antônio de Jesus, Bahia	Brazil	EMBRAPA
BGP336	<i>P. edulis</i> Sims	Yellow	Itamarajú, Bahia	Brazil	EMBRAPA
BGP340	<i>P. edulis</i> Sims	Yellow	Rio Real, Bahia	Brazil	EMBRAPA
BGP341	<i>P. edulis</i> Sims	Yellow	Itamarajú, Bahia	Brazil	EMBRAPA
BGP342	<i>P. cincinnata</i> Mast	Does not apply	Rio Real, Bahia	Brazil	EMBRAPA
BGP343	<i>P. edulis</i> Sims	Purple	Cruz das Almas, Bahia	Brazil	EMBRAPA
BGP354	<i>P. edulis</i> Sims	Yellow	Itiúba, Bahia	Brazil	EMBRAPA
BGP407	<i>P. laurifolia</i> L	Does not apply	Unavailable	Brazil	EMBRAPA
BGP412	<i>P. watsoniana</i> Mast	Does not apply	Unavailable	Brazil	EMBRAPA
BRS Gigante Amarelo	<i>P. edulis</i> Sims	Does not apply	CPAC/EMBRAPA	Brazil	EMBRAPA
HFOP05	<i>P. edulis</i> Sims	Does not apply	CPAC/EMBRAPA	Brazil	EMBRAPA
HFOP09	<i>P. edulis</i> Sims	Does not apply	CPAC/EMBRAPA	Brazil	EMBRAPA
BRS Ouro Vermelho	<i>P. edulis</i> Sims	Does not apply	CPAC/EMBRAPA	Brazil	EMBRAPA
Pc-Anagé01	<i>P. cincinnata</i> Mast	Does not apply	Brasil	Brazil	UESB
Pe-Anagé01	<i>P. edulis</i> Sims	Does not apply	Anagé, Bahia	Brazil	UESB
Ps-VCA01	<i>P. setacea</i> DC	Does not apply	Vitoria da Conquista, Bahia	Brazil	UESB
BRS Rubi do Cerrado	<i>P. edulis</i> Sims	Does not apply	CPAC/EMBRAPA	Brazil	EMBRAPA
BRS Sol do Cerrado	<i>P. edulis</i> Sims	Does not apply	CPAC/EMBRAPA	Brazil	EMBRAPA

Characterization and selection of passion fruit (yellow and purple) accessions based on molecular markers and disease reactions for use in breeding programs

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Abstract *Passiflora edulis* Sims, which is native to South America, stands out as a passion fruit species with major potential for fruit production and marketing. This species is popularly known as yellow or purple passion fruit, depending on the color of the fruits produced. Brazil is the major worldwide producer of passion fruit; however, the average productivity of the country is low compared with its potential for culture. Fungal, bacterial and viral pathogens are among the factors limiting the productivity of passion fruit. Furthermore, no existing

cultivars exhibit both productivity and resistance to disease. To select genetic material that will be useful for core collections and for increasing the genetic resistance of passion fruit cultivars to pathogens, we characterized 36 accessions based on 23 microsatellite loci and six variables related to the reactions to three diseases (woodiness virus, scab and anthracnose). We identified 127 alleles (an average of 5.52 alleles per locus), 30 % of which were private for yellow or purple passion fruit accessions. Analysis of variance and mean comparison tests indicated differences in five of the six variables ($p < 0.05$, Scott-Knott test). Differences between the average reactions of the yellow and purple passion fruit accessions were also observed for the symptoms of woodiness virus and anthracnose ($p < 0.05$, Mann–Whitney test). Together with these results, molecular and phenotypic estimates allowed the identification of groups of preferential accessions for use in breeding programs, for example, accessions BGP029, 071, 168, 205 and 277.

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Keywords Germplasm bank · Microsatellite marker · *Passiflora* · Pre-breeding · Resistance

Introduction

Passion fruit (*Passiflora* spp.) is grown in different parts of the world, primarily in tropical regions, with Brazil, Colombia, Peru and Ecuador representing the

major producers of these fruits (Borges et al. 2005). Although Brazilian production has varied, it has generally increased in recent decades, with an average production of 676,000 tons being recorded during the last 10 years (IBGE 2012). However, despite dominating global passion fruit production, Brazilian productivity has remained stable (approximately $14 \text{ t ha}^{-1} \text{ year}^{-1}$) and well below this crop's productivity potential, which is estimated at approximately $50 \text{ t ha}^{-1} \text{ year}^{-1}$ (Meletti et al. 2005).

Different factors contribute to the low productivity of passion fruit in Brazil, such as the occurrence of fungal, bacterial and viral pathogens. Records in the literature indicate the occurrence of numerous pathogens associated with passion fruit crops. Discussions of advances and perspectives related to the primary diseases that affect passion fruit cultivation (such as fusariosis, scab, anthracnose, bacteriosis, and woodiness virus) are also available in the literature (Cerqueira-Silva et al. 2014a; Silva et al. 2013; Faleiro et al. 2011; Meletti et al. 2005; Faleiro et al. 2005).

Recent and ongoing research has aimed to identify or to generate genotypes and hybrids with greater resistance to the diseases that affect passion fruit. To identify sources of resistance to diseases, we highlight studies addressing the characterization of passion fruit varieties and cultivars (Batistti et al. 2013; Fischer et al. 2010; Junqueira et al. 2003), as well as accessions maintained in collections and germplasm banks (Oliveira et al. 2013; Silva et al. 2013; Cerqueira-Silva et al. 2008; Junqueira et al. 2006). In general, the results presented in the published literature vary, indicating the differing susceptibility of the evaluated cultivars and the broad genetic variability of the specimens in germplasm banks, for both *P. edulis* and wild passion fruit species.

Various investigators have indicated a need for research that contributes to advancing genetic breeding programs for passion fruit and to releasing cultivars that combine resistance characteristics (Faleiro et al. 2011; Meletti et al. 2005; Faleiro et al. 2005; Junqueira et al. 2003). Promising results associated with identifying potential sources of resistance have been observed in some accessions of *P. edulis* for resistance to bacteriosis and anthracnose; in accessions of wild species such as *P. actinia*, *P. setacea* and *P. coccinea* for resistance to virosis; in *P. odontophylla*, *P. gibertii*, *P. caerulea*, *P. actinia*, and *P. mucronata* for resistance to bacteriosis; and in *P. serrato-digitata*, *P. gibertii*, *P.*

coccinea, *P. actinia*, *P. setacea*, *P. nitida*, and *P. caerulea* for resistance to anthracnose (Oliveira et al. 2013; Faleiro and Junqueira 2009; Faleiro et al. 2005). However, basic information associated with agronomic evaluations and the available estimates of genetic variability remain limited for many of these materials. Therefore, pre-breeding activities, such as the prospecting and characterization of accessions, are required to maintain the variability in collections, and germplasm banks could be effectively used as a genetic resource allowing expansion of the genetic basis of passion fruit breeding programs (Faleiro et al. 2011; Meletti et al. 2005; Faleiro et al. 2005).

Costs are generally high for research activities specifically associated with pre-breeding (such as performing agronomic characterizations, determining diversity estimates and identifying preferred crossings), and the time required to obtain results is considerable (Nass 2011; Nass and Paterniani 2000). The use of molecular markers is fundamentally important for enhancing the amount of information generated and for reducing the time required to obtain results (Ferreira and Rangel 2011). In this context, we note a considerable increase in research devoted to obtaining estimates of genetic variability using molecular markers in both commercial (Ortiz et al. 2012; Costa et al. 2012; Cerqueira-Silva et al. 2010a; Bellon et al. 2007; Viana et al. 2003) and wild passion fruit species (Cerqueira-silva et al. 2010b, c, 2012a; Junqueira et al. 2007). These studies have identified low variability among commercial accessions of *P. edulis* and generally wide variability among wild accessions of passion fruit, reinforcing the potential for the use of these materials as a resource in breeding programs.

Significant advances in research devoted to constructing genetic maps and identifying regions associated with resistance control, at least for *Xanthomonas axonopodis* pv. *passiflorae* yellow passion fruit, have occurred since 2002 (Lopes et al. 2006; Carneiro et al. 2002). In this context, with the addition of a new set of microsatellite markers, the first integrated map of passion fruit was published for *P. edulis* (Oliveira et al. 2008), providing the ability to use this information for identifying quantitative trait loci (QTLs). The construction of a genetic map using multiple markers, such as amplified fragment length polymorphisms (AFLPs), microsatellite-AFLPs (M-AFLPs), simple sequence repeats (SSRs), resistance gene analogs (RGAs) and

single nucleotide polymorphisms (SNPs), has also been reported for *P. alata* (Pereira et al. 2013). In addition, recent efforts associated with developing and characterizing bacterial artificial chromosomes (BACs) have led to the construction of physical maps for passion fruit (Santos 2013).

Although recent, the inclusion of molecular markers in breeding programs for passion fruit is already occurring at different stages (Faleiro et al. 2012). Molecular markers are currently being employed for purposes such as identifying ornamental hybrids and improving fruit production, using both dominant and co-dominant markers (Santos et al. 2012; Conceição et al. 2011; Junqueira et al. 2008). Molecular markers have also contributed to the monitoring of genetic variability in segregant populations subjected to cycle selection (Reis et al. 2012, 2011; Costa et al. 2012) and backcrossing (Fonseca et al. 2009). These referenced works, together with the growing number of results concerning the identification and characterization of microsatellite markers in passion fruit species (Cerqueira-Silva et al. 2014b; Penha et al. 2013; Cazé et al. 2012; Cerqueira-Silva et al. 2012b; Padua et al. 2005; Oliveira et al. 2005) should effectively contribute to advancing breeding programs and to proposing strategies for managing and conserving passion fruit.

Considering the need for research that contributes to the genetic improvement of passion fruit cultivars, thereby increasing resistance to major diseases affecting the fruits, in the present study, we characterized *P. edulis* (yellow and purple shell) accessions based on molecular markers and their reactions to diseases to select genetic material that will be useful for establishing core collections and working populations dedicated to understanding and increasing genetic resistance in passion fruit cultivars.

Materials and methods

Biological material

Thirty-six accessions were used in this study, one of which was a cultivated passion fruit variety (BRS Gigante Amarelo). Most of the accessions (34) originated from 18 cities distributed across five Brazilian states, and the remaining two accessions originated from Venezuela and Portugal (Fig. 1) (Supplementary Material—SM 1). The accessions encompassed

popularly known variations, such as purple and yellow passion fruits, both of which are found in *P. edulis* Sims (Bernacci et al. 2008). All the accessions belong to the Active Germplasm Bank for *Passiflora* of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) Cassava and Fruits (CNPMP/EMBRAPA), Cruz das Almas, Bahia, Brazil (12°48'38"S and 39°06'26"W).

Characterization of microsatellite locus polymorphisms

Genomic DNA was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990) and stored in duplicate samples in an ultra-cold freezer at the Laboratory of Applied Molecular Genetics of the Universidade Estadual do Sudoeste da Bahia (UESB), Itapetinga, Bahia, and at the Laboratory of Molecular Genetic Analysis of the Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo. Every accession was represented by three plants (grown from seeds of fruits obtained through controlled pollination). DNA sample quality was assessed via electrophoresis (90 V electric current for 90 min) in 1 % (m v⁻¹) agarose gels and staining with ethidium bromide. DNA concentrations (ng μL⁻¹) were estimated based on comparisons with known quantities of lambda phage DNA (undigested lambda DNA).

A set of 23 SSR loci was used, 11 of which came from the series *mPc-UNICAMP*, *mPe-UNICAMP* and *mPs-UNICAMP* (Cerqueira-Silva et al. 2012b, 2014b) and 12 from the series *USP* (Oliveira 2006). Polymerase chain reaction (PCR) assays to obtain diversity estimates were conducted in a final volume of 15 μL, containing 12 ng of template DNA, 0.4 mM each primer (reverse and forward) and 1.0 U of Taq DNA polymerase (Invitrogen Co, Carlsbad, CA, USA). For other reagents (dNTP concentrations, MgCl₂ and buffers) and amplification conditions (annealing temperatures and characteristics of PCR cycling), the exact procedures described in the original articles addressing the identification and characterization of the loci were used.

The products were initially visualized through horizontal electrophoresis in 3 % (m/v) agarose gels run in 0.5× TBE and stained with ethidium bromide to confirm amplification and subsequently through vertical electrophoresis in denaturing 6 % (m v⁻¹) polyacrylamide gels run in 1× TBE and stained with silver

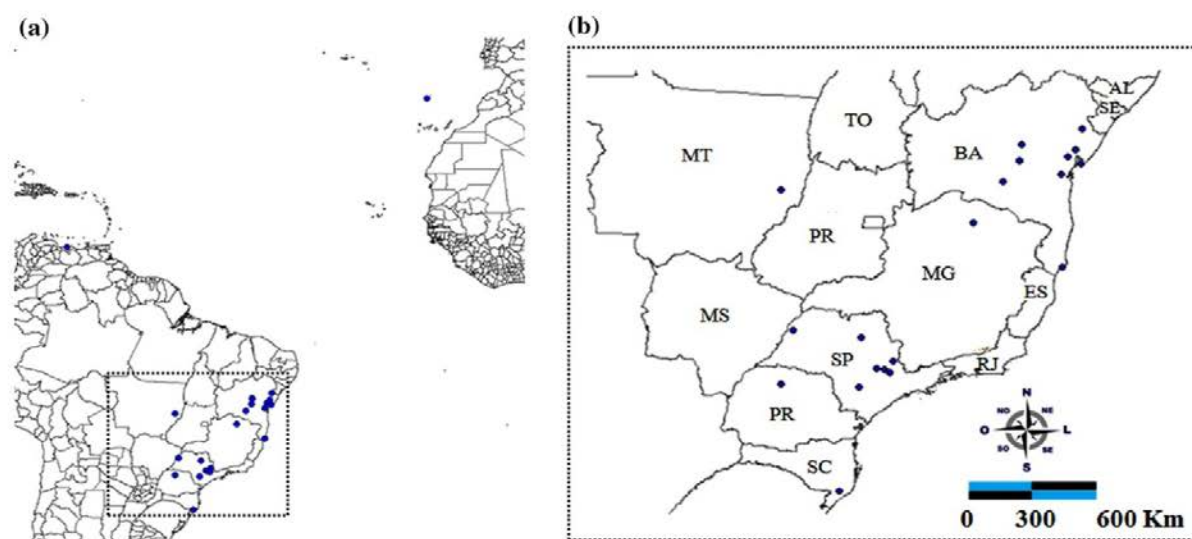


Fig. 1 Geographical areas where passion fruit (*Passiflora edulis* Sims) accessions were collected (a), highlighting areas of the Brazilian territory where most of the collections were performed (b). The blue dots correspond to sampled areas. (Color figure online)

nitrate to characterize the amplification profiles. The sizes of the products were determined using a 10-bp DNA ladder (Invitrogen), and the repeatability of the electrophoretic profiles was evaluated by repeating the vertical electrophoresis runs for a minimum of 10 % of the samples considered.

Characterization of the disease reactions of the passion fruit accessions

Experimental field data were collected as part of the characterization activities (and subsequent stages of the pre-breeding program) conducted on the *Passiflora* Germplasm Bank of EMBRAPA Cassava and Fruits from April to July in 2009, which are the months with the highest natural incidence of disease. Artificial inoculations had not been previously performed, and a randomized block design was adopted, with four repeats and ten plants per parcel. The spacing between rows was 2.6 m, while that between plants was 3.7 m, and a system of vertical trellises was used for the conduction of branches.

The evaluation of symptoms was based on previously developed note scales that are routinely used to characterize the reactions of passion fruit to woodiness virus (Table 1) (Novaes and Rezende 1999), scab and anthracnose (Table 2) (Junqueira et al. 2003). Then,

Table 1 Scale used to quantify symptoms of woodiness virus in the leaves and fruits of 36 passion fruit (*Passiflora edulis* Sims) accessions from the active germplasm bank of EMBRAPA Cassava and Fruits, Cruz das Almas, Bahia, Brazil

Note scale	Symptoms of woodiness disease in passion fruit		
	Leaves	Fruits	Plants
1	No symptoms	No symptoms	Few leaves with mild mosaic and wrinkling
2	Mild mosaic, no deformation of leaves	Mildly deformed	Moderate quantity of leaves with mild mosaic and wrinkling
3	Mild mosaic, blisters and deformation of leaves	Totally deformed, fruit woodiness, spots	Large quantity of leaves with mosaic and severe wrinkling
4	Severe mosaic, blisters and deformation of leaves	—	—

the observed data were employed to quantify the reactions of the accessions to each of the three diseases considered in this study.

To evaluate disease symptoms caused by woodiness virus, scab and anthracnose on fruits, 15 fruits

Table 2 Scale used to quantify symptoms of scab on the fruits and branches and anthracnose on the fruits in 36 passion fruit (*Passiflora edulis* Sims) accessions from the active germplasm bank of EMBRAPA Cassava and Fruits, Cruz das Almas, Bahia, Brazil

Note scale	Symptoms of scab and anthracnose on passion fruit scale
1	No symptoms
2	Surface of the fruit/branch covered with lesions ≤ 10 %
3	Surface of the fruit/branch covered with lesions between 11 and 30 %
4	Surface of the fruit/branch covered with lesions >30 %

were randomly collected at the beginning of the maturation process. To assess the severity of scab on branches, we examined three young branches on each of five central plants in the plot. Finally, to assess the severity of woodiness virus symptoms in leaves, the fifth leaf of young branches and the overall distribution of woodiness virus symptoms were evaluated in five central plants in the plot.

Data analysis and diversity estimates

Initially, we performed a descriptive statistical analysis of the 23 SSR loci considering all 36 accessions (with each accession represented by three plants). The number of alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and fixation index (F) were calculated using the GenAlEx v. 6.5.3 program (Peakall and Smouse 2012), and the polymorphic information content (PIC) of each locus was calculated using the macro MSTools application (Excel Microsatellite Toolkit v. 3.1) in Microsoft Excel 2007. The data obtained from the assessment of disease symptoms based on the note scales were standardized (each observed value was divided by the highest value in each scale) such that all observations ranged from 0 (resistant) to 1 (susceptible), regardless of the disease evaluated. The data were analyzed using descriptive statistics by estimating the mean and extreme values and the variance of the samples using BIOSTAT 5.3 software (Ayres et al. 2007).

Diversity estimates were performed for the molecular data based on the modified Rogers' distance (Wright 1978), with each accession being examined as a group composed of three plants, and the agronomic

data concerning disease reactions were assessed based on the Mahalanobis distance; these analyses were conducted using the TFPGA (Miller 1997) and GENES (Cruz 2008, 2006) programs, respectively. Clusters were graphically represented as dendrograms using the unweighted pair-group method with arithmetic mean (UPGMA) clustering algorithm, which was implemented in DARwin software (Perrier and Jacquemoud-Collet 2006). The efficiencies of the cluster matrices were evaluated through bootstrap analysis (1,000 resampling values), and the correlation between the matrices was verified using the Mantel test (with 10,000 simulations), which was implemented in the GENES program (Cruz 2006).

Comparisons regarding the intensity of the symptoms presented by the yellow and purple passion fruit accessions for each of the evaluated diseases were performed based on the Mann–Whitney test using BIOSTAT 5.3 software (Ayres et al. 2007). Diversity and genetic structure were also assessed between the yellow and purple passion fruit accessions. Both parameters were estimated using the GenAlEx v. 6.5.3 program (Peakall and Smouse 2012) based on the occurrence of private alleles, the value of N_e 's G_{st} and the percentage of variation between groups through analysis of molecular variance (AMOVA).

Selection of accessions and establishment of core and working collections

The accessions were sorted based on the intensity of the observed symptoms for all six studied variables and subjected to analyses of variance (ANOVAs), and univariate grouping tests (Scott-Knott), which were implemented in the GENES program (Cruz 2006, 2008). Using the ANOVA results as a basis, the coefficient of variation (CV) and the broad-sense heritability (BSH) for each variable were also estimated. The same analysis procedures were applied jointly considering the variables related to each disease, where the values used in the analysis represented the average values presented by the accessions for the three variables related to woodiness virus (in leaves, fruits and plants) and the average values for the two variables related to scab (on fruits and branches).

Considering the ranks of the accessions, which were based on the severity of symptoms for the three diseases, groups were formed that encompassed 25, 50, 75 and 100 % of the accessions in ascending order

Table 3 Characteristics of the microsatellite markers used in the genotyping of 36 passion fruit (*Passiflora edulis* Sims) accessions from the germplasm bank of EMBRAPA Cassava and Fruits, Cruz das Almas, Bahia, Brazil

Markers	Na	H _o	H _E	PIC	F
mPc-UNICAMP11	2	0.15	0.14	0.13	-0.08
mPs-Unicamp01	7	0.62	0.73	0.68	0.15
mPs-Unicamp02	7	0.58	0.73	0.68	0.2
mPs-UNICAMP05	3	0.21	0.29	0.27	0.3
mPs-Unicamp06	4	0.82	0.66	0.59	-0.25
mPs-UNICAMP09	2	0.01	0.01	0.01	0
mPs-Unicamp10	4	0.08	0.17	0.16	0.49
mPs-UNICAMP15	2	0.14	0.13	0.12	-0.08
mPs-UNICAMP16	3	0.45	0.53	0.45	0.15
mPe-UNICAMP01	2	0.03	0.05	0.04	0.39
mPe-Unicamp02	4	0.44	0.43	0.37	-0.02
USP 07	16	0.81	0.87	0.86	0.06
USP 08	6	0.36	0.57	0.52	0.36
USP 15	8	0.6	0.69	0.64	0.13
USP 16	9	0.62	0.77	0.75	0.19
USP 17	4	0.48	0.51	0.46	0.06
USP 18	9	0.69	0.68	0.64	-0.02
USP 19	9	0.52	0.8	0.77	0.34
USP20	3	0.41	0.39	0.32	-0.06
USP 23	6	0.35	0.75	0.72	0.54
USP 24	8	0.66	0.73	0.68	0.09
USP 42	3	0.07	0.08	0.08	0.19
USP 58	6	0.59	0.62	0.55	0.04
Average	5.52	0.42	0.49	0.46	0.14
Confidence interval ^a	4.3–6.7	0.32–0.51	0.38–0.58	0.34–0.54	0.06–0.21

Na number of alleles, H_o observed heterozygosity, H_E expected heterozygosity (Nei's genetic diversity), PIC polymorphic information content, F fixation index
^a Confidence interval estimated through bootstrapping (100,000 simulations)

of symptom intensity. The number of microsatellite alleles maintained in each group that was formed was estimated using the GenAlEx v. 6.5.3 program (Peakall and Smouse 2012). Estimations for the core collections based only on molecular data were also performed with the COREFINDER program using 99 interactions (Cipriani et al. 2010). Statistical analyses were conducted considering the probability level of significance to be 5 % ($p < 0.05$).

Results

Genetic diversity based on microsatellite markers

We identified 127 alleles among the 36 passion fruit accessions (with an average of 5.52 alleles per assessed locus), and an excess of homozygotes was detected based on the observed heterozygosities (H_o; mean value = 0.42) and the expected heterozygosities

(H_E, Nei's genetic diversity; mean value = 0.49) (Table 3). The PIC presented a mean value of 0.46 (ranging from 0.01 to 0.86), and the fixation index (F) values were positive for most of the SSR loci (70 %), with a mean value of 0.14 being obtained (ranging from -0.25 to 0.54). No genetic structure was observed between the purple and yellow passion fruit accessions (Nei's G_{st} = 0.012), and only 1 % of the existing diversity occurred among the groups of yellow and purple passion fruit (AMOVA based on 999 permutations). Approximately 30 % of the alleles were identified as private, corresponding to 34 and five alleles restricted to accessions of yellow and purple passion fruit, respectively.

Characterization of the disease reactions of the passion fruit accessions

The symptom-associated values observed for the six variables related to the reactions of the accessions to

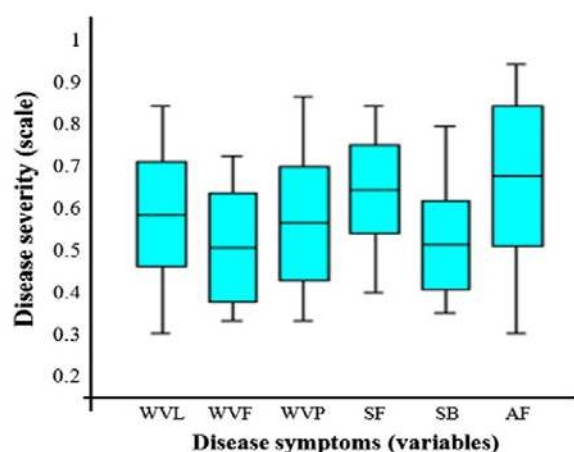


Fig. 2 Box-plot representing the average, standard deviation and extreme values observed for the symptoms of woodiness virus in leaves (WVL), woodiness virus in fruits (WVF), woodiness virus in plants (WVP), scab on fruit (SF), scab on branches (SB), and anthracnose on fruits (AF) in 36 passion fruit (*P. edulis* Sims) accessions

the three analyzed diseases ranged from 0.30 to 0.95 (when the data were standardized to range from 0 to 1). The average values observed in the 36 accessions for each variable were as follows: 0.58 for woodiness virus in leaves, 0.50 for woodiness virus in fruits, 0.57 for woodiness virus in plants, 0.65 for scab on fruit, 0.51 for scab on branches, and 0.68 for anthracnose on fruits (Fig. 2).

The ANOVA identified significant differences for all six variables ($p < 0.05$), and the univariate test for the comparison of means (Scott-Knott) allowed the accessions to be classified into two distinct groups in terms of the severity of symptoms (identified with the letters a and b) for four of the six variables considered (symptoms of woodiness virus in leaves, woodiness virus in fruits, woodiness virus in plants and scab on fruit) (Table 4). No significant difference was observed between the average values of the variables related to scab on branches and anthracnose. The correlations between the six variables were extremely low ($-0.12 < r < 0.36$), with the only significant correlations being detected between the symptoms of scab on fruits and woodiness virus on fruits (Supplementary Material—SM 2). Finally, when the variables related to each disease were analyzed together, we identified a significant difference between the groups for the symptoms related to woodiness virus ($p < 0.05$, Scott-Knott test), but not for those related to scab (Table 4).

Estimation of diversity, grouping and selection of accessions

The modified Rogers' distance (Wright 1978) presented a mean value of 0.41 ± 0.07 among the pairs of accessions evaluated using SSR markers, with the values ranging from 0.21 to 0.62. Based on the disease reaction data, a mean value of 5.06 ± 3 was observed for the Mahalanobis distance among the pairs of accessions, with a range of 0.38–22.9. Details regarding the distances observed between pairs of accessions for each of the estimations are presented in dendrograms (Fig. 3). The Mantel correlation test was significant for the two distance matrices ($p < 0.001$); however, the value of the correlation coefficient was low ($r = 0.16$ with 10,000 simulations).

A significant difference in the mean values of symptoms was observed between the groups formed based on different percentages of selection (25, 50 and 75 % of accessions per group) and based on the total set of accessions evaluated ($p < 0.05$; Mann-Whitney test) (Table 5). The number of alleles present in each group ranged from 98 (in the group formed by the nine accessions showing the lowest intensity of symptoms for anthracnose) to 125 (in the group formed by the 27 accessions showing the lowest intensity of symptoms for scab), representing 77 and 98 %, respectively, of the total alleles identified in the 36 characterized accessions. For all groups, the values of Nei's genetic diversity were similar, ranging from 0.48 to 0.52 (Table 5). Additionally, five accessions were identified (BGP029, 071, 168, 205, 277) as being present in both of the groups displaying lower intensities of symptoms (groups 1 and 2) for the three evaluated diseases. The average values for the observed symptoms differed significantly between the accessions of yellow passion fruit (0.57 for woodiness virus, 0.59 for scab, and 0.66 for anthracnose) and purple passion fruit (0.51 for woodiness virus, 0.58 for scab, and 0.72 for anthracnose) for woodiness virus ($p < 0.05$, Mann-Whitney test), but not for scab and anthracnose ($p < 0.1$, Mann-Whitney test) (Table 6).

The estimates performed for the core collections with the COREFINDER program (Cipriani et al. 2010) indicated that four and 16 accessions represented 70 and 100 % of the genetic diversity of alleles, respectively (Fig. 4).

Table 4 Average values observed in 36 passion fruit (*P. edulis* Sims) accessions for symptoms of woodiness virus in leaves (WVL), woodiness virus in fruits (WVF), woodiness virus in plants (WVP), scab on fruit (SF), scab on branches (SB), and anthracnose on fruits (AF) and the average symptoms observed for woodiness virus and scab

Accessions ^a	Woodiness virus				Scab			Anthracnose
	WVL	WVF	WVP	Average	SF	SB	Average	AF
BGP007	0.65a	0.60a	0.60b	0.62a	0.85a	0.35a	0.60a	0.70a
BGP009	0.65a	0.73a	0.53b	0.64a	0.60b	0.40a	0.50a	0.55a
BGP017	0.65a	0.67a	0.47b	0.59a	0.50b	0.55a	0.53a	0.85a
BGP022	0.75a	0.33b	0.53b	0.54b	0.45b	0.40a	0.43a	0.90a
BGP023	0.65a	0.40b	0.40b	0.48b	0.70a	0.55a	0.63a	0.75a
BGP028	0.70a	0.60a	0.60b	0.63a	0.60b	0.55a	0.58a	0.70a
BGP029	0.55b	0.40b	0.60b	0.52b	0.45b	0.60a	0.53a	0.60a
BGP033	0.55b	0.60a	0.53b	0.56a	0.75a	0.55a	0.65a	0.95a
BGP034	0.70a	0.60a	0.73a	0.68a	0.80a	0.60a	0.70a	0.70a
BGP041	0.80a	0.60a	0.53b	0.64a	0.65a	0.50a	0.58a	0.45a
BGP043	0.70a	0.40b	0.60b	0.57a	0.80a	0.55a	0.68a	0.85a
BGP044	0.40b	0.40b	0.67a	0.49b	0.60b	0.65a	0.63a	0.70a
BGP048	0.55b	0.67a	0.53b	0.58a	0.70a	0.60a	0.65a	0.50a
BGP051	0.60a	0.73a	0.80a	0.71a	0.70a	0.50a	0.60a	0.85a
BGP064	0.50b	0.60a	0.80a	0.63a	0.80a	0.80a	0.80a	0.30a
BGP071	0.50b	0.47b	0.47b	0.48b	0.65a	0.50a	0.58a	0.50a
BGP078	0.50b	0.73a	0.53b	0.59a	0.70a	0.40a	0.55a	0.65a
BGP079	0.65a	0.47b	0.47b	0.53b	0.80a	0.40a	0.60a	0.85a
BGP092	0.50b	0.33b	0.67a	0.50b	0.55b	0.50a	0.53a	0.85a
BGP094	0.85a	0.40b	0.80a	0.68a	0.70a	0.60a	0.65a	0.55a
BGP116	0.70a	0.33b	0.47b	0.50b	0.60b	0.55a	0.58a	0.80a
BGP121	0.55b	0.53a	0.47b	0.52b	0.55b	0.65a	0.60a	0.65a
BGP123	0.50b	0.47b	0.53b	0.50b	0.75a	0.50a	0.63a	0.70a
BGP158	0.30b	0.53a	0.33b	0.39b	0.65a	0.65a	0.65a	0.65a
BGP168	0.45b	0.33b	0.33b	0.37b	0.65a	0.40a	0.53a	0.35a
BGP181	0.75a	0.73a	0.53b	0.67a	0.75a	0.35a	0.55a	0.85a
BGP185	0.50b	0.60a	0.53b	0.54b	0.70a	0.50a	0.60a	0.70a
BGP186	0.45b	0.40b	0.67a	0.51b	0.65a	0.70a	0.68a	0.80a
BGP188	0.45b	0.40b	0.40b	0.42b	0.40b	0.50a	0.45a	0.95a
BGP205	0.45b	0.47b	0.47b	0.46b	0.65a	0.35a	0.50a	0.65a
BGP208	0.45b	0.33b	0.73a	0.51b	0.60b	0.35a	0.48a	0.90a
BGP222	0.65a	0.67a	0.53b	0.62a	0.65a	0.45a	0.55a	0.70a
BGP227	0.80a	0.40b	0.73a	0.64a	0.55b	0.60a	0.58a	0.45a
BGP277	0.60a	0.40b	0.33b	0.44b	0.55b	0.45a	0.50a	0.45a
BGP311	0.50b	0.47b	0.87a	0.61a	0.55b	0.45a	0.50a	0.65a
BRS Gigante Amarelo	0.65a	0.47b	0.60b	0.57a	0.70a	0.50a	0.60a	0.50a
CV	35 %	40 %	36 %	23 %	28 %	37 %	22 %	33 %
BSH	46 %	50 %	57 %	54 %	45 %	36 %	41 %	65 %

Averages followed by the same letter within a column do not significantly differ by the Scott-Knott test at a 5 % probability

CV coefficient of variation, BSH broad-sense heritability

^a Details of the accessions are available in the Supplementary Material - SM 1

Discussion

Considering the need to release cultivars exhibiting combined resistance to the major diseases that affect

passion fruit, the results obtained in this study will contribute to passion fruit pre-breeding activities aimed at intensifying the use of the accessions available in the active germplasm bank of EMBRAPA. From this

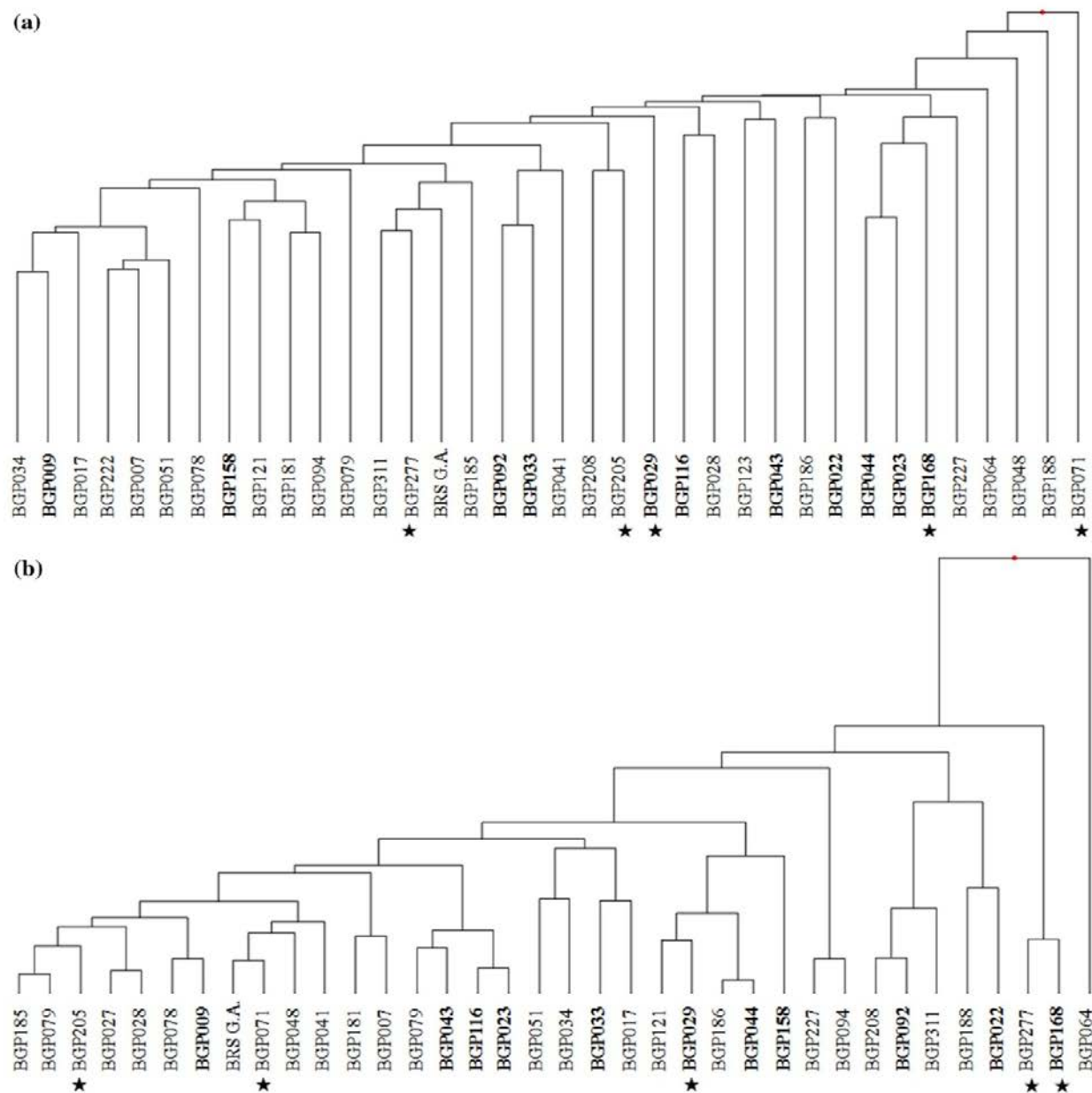


Fig. 3 Dendrograms obtained through UPGMA cluster construction based on the modified Rogers' distance (Wright 1978) for microsatellite data (a) and for the Mahalanobis distance for disease reaction data (b), considering 36 passion fruit accessions

from the germplasm bank of EMBRAPA Cassava and Fruits, Cruz das Almas, Bahia, Brazil. *Note* Accessions that presented a lower intensity of symptoms for the three diseases evaluated in this study are identified with a star

perspective, the estimates of molecular genetic variation and of the gradient of disease resistance observed for the accessions enable the identification of preferred crossings to establish populations that are useful in both

recurrent selection cycles and for genetic map construction and QTL identification.

The average number of alleles (5.52 per locus) observed in the 36 accessions (Table 3) is consistent with

Table 5 Descriptive presentation of the average intensity of symptoms, number of microsatellite alleles (Na) and Nei's genetic diversity (H_E) for the different groups formed after classification of the severity of symptoms in 36 passion fruit accessions for the three evaluated diseases

Diseases	Percentage of accessions selected ^a	Symptoms	Na	H_E	Set of accessions ^b
Woodiness virus	Group 1 (25 %)	0.45	105	0.52	BGP023, 044, 071, 092, 158, 168, 188, 205, 277
	Group 2 (50 %)	0.48	119	0.51	BGP022, 029, 079, 116, 121, 123, 185, 186, 208
	Group 3 (75 %)	0.52	121	0.50	BGP017, 028, 033, 043, 048, 078, 222, 311, BRS-GA
	Group 4 (100 %)	0.55	127	0.49	BGP007, 009, 034, 041, 051, 064, 094, 181, 227
Scab	Group 1 (25 %)	0.49	99	0.48	BGP009, 022, 029, 092, 188, 205, 208, 277, 311
	Group 2 (50 %)	0.53	118	0.50	BGP017, 028, 041, 071, 078, 116, 168, 181, 222
	Group 3 (75 %)	0.55	125	0.50	BGP007, 023, 044, 051, 079, 121, 185, 227, BRS-GA
	Group 4 (100 %)	0.58	127	0.49	BGP033, 034, 043, 048, 064, 094, 123, 158, 186
Anthracnose	Group 1 (25 %)	0.45	98	0.48	BGP009, 041, 048, 064, 071, 168, 227, 277, BRS-GA
	Group 2 (50 %)	0.55	113	0.49	BGP007, 028, 029, 078, 094, 121, 158, 205, 311
	Group 3 (75 %)	0.61	120	0.49	BGP017, 023, 034, 044, 116, 123, 185, 186, 222
	Group 4 (100 %)	0.68	127	0.49	BGP022, 033, 043, 051, 079, 092, 181, 188, 208

^a Accessions of group 1 are also part of the accession list of group 2, accessions of group 1 and 2 are part of the accession list of group 3, as well as accessions from group 1, 2 and 3 are part of accessions list of the group 4

BRS-GA BRS Gigante Amarelo

^b Details of the accessions are available in the Supplementary Material - SM 1

Table 6 Presentation of the symptoms of three diseases in 25 yellow passion fruit and 11 purple passion fruit accessions, including the average intensity, standard deviation and results of the Mann–Whitney test

Disease	Average intensity of the symptoms		Mann–Whitney
	Yellow	Purple	
Woodiness virus	0.57 (± 0.08)	0.51 (± 0.07)	Z value = 2.18*
Scab	0.59 (± 0.07)	0.58 (± 0.08)	Z value = 0.11
Anthracnose	0.66 (± 0.17)	0.72 (± 0.18)	Z value = 1.04

* Differ significantly by the Mann–Whitney test at a 5 % probability (p value observed = 0.014)

the values obtained in previous genetic characterizations performed in passion fruit using microsatellite markers (Cerqueira-Silva et al. 2014c). The few such studies conducted to date have indicated a low number of alleles at the loci characterized for the commercial species *P. edulis* (4.7) (Oliveira et al. 2005; Cerqueira-Silva et al. 2014b) and *P. alata* (3.1) (Padua et al. 2005).

For wild passion fruit species, such as *P. contracta*, *P. cincinnata* and *P. setacea*, the average number of alleles per locus has also been found to be low, ranging from 2.8 to 4.9 (Cerqueira-Silva et al. 2014b; Cazé et al. 2012). In addition, the number of alleles detected in passion fruit undergoing recurrent selection has been reported to be fewer than three alleles per locus (Reis et al. 2011). Considering the apparent pattern of low diversity at SSR loci observed in passion fruit (Cerqueira-Silva et al. 2014c), the number of alleles identified in the set of evaluated accessions is considered favorable for establishing strategies for selecting and forming base populations for breeding.

The obtained Nei's genetic diversity values (average of 0.49) were consistent with the expectation for outcrossing species and with the values observed in a previous study for *P. edulis* (Oliveira et al. 2005). The mean value of F (0.14) can be interpreted as indicating a loss of heterozygotes and corroborates the higher values obtained for H_E than for H_0 . These results may be partly explained by the origins of the accessions

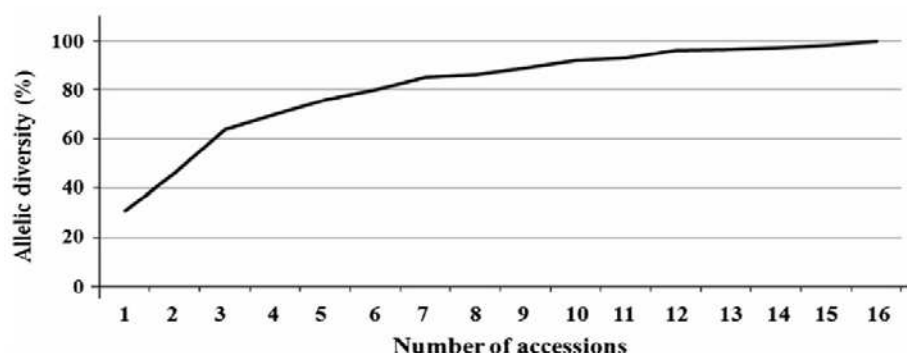


Fig. 4 Allelic diversity as a function of the number of accessions included in the core collection of passion fruit (*Passiflora edulis* Sims). Note The order of the accessions included in the core collection was as follows: BGP007;

BGP208; BGP041; BGP023; BGP028; BGP123; BGP071; BGP116; BGP188; BGP185; BGP181; BGP051; BGP168; BGP064; BGP017; BGP079

found in germplasm banks; i.e., the transport of seeds among production regions and the use of seeds produced by a given plantation for its next generation of seedlings are common practices, particularly for commercial passion fruit species (Santos et al. 2011).

The amplitude of the observed values for the six measured variables (0.65, considering a scale from 0 to 1) (Fig. 2) and the significant results of ANOVA ($p < 0.05$) for all six variables indicate the existence of variability that is potentially useful for breeding programs, particularly in the early stages of mass selection and the establishment of progenies for recurrent selection. The results obtained from the univariate test for the comparison of means (Scott-Knott) corroborate the existence of variability in the reaction of the passion fruit accessions to at least four of the six considered variables (Table 4).

Descriptions of the reactions of *P. edulis* (yellow and purple shell) to different pathogens are available in the literature and indicate the existence of both variability available for selection in collections and accessions maintained in germplasm banks (Oliveira et al. 2013; Silva et al. 2013; Cerqueira-Silva et al. 2008; Junqueira et al. 2006) and low disease resistance or tolerance in passion fruit cultivars (Batistti et al. 2013; Fischer et al. 2010; Junqueira et al. 2003).

The low correlations observed among the six variables considered in evaluating the reactions of the passion fruit accessions to the three studied diseases ($-0.12 < r < 0.36$) indicate possible difficulties in obtaining balanced genetic gains using direct selection procedures, given the trend toward unwanted losses of

genetic gains due to the existence of uncorrelated features. Similar correlation results were observed by Freitas et al. (2012) when evaluating the same six variables among 43 accessions of yellow passion fruit. However, the obtained correlation values do not prevent the use of selection indices, particularly because no significant negative correlations were found. The application of different selection indices for agronomic production traits and disease resistance was recently evaluated in passion fruit accessions (Freitas et al. 2012), and an imbalance was observed in the genetic gains associated with production characteristics and with levels of disease resistance when using the strategy of direct selection. In this work, Freitas et al. (2012) also detected more balanced genetic gains using the Mulamba and Mock index.

The variability estimates obtained from both the modified Rogers' distance (Wright 1978) and the Mahalanobis distance allowed genetic differentiation of all the evaluated accessions (Fig. 3). Estimates of genetic distance between passion fruit accessions or within natural passion fruit populations based on microsatellite loci are in their incipient stages, with the results being restricted to recent diversity estimates, such as estimates from the SSR locus cross-amplification (Oliveira et al. 2013). The use of these markers has also contributed to the monitoring of the variability present in segregating populations undergoing recurrent selection (Reis et al. 2012, 2011) and to developing genetic maps for *P. edulis* (Oliveira et al. 2005) and for *P. alata* (Pereira et al. 2013).

Based on the obtained estimates of the modified Rogers' distance, it is possible to identify pairs of

convergent and divergent accessions, highlighting the pair BGP009 and BGP034 (or BGP051), with a distance value of 0.21, and the pair BGP071 and BGP186, with a distance value of 0.62. Characterizing the genetic distance within collections and germplasm banks based on molecular markers contributes to both the monitoring of variability and the definition of preferred crosses to be performed at different stages of a breeding program. Genetically divergent accessions, such as BGP071 and BGP186, may be useful in establishing segregating populations and in producing hybrids with a potential heterosis effect. In turn, identifying genetically similar accessions can reduce the time and number of crossings required in back-crossing cycles whose goal is to introgress specific traits into cultivars of interest.

In turn, extreme values were observed for the Mahalanobis distance between the accession pair BGP044 and BGP186 (0.38) and the accession pair BGP064 and BGP188 (22.9). Considering the low correlations found among the reactions of the accessions to the different diseases and the consequent difficulty of applying a direct selection strategy grounded in individual characteristics, we understand that identifying convergent crosses potentiates the average gain of resistance to diseases. In turn, the use of divergent accessions, based on the Mahalanobis distance, can be useful in generating mapping populations, which increases the possibility of identifying QTLs for resistance, at least for the studied diseases.

Variability data based on molecular markers and the reactions of accessions to diseases potentiate the establishment of populations showing high genetic variability (considering divergent values for the modified Rogers' distance) and displaying reduced variance in their reactions to the evaluated diseases (considering convergent values for the Mahalanobis distance). Freitas et al. (2012) indicated that maximizing the variability among the accessions used in the composition of base populations undergoing recurrent selection is a strategy employed to avoid unwanted narrowing of the available genetic base in the initial selection cycles.

The low correlations observed between the matrices of distance estimates obtained from the molecular data (such as microsatellite markers) and the agronomic data (such as the reactions of the accessions to disease) ($r = 0.16$; Mantel test with 10,000 simulations) are expected due to the nature of these data.

Most of the observed variability in the molecular markers is, by definition, nonadaptive, unlike phenotypic characteristics, which are affected by selection and influenced by interactions with the environment. Reis et al. (2012) reported similar results when comparing data from the genotyping and phenotyping of passion fruit undergoing recurrent selection cycles.

Classification of the accessions into groups according to the percentages of selection (25, 50, 75 and 100 %) considering the rank of the accessions based on the ascending order of symptom intensity for the three diseases (Table 5), contributed to defining the accessions to be prioritized in breeding programs dedicated to resistance. As the accessions classified as belonging to groups 1 and 2 (Table 5) exhibited the lowest intensities of symptoms (average symptom values ranging from 0.48, for woodiness virus, to 0.55, for anthracnose) and retained, on average, 85 % of the alleles identified in the microsatellite loci, these accessions should be prioritized in the composition of breeding populations. These results hold greater relevance when compared with the set of accessions suggested for use in a core collection considering only genotyping data from microsatellite loci (Fig. 4). The average values of symptoms observed in this accession collection varied between 0.55, for woodiness virus, and 0.70, for anthracnose.

Considering that a core collection should retain from 5 to 20 % of the accessions available in a collection and should represent at least 70 % of the existing diversity (Brown et al. 1987; Nass 2011), both the set of accessions suggested by the COREFINDER program (Fig. 4) and the accessions from groups 1 and 2 (Table 5) are suitable for forming core and working collections. Identifying accessions with favorable characteristics for passion fruit genetic breeding is of great importance because studies conducted by Freitas et al. (2011) on *Passiflora* genetic resources demonstrated the need for genetic recombination among accessions to further explore and select superior progenies, as accessions of passion fruit combining the quantitative and qualitative characteristics favorable for production were not identified by these authors.

Notably, BGP029, 071, 168, 205 and 277 were ranked among the accessions displaying weaker symptoms for the three evaluated diseases when considering a selection percentage of 50 %. Assuming a more restrictive selection percentage (25 %),

accessions BGP029 and BGP205 were both classified into group 1, displaying a low intensity of symptoms for woodiness virus and scab. In turn, accession BGP168 was classified into group 1 based on its symptoms related to woodiness virus and anthracnose. Finally, accession BGP277 was the only accession classified into group 1 for all three evaluated diseases.

Although few studies have focused on the agronomic characterization of accessions maintained in collections and germplasm banks, important associations can be identified based on the available publications. Data related to the resistance of passion fruit to *Fusarium oxysporum* f. sp. *passiflorae* (Silva et al. 2013) indicate that the BGP208 and BGP029 accessions, which were classified into the groups showing weaker symptoms for woodiness virus and scab, are also resistant to *F. oxysporum*. In turn, agronomic characterizations performed by Reis et al. (2011) indicate that BGP208 presents high fruit production (80–98 fruit per plant) compared with other accessions in the germplasm bank.

The 'BRS Gigante Amarelo' cultivar was classified into group 3 due to its reactions to both woodiness virus and scab because this cultivar was more susceptible to these diseases than most of the accessions included in this study (Table 5). Considering anthracnose, however, the 'BRS Gigante Amarelo' cultivar was classified into group 1 because of being less susceptible to this disease. These results are consistent with the data available in the literature (Batisti et al. 2013; Fischer et al. 2010; Junqueira et al. 2003) indicating the susceptibility of passion fruit cultivars to the most important diseases related to fruit culture. In this context, different authors have indicated the need for research focused on increasing the resistance of existing cultivars or on developing new cultivars using both intraspecific and interspecific crosses (Faleiro et al. 2011; Meletti et al. 2005; Faleiro et al. 2005). The genetic variability and the gradient of resistance identified in this study reinforce the possibility, which was also presented by Oliveira et al. (2013), of employing intraspecific crossing as a strategy to reduce the susceptibility of passion fruit cultivars to the primary diseases that affect passion fruit culture.

Although significant genetic structure was not observed among the purple and yellow passion fruit accessions (Nei's $G_{st} = 0.012$) and although no clear evidence of subgroups (Fig. 3) was observed, the private alleles identified among these groups of

accessions could be useful in monitoring hybrids between these variants of *P. edulis*. In addition, the results of the comparison of averages (Table 6) suggest that the purple passion fruit accessions can be prioritized in the search for resistance to woodiness virus. Additionally, a tendency toward reduced anthracnose symptoms was observed in the yellow passion fruit accessions.

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SUPPLEMENTARY MATERIAL

SM 1 List of the yellow and purple passion fruit accession (*Passiflora edulis* Sims) used in this study and present in the active germplasm bank of EMBRAPA Cassava & Fruits, Cruz das Almas, Bahia, Brazil.

Code	Species	Variety	City, State	Country	Germplasm Bank
BGP007	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP009	<i>P. edulis</i> Sims	Purple	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP017	<i>P. edulis</i> Sims	Yellow	Andradina, São Paulo	Brazil	EMBRAPA
BGP022	<i>P. edulis</i> Sims	Purple	Cruz das Almas, Bahia	Brazil	EMBRAPA
BGP023	<i>P. edulis</i> Sims	Purple	Mogi-Guaçu, São Paulo	Brazil	EMBRAPA
BGP028	<i>P. edulis</i> Sims	Yellow	Nova Soure, Bahia	Brazil	EMBRAPA
BGP029	<i>P. edulis</i> Sims	Purple	Urussanga, Santa Catarina	Brazil	EMBRAPA
BGP033	<i>P. edulis</i> Sims	Purple	Maringá, Paraná	Brazil	EMBRAPA
BGP034	<i>P. edulis</i> Sims	Yellow	Maringá, Paraná	Brazil	EMBRAPA
BGP041	<i>P. edulis</i> Sims	Yellow	Monte Alegre do Alto, São Paulo	Brazil	EMBRAPA
BGP043	<i>P. edulis</i> Sims	Purple	Funchal, Ilha da Madeira	Portugal	EMBRAPA
BGP044	<i>P. edulis</i> Sims	Purple	Unavailable	Brazil	EMBRAPA
BGP048	<i>P. edulis</i> Sims	Yellow	Caracas	Venezuela	EMBRAPA
BGP051	<i>P. edulis</i> Sims	Yellow	Janauba, Minas Gerais	Brazil	EMBRAPA
BGP064	<i>P. edulis</i> Sims	Yellow	Conceição do Jacuípe, Bahia	Brazil	EMBRAPA
BGP071	<i>P. edulis</i> Sims	Yellow	Presidente Tancredo Neves, Bahia	Brazil	EMBRAPA
BGP078	<i>P. edulis</i> Sims	Yellow	Andaraí, Bahia	Brazil	EMBRAPA
BGP079	<i>P. edulis</i> Sims	Yellow	Andaraí, Bahia	Brazil	EMBRAPA
BGP092	<i>P. edulis</i> Sims	Purple	Livramento Nossa Senhora, Bahia	Brazil	EMBRAPA
BGP094	<i>P. edulis</i> Sims	Yellow	Livramento Nossa Senhora, Bahia	Brazil	EMBRAPA
BGP116	<i>P. edulis</i> Sims	Purple	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP121	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP123	<i>P. edulis</i> Sims	Yellow	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP158	<i>P. edulis</i> Sims	Purple	Jaboticabal, São Paulo	Brazil	EMBRAPA
BGP168	<i>P. edulis</i> Sims	Purple	Nova Odessa, São Paulo	Brazil	EMBRAPA
BGP181	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP185	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP186	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP188	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP205	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP208	<i>P. edulis</i> Sims	Yellow	Piracicaba, São Paulo	Brazil	EMBRAPA
BGP222	<i>P. edulis</i> Sims	Yellow	Salvador, Bahia	Brazil	EMBRAPA
BGP227	<i>P. edulis</i> Sims	Yellow	Campinas, São Paulo	Brazil	EMBRAPA
BGP277	<i>P. edulis</i> Sims	Yellow	Ruy Barbosa/Utinga, Bahia	Brazil	EMBRAPA
BGP311	<i>P. edulis</i> Sims	Yellow	Nova Viçosa, Bahia	Brazil	EMBRAPA
BRS Gigante Amarelo	<i>P. edulis</i> Sims	Not apply	CPAC/EMBRAPA	Brazil	EMBRAPA

SM 2 Correlation matrix between the observed symptoms of woodiness virus in leaves (WVL), woodiness virus in fruits (WVF), woodiness virus in plants (WVP), scab on fruit (SF), scab on branches (SB), and anthracnose on fruits (AF) in the 36 passion fruit (*Passiflora edulis* Sims) accessions present in the active germplasm bank of EMBRAPA Cassava & Fruits, Cruz das Almas, Bahia, Brazil.

Symptoms	WVL	WVF	WVP	SF	SB	AF
WVL	---					
WVF	0,129	---				
WVP	0,190	0,051	---			
SF	0,118	0,3673*	0,157	---		
SB	-0,118	-0,067	0,249	-0,003	---	
AF	-0,022	-0,056	0,000	-0,101	-0,236	---

*Significant correlation at a 5% probability.

RESULTADOS COMPLEMENTARES

Considerando que o cruzamento interespecífico é uma alternativa para o incremento da diversidade genética em etapas iniciais de um programa de melhoramento genético, bem como indicado por diversos autores como alternativa para incremento de resistência em variedades cultivadas ou mesmo para obtenção de novas variedades de maracujazeiro (FALEIRO et al., 2011; WETZEL et al., 2011; MELETTI et al., 2005; FALEIRO et al., 2005), caracterizações genéticas interespecíficas contribuem para o sucesso dessas atividades. Nesse contexto, o conjunto de marcadores microsatélites caracterizados na presente Tese (especificamente no primeiro artigo do Capítulo 3) deverá servir como ponto de partida para estudos dessa natureza.

A ocorrência, ao menos em potencial, de cruzamentos interespecíficos entre espécies que convivem em simpatria (seja no ambiente natural, seja em ambientes de conservação *ex-situ*, como os bancos de germoplasma), pode mascarar e comprometer estimativas de diversidade genética. Além do que, se não monitorado ao longo do processo de manutenção e regeneração do banco de germoplasma, a ocorrência de fluxo gênico entre acessos intra e interespecíficos, poderá comprometer a identidade genética dos acessos e por consequência a sua representatividade em relação aos locais de coleta.

Com intuito de fomentar a realização de estudos interespecíficos para o gênero *Passiflora*, bem como contribuir com informações preliminares para o entendimento da diversidade e estruturação genética existente entre 116 acessos de maracujazeiro avaliados (24 referentes a *P. cincinnata*, 85 referentes a *P. edulis* e 7 referentes a *P. setacea*), nós utilizamos os dados de genotipagem obtidos a partir de oito locos microsatélites que foram comuns aos estudos de diversidade intraespecífica realizados no âmbito do segundo artigo do Capítulo 3. Assim, foram considerados oito locos (a saber: Pe-07, 08, 15, 18 e 19; mPs-Unicamp05, 10 e 16) e realizadas estimativas de diversidade (número de alelos, distância genética, análise de coordenadas principais,

estimativa de alelos privados e nível de estrutura genética). Todas as análises foram realizadas com auxílio do programa GenAEx 6.5 (PEAKALL e SMOUSE, 2012).

Foram identificados um total de 108 alelos microssatélites e uma média de 6,5 alelos por loco. Todos os locos foram polimórficos nas três espécies avaliadas, mas as estimativas de diversidade (H_E) e os índices de fixação foram bem diferentes para cada uma das espécies (Tabela 2). Estes resultados indicam a menor diversidade presente na coleção de acessos de *P. setacea*, em relação a coleção de acessos de *P. cincinnata* e *P. edulis* e estão de acordo com as estimativas apresentadas no Capítulo 3. A semelhança observada nos resultados gerais, para uma análise descritiva da diversidade, entre os dados do Capítulo 3 e os dados aqui apresentados com base em apenas oito locos microssatélites atestam a eficiência destes marcadores para estimativas genéticas.

Tabela 2. Resultado descritivo da caracterização dos acessos de *P. cincinnata* (24 acessos), *P. edulis* (85 acessos) e *P. setacea* (7 acessos) com base em oito locos microssatélites previamente desenvolvidos por OLIVEIRA (2006) e CERQUEIRA-SILVA et al. (2012b; 2014).

Espécie	Na	H _o	H _E	F
<i>P. cincinnata</i> Mast	8	0.54	0.69	0.23
<i>P. edulis</i> Sims	8.2	0.48	0.59	0.19
<i>P. setacea</i> DC	3.4	0.20	0.33	0.41
Intervalo de confiança*	4,8 -- 8,2	0,29 -- 0,49	0,44 -- 0,61	0,18 -- 0,27

Nota: Na = número de alelos; H_o = heterozigosidade observada; H_E = heterozigosidade esperada; F = índice de fixação; *Intervalo de confiança estimado por *bootstrap* de 100 mil simulações.

Do total de alelos identificados aproximadamente 60% foram observados em *P. cincinnata* e *P. edulis* e apenas 25% foram observados em *P. setacea*. Considerando que o número de acessos e plantas disponíveis para cada espécie foi desigual e que o número amostral foi o de *P. setacea* os resultados estão de acordo com o esperado. Também é importante registrar que as estimativas de diversidade e estrutura genética apresentadas no primeiro artigo do Capítulo 2 indicaram menor diversidade entre os acessos de *P. setacea*, quando comparada a diversidade observada entre os acessos das demais espécies. De forma similar, a estrutura observada para os acessos de *P. setacea* foi também a mais elevada entre as espécies avaliadas.

Como esperado, por se tratar de espécies distintas, foi observado um elevado percentual de alelos privados entre os grupos de acessos representativos de cada espécie (aproximadamente 62%) e apenas um baixo número de alelos comuns às três espécies (aproximadamente 7%) (Figura 4). O monitoramento da frequência dos alelos identificados, bem como dos alelos privados, ao longo dos ciclos de regeneração do banco de germoplasma possibilitarão ao melhorista identificar e quantificar eventuais perdas de alelos, bem como a ocorrência de fluxo (contaminação) entre acessos intra e interespecíficos. A perda de alelos ao longo dos ciclos de regeneração do banco de germoplasma considerado neste estudo foi evidenciada por CASTRO (2012) e o monitoramento da variabilidade através do uso de marcadores foi sugerido pela autora. Nossos resultados deverão contribuir para o monitoramento da diversidade genética do banco, especialmente por disponibilizar informações a respeito de potenciais alelos espécie-específicos.

A dispersão realizada com base na análise de coordenadas principais para todas as 364 plantas avaliadas permitiu a visualização de três grupos, correspondendo às três espécies avaliadas (Figura 5), sendo evidenciada maior distância entre *P. edulis* e *P. setacea*. Embora não sejam observadas nítidas misturas entre conjuntos de indivíduos de espécies diferentes, é possível identificar (circunscrito em linha pontilhada na Figura 4) maior proximidade entre alguns indivíduos de *P. setacea* e *P. cincinnata* (sendo estes indivíduos pertencentes ao acesso Ps-VCA de *P. setacea* mantido na coleção da UESB

e o acesso BGP273 de *P. cincinnata* mantido na EMBRAPA), bem como entre indivíduos de *P. cincinnata* e *P. edulis* (sendo estes indivíduos pertencentes aos acessos BGP275, 280, 296 e 297 de *P. cincinnata* e BGP071 e 188 de *P. edulis*, todos mantidos na EMBRAPA Mandioca e Fruticultura).

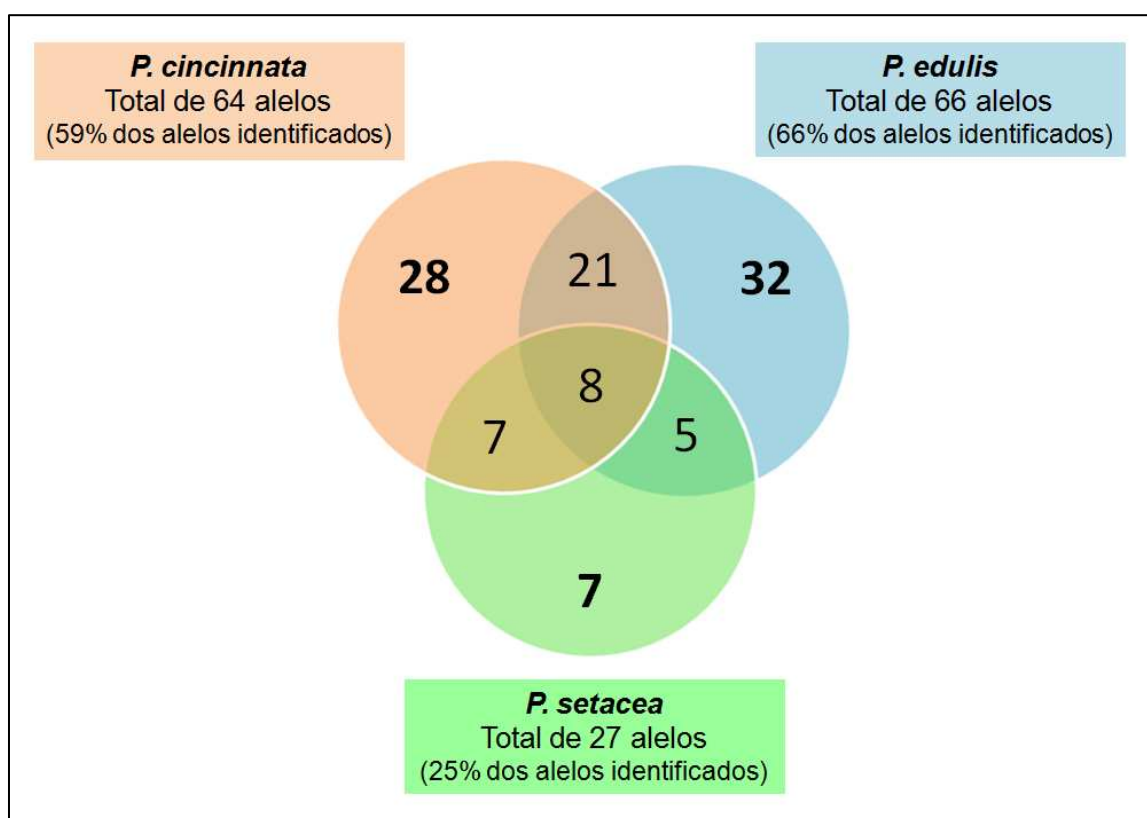


Figura 4. Apresentação descritiva do número absoluto e percentual de alelos em cada espécie e representação dos alelos privados e compartilhados entre as três espécies de *Passiflora* mediante diagrama de Venn.

Resultados desta natureza evidenciam variações significativas na distancia interespecífica entre os acessos, indicando que a escolha do acesso a ser utilizado em cruzamentos interespecíficos pode ser determinante para futuras etapas de um programa de melhoramento. O uso de acessos muito distantes, por exemplo, pode levar

a necessidade de maior número de ciclos de retro-cruzamento, quando comparado a cruzamentos realizados a partir de acessos mais similares geneticamente. O uso de mais locos microssatélites em caracterizações interespecífica permitirá uma maior cobertura do genoma e por conseguinte maior segurança para os resultados obtidos. Também é importante o uso de dados agrônômicos para auxiliar na definição dos acessos a serem priorizados em cruzamentos interespecíficos, a exemplo da identificação de cruzamentos interespecíficos convergentes e divergentes realizadas por CERQUEIRA-SILVA et al. (2009) com base na dissimilaridade genética, estimada a partir de características físico-químicas de frutos de maracujazeiros amarelo (*P. edulis*) e do sono (*P. setacea*).

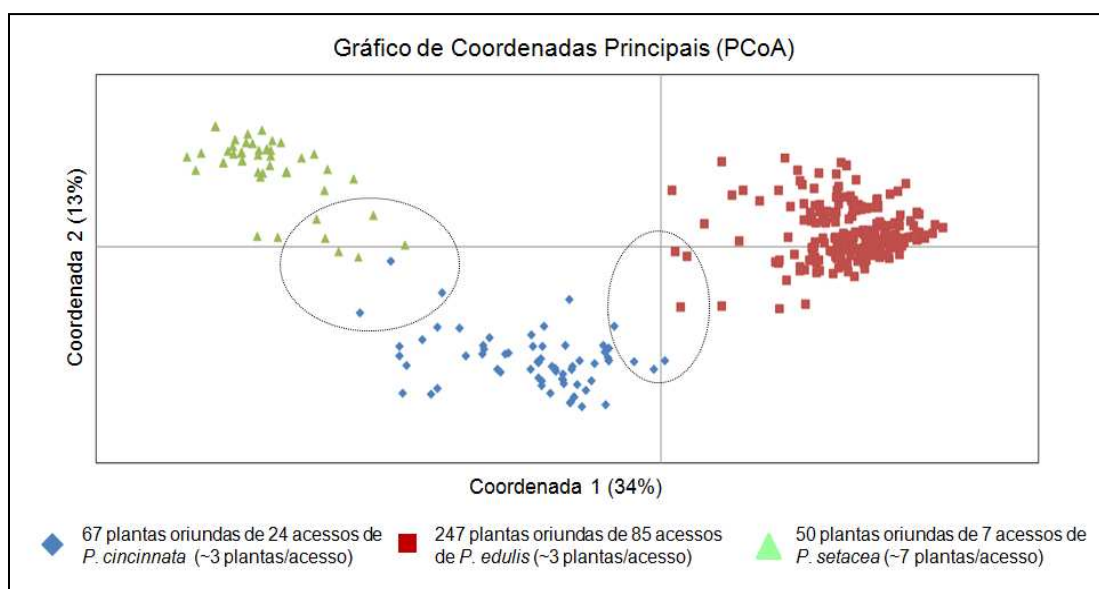


Figura 5. Gráfico de dispersão com base em coordenadas principais para 364 plantas de maracujá, referentes a 116 acessos e três espécies de *Passiflora*, caracterizados com base no polimorfismo genético de oito locos microssatélites.

CONSIDERAÇÕES FINAIS

O desenvolvimento do projeto de doutorado que possibilitou a apresentação da presente Tese logrou êxito, sendo alcançados todos os objetivos inicialmente traçados. Os resultados obtidos e os desafios enfrentados foram apresentados e discutidos em eventos e reuniões científicos (seminários e palestras) e publicações (resumos, relatórios e artigos). Didaticamente as contribuições oriundas da Tese podem ser apresentadas desde o ponto de vista institucional até a indicação de ações práticas passíveis de serem aplicadas em programas de pré e ou melhoramento genético.

No que diz respeito às relações interinstitucionais, o desenvolvimento da presente Tese de doutorado possibilitou o fortalecimento de parcerias institucionais já existentes no momento da proposição deste trabalho (a exemplo das parcerias entre pesquisadores da UNICAMP, ESALQ/USP e UESC), bem como possibilitou a formação de novas parcerias (a exemplo da rede de colaboração estabelecida entre pesquisadores da UNICAMP, UESB e CNPMF/EMBRAPA).

Em se tratando de produtos científicos, entendemos que os trabalhos desenvolvidos ao longo do doutorado contribuíram para o avanço das discussões a respeito da variabilidade genética dos maracujazeiros e suas relações com os desafios inerentes à conservação e especialmente ao melhoramento genético. Neste sentido, foram apresentadas à comunidade científica desde uma visão crítica a respeito do uso e das contribuições dos marcadores moleculares em relação as pesquisas desenvolvidas com espécies do gênero *Passiflora*, até a indicação de perspectivas de curto a longo prazo para pesquisas moleculares no gênero. De maneira similar, foi apresentado a sociedade científica nossa visão a respeito dos avanços e dos desafios relacionados ao principal patossistema associado a passicultura, qual seja a relação maracujazeiro vs. endurecimento dos frutos.

Considerando que uma das justificativas para o reduzido número de estudos moleculares com marcadores microssatélites em maracujazeiro é a ausência de locos caracterizados disponíveis para a maioria das espécies, e que o custo associado ao

desenvolvimento destes marcadores é relativamente alto, especialmente quando não estão disponíveis informações e sequências genômicas adequadas para identificação de microssatélites em bancos de dados públicos, acreditamos que os resultados apresentados nos Capítulos 2 e 3 contribuirão efetivamente para o aumento no número de pesquisas genéticas sobre o gênero *Passiflora*, especialmente para estudos relacionados a espécies silvestres.

As informações relativas a variabilidade e estruturação genética disponibilizadas para os acessos de *P. cincinnata*, *P. edulis* e *P. setacea*, no primeiro artigo do terceiro Capítulo da Tese, além de possibilitar uma visualização geral da variabilidade mantida no banco de germoplasma da EMBRAPA (CNPMPF, Cruz das Almas, Bahia), contribui para o direcionamento de novas coletas e auxilia nas discussões de cruzamentos (divergentes e convergentes) passíveis de serem explorados nas diferentes etapas do pré-melhoramento do maracujá. Além do que, a identificação de um conjunto de locos microssatélites que não apresentaram amplificação cruzada, bem como os que apresentaram 100% de amplificação cruzada entre as 14 espécies avaliadas, possuem uso prático na identificação de híbridos interespecíficos e em estudos de diversidade interespecífica.

O esforço realizado no sentido de associar e discutir os resultados oriundos da genotipagem com marcadores microssatélites e da caracterização da reação de acessos à importantes doenças que acometem a passicultura, potencializa o uso racional da variabilidade genética durante etapas de seleção em materiais de interesse. Neste contexto os resultados apresentados no segundo artigo do terceiro Capítulo, contribuem para o estabelecimento de coleções nucleares e de trabalho, a serem priorizadas em avaliações de características agrônomicas de interesse ou, utilizadas em cruzamentos dirigidos que objetivem não apenas ganho de produção, mas também resistência/tolerância a patógenos.

CONCLUSÕES

São notórios os avanços obtidos para a cultura do maracujazeiro nas últimas décadas, destacando-se o crescente uso de técnicas moleculares para a obtenção de conhecimento básico a respeito da diversidade genética do gênero, bem como o emprego do conhecimento e de técnicas moleculares que contribuam para o estabelecimento de um programa de melhoramento genético assistido por marcadores moleculares.

Ao menos para as espécies avaliadas neste estudo, a variabilidade genética presente nos locos microssatélite mostrou-se baixa para os maracujazeiros, tanto em relação ao percentual de locos microssatélites polimórficos, quanto em relação ao número de alelos presentes nestas regiões do genoma. O crescente uso destes marcadores em estudos genéticos do gênero e o consequente aumento no número de espécies e locos avaliados permitirão verificar se o padrão de baixa variabilidade observado é mesmo regra entre os maracujazeiros (*Passiflora* spp).

A estratégia de amplificação cruzada mostrou-se eficiente para identificação e caracterização de locos microssatélites entre espécies do gênero *Passiflora*, sendo possível utilizar essa metodologia para subsidiar estudos genéticos intra e interespecíficos em maracujazeiros.

A estrutura e a variabilidade genética das três espécies de maracujazeiro avaliadas (*P. cincinnata*, *P. edulis* e *P. setacea*) são distintas, ao menos para os acessos considerados neste estudo, devendo ser priorizado devido ao elevado valor de estruturação e reduzido número de acessos, a prospecção de material biológico referente ao maracujazeiro do sono (*P. setacea*). Por sua vez, a prospecção e a caracterização de novos acessos de *P. cincinnata* e *P. edulis* é também justificada, principalmente pela baixa representatividade de algumas regiões de ocorrência destas espécies no banco de germoplasma estudado.

Os grupos genéticos identificados com auxílio das estimativas Bayesianas e frequentistas contribuem para o direcionamento de coletas, bem como para o estabelecimento de cruzamentos preferenciais a serem realizados para o

estabelecimento de populações úteis aos programas de melhoramento genético do maracujá (passíveis de seleção massal, seleção recorrente, obtenção de híbridos sujeitos a heterose), bem como úteis à construção de mapas genéticos e identificação de QTLs (*Quantitative Trait Loci*).

O gradiente de resistência observado para os acessos de maracujazeiro (amarelo e roxo) em relação as três doenças avaliadas, assim como a variabilidade genética mensurada com base no polimorfismo genético observado com marcadores microssatélites, atestam à possibilidade de ganhos genéticos a partir de cruzamentos intraespecíficos para introgressão de genes de resistência nas cultivares existentes, bem como para lançamento de novas cultivares.

PERSPECTIVAS

Tendo em vista o caráter multi-institucional das atividades realizadas e os desdobramentos práticos de muitos dos resultados obtidos com a presente Tese de doutorado, é possível estabelecer metas de pesquisa a curto, médio e longo prazo. Em parte, as metas apresentadas a seguir estão contempladas nas perspectivas de trabalho traçadas pelo grupo de pesquisa e coordenadas por pesquisadores da Universidade Estadual do Sudoeste da Bahia - UESB (*campus* de Itapetinga, Bahia) e EMBRAPA Mandioca e Fruticultura (Cruz das Almas, Bahia), tendo como parceiros pesquisadores da Universidade Estadual de Santa Cruz - UESC, Universidade Estadual de Campinas - UNICAMP e dos demais *campi* da UESB e das unidades da EMBRAPA.

Em curto e médio prazo deverão ser intensificadas ações de pesquisa objetivando ampliar o número de espécies silvestres de maracujazeiro (*Passiflora* spp.) com locos microssatélites disponíveis e caracterizados. Para tanto, deverão ser utilizados acessos de espécies silvestres mantidas em bancos de germoplasma de maracujá (a exemplo dos bancos e coleções mantidos pela EMBRAPA, IAC, IAPAR, UNESP, ESALQ, entre outros). Como estratégia para identificação e caracterização dos locos microssatélites será utilizada, prioritariamente, a amplificação cruzada com base nos conjuntos de marcadores já disponibilizados e caracterizados para espécies comerciais e silvestres de maracujazeiro (Tabela 3).

Ainda no contexto da caracterização de germoplasma, pretende-se ampliar as estimativas de diversidade genética entre acessos de maracujazeiros mantidos em bancos e coleções, utilizando-se os marcadores microssatélites previamente avaliados. Inicialmente deverão ser considerados os acessos mantidos pela UESB e EMBRAPA, cujas avaliações já foram iniciadas pelo grupo de pesquisa.

Tomando por base as informações a serem obtidas com a realização das estimativas de diversidade presente nos bancos de germoplasma, bem como as estimativas de diversidade publicadas com base em marcadores dominantes para espécies silvestres (*P. cincinnata*, *P. setacea*, *P. trintae* e *P. nítida*) (CERQUEIRA-

SILVA et al., 2012a; 2010bc; JUNQUEIRA et al., 2007), e os resultados disponíveis a cerca dos estudos de filogeografia e variabilidade observada a partir da análise de sequências de DNA (MUSCHNER et al., 2012; MADER et al., 2010), pretende-se desenvolver projetos que permitam a realização dos primeiros estudos de genética de populações, baseados em marcadores microssatélites, para espécies silvestres de maracujazeiros.

Tabela 3. Número de locos microssatélites (SSR) desenvolvidos e caracterizados para diferentes espécies de maracujazeiro (*Passiflora* spp.).

Espécies (<i>Passiflora</i> spp.)	Número de locos SSR	Referências
<i>P. alata</i> Curtis	237	Pádua et al. 2005; Penha et al. 2013.
<i>P. cincinnata</i> Mast	25	Cerqueira-Silva et al. 2012b; Cerqueira-Silva et al. 2014.
<i>P. contracta</i> Vitta	7	Cazé et al. 2012.
<i>P. edulis</i> Sims	124	Oliveira et al. 2005; Oliveira et al. 2008. Cerqueira-Silva et al. 2014.
<i>P. pohlii</i> Mast	5	Pádua 2004.
<i>P. setacea</i> DC	52	Cerqueira-Silva et al. 2014.

É possível que a médio e longo prazo os estudos populacionais contribuam efetivamente para o entendimento da variabilidade genética presente em populações naturais de maracujazeiros e possibilitem a compreensão da estrutura desta variabilidade. Estas informações populacionais devem permitir a indicação de áreas prioritárias para conservação da variabilidade genética do grupo e, conseqüentemente, o direcionamento de ações de manejo e conservação. A disponibilidade de informações

a respeito da variabilidade genética das passifloras em populações naturais também permitirá uma estimativa mais realista a respeito da representatividade da variabilidade mantida nos bancos de germoplasma. Além do que, em conjunto, as informações a serem geradas com as caracterizações genéticas possibilitarão o direcionamento de pontos estratégicos para coleta de novos acessos a serem inseridos, conservados e caracterizados nos bancos de germoplasma.

A médio e longo prazo, também considerando os avanços das pesquisas genômicas relacionadas aos maracujazeiros, a exemplo da construção de bibliotecas de *Bacterial Artificial Chromosome* - BAC (PENHA, 2012), identificação de variantes *single nucleotide polymorphism* - SNPs (PEREIRA et al., 2013; PEREIRA, 2010) e desenvolvimento e caracterização de bibliotecas de sequências expressas (Cutri e DORNELAS, 2012; YOTOKO et al., 2011), têm-se como perspectivas o estabelecimento de plataformas para efetivo uso das técnicas de genotipagem-por-sequenciamento - GBS (ELSHIRE et al., 2011) em programas de seleção genômica e em avaliações de variabilidade populacional.

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ARTIGO 1.

SANTOS E.S.L., CERQUEIRA-SILVA C.B.M., MORI G.M., AHNERT D., CORRÊA R.X., SOUZA A.P (2012) **New polymorphic microsatellite loci for Theobroma cacao: isolation and characterization of microsatellites from enriched genomic libraries.** *Biologia Plantarum*, 1-4.

ARTIGO 2.

CONCEIÇÃO L.C.D.H., BELO G.O., SOUZA M.M., SANTOS S.F., CERQUEIRA-SILVA C.B.M., CORRÊA R.X (2011). **Confirmation of cross-fertilization using molecular markers in ornamental passion flower hybrids.** *Genetics and Molecular Research* 10 (1), 47-52.

DECLARAÇÃO - Comissão Interna de biossegurança

BRIEF COMMUNICATION

New polymorphic microsatellite loci for *Theobroma cacao*: isolation and characterization of microsatellites from enriched genomic libraries

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Abstract

Seventeen polymorphic microsatellite markers were isolated from enriched genomic libraries for *Theobroma cacao*, providing additional tools for studying the genetic diversity and map saturation of this species. These markers were characterized in 32 accessions of the *T. cacao* germplasm collection from the Centro de Pesquisas do Cacau. The number of alleles at each locus varied from 2 to 8, with an average of 4.41 alleles per locus. The polymorphism information content varied from 0.060 to 0.695, with an average of 0.333. The markers characterized in this study will be employed in map saturation studies and diversity assessments of cacao genotypes.

Additional key words: cacao, genetic diversity, molecular markers, PCR.

Theobroma cacao L. (2n=20) is a neotropical plant that is native to the northern regions of South America and has been domesticated in Central America (Motamayor *et al.* 2002). Among the 22 species of the *Theobroma* genus cacao is the only species cultivated on a large scale for chocolate production (Luz and Silva 2001). International genetic breeding programs for cacao have been developed as a means of obtaining cultivars with improved productivity, increased resistance and good industrial quality (Guiltinan *et al.* 2008). As a useful tool for genetic studies, molecular markers, especially microsatellite markers, have been developed for different species, such as *Jatropha curcas* (Phumichai *et al.* 2011) and *Aegiphila sellowiana* (Ruas *et al.* 2011). Specifically

in cacao, some simple sequence repeat (SSR) markers have already been developed (Lanaud *et al.* 1999, Pugh *et al.* 2004, Araújo *et al.* 2007, Lima *et al.* 2008) and have been used for diversity studies and breeding programs (Aikpokpodion *et al.* 2009, Lanaud *et al.* 2009, Yamada *et al.* 2009). To study the genetic diversity and genetic map saturation of *T. cacao*, we isolated and characterized a new set of polymorphic microsatellites from *T. cacao*. These markers may also be used for future genetic studies of this species.

To identify and characterize microsatellites, two microsatellite-enriched libraries for *T. cacao* were developed following the protocol described by Billotte *et al.* (1999) from two phenotypically and genetically

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Abbreviations: CTAB - cetyltrimethylammonium bromide; HWE - Hardy-Weinberg equilibrium; LD - linkage disequilibrium; PCR - polymerase chain reaction; PIC - polymorphism information content; SSR - simple sequence repeat; TBE - Tris/borate/EDTA.
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distinct clones: TSH 1188 (Upper Amazon rainforest of Trinidad) and GU 261 (Lower Amazon rainforest of French Guiana). These clones were sampled from the germplasm collection at the Centro de Pesquisas do Cacau/Comissão Executiva do Plano da Lavoura Cacaueira (CEPEC/CEPLAC) in Itabuna, Bahia, Brazil. Genomic DNA was isolated from fresh leaves from each clone using a cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990) with modifications specific for cacao (Faleiro *et al.* 2002). The DNA samples (5 µg) were digested with *AfaI* and ligated to the double-stranded adapters 5'-CTCTTGCTTACGCGTGGACTA-3' and 5'-TAGTCCACGCGTAAGCAAGAGCACA-3'. The enrichment was performed using a hybridization-based capture with (GT)₈ and (CT)₈ biotin-linked probes and magnetic-coated streptavidin beads (streptavidin *MagneSphere* paramagnetic particles, *Promega*, Madison, WI, USA). Selected fragments were then cloned into a *pGEM-T Easy* (*Promega*) plasmid vector and transformed into *Escherichia coli* XL1-Blue competent cells (*Stratagene*, La Jolla, CA, USA). Recombinant

colonies were selected by blue/white screening. Clones were randomly selected and were double-sequenced using the T7 and SP6 primers and the *v3.1 Big Dye* terminator kit (*Applied Biosystems*, Foster City, CA, USA) on an *ABI PRISM 377* automated DNA sequencer (*Applied Biosystems*). Every obtained sequence was aligned, edited and eliminated if redundant using *SeqMan* software (*DNASTAR*, Madison, WI, USA). *MICROSAT* software (A.M. Risterucci, CIRAD, personal communication) was used to eliminate adapters and restriction sites from the sequences. The simple sequence repeat identification tool (*SSRIT*) (Temnykh *et al.* 2001) was used to identify the following microsatellites: di- and trinucleotides with four or more repeats; and tetra- and pentanucleotides with three or more repeats.

Primer pairs were designed using the *PrimerSelect* (*DNASTAR*) and *Primer3Plus* (Untergasser *et al.* 2007) programs. The polymerase chain reaction (PCR) was conducted in a final volume of 0.015 cm³ containing 15 ng of template DNA, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 0.2 µM of

Table 1. Characteristics of the forward (F) and reverse (R) primers, repeat motif, and amplification conditions (AC) of 17 microsatellite loci from *Theobroma cacao* L. * - TD65-55 indicates touchdown PCR with temperatures ranging from 65 to 55 °C.

Locus	GenBank acc.	Primer sequence (5'-3')	Repeat motif	AC*
mTc-UNICAMP01	JF812968	F: TCATGCAAAGCAAAGTGAAG R: ACGGGAAACTCATCATTACA	(AG) ₁₁	TD65-55
mTc-UNICAMP02	JF812969	F: GGTCCCTCCAAGCTGAGTAACA R: CTCCCTATTTGCATCGCATT	(TC) ₁₅	TD65-55
mTc-UNICAMP03	JF812970	F: GCGATGCCAAAAGTTGTGTA R: AGAGAAGGGATGGGTGTGTG	(AG) ₁₆	TD65-55
mTc-UNICAMP04	JF812971	F: GCAGCGAGAGACAAAGATA R: TTTGACTGAAATGGTGGTAA	(TC) ₂₁	60 °C
mTc-UNICAMP05	JF812972	F: AGCTGTTATGATTACATCC R: GAAGCAGCAATTGTAACCAC	(TA) ₇ (CA) ₆	60 °C
mTc-UNICAMP06	JF812973	F: AACTCTGGCATTTCATTGG R: GTGATGGATGCAGGTGGAAT	(TC) ₁₆ TGT(CA) ₁₀	60 °C
mTc-UNICAMP07	JF812974	F: CATGCCTGAGCTATGAACA R: TCAGATATGGGTAAAGGAAGAG	(GA) ₁₁ ...(GA) ₅	TD65-55
mTc-UNICAMP08	JF812975	F: GGGAAACTGGGCATCACTTA R: TTAATGGGTGAGGAGAGCAT	(AC) ₅ AG(AT) ₉	60 °C
mTc-UNICAMP09	JF812976	F: TTCGGCAGTTCGATCTATGA R: ATCCACCGTAAGCCTTTCCT	(TAAAG) ₇	TD65-55
mTc-UNICAMP10	JF812977	F: AACTGTCGCATACGCTCATC R: TCAAAAAGTGAAGCCCACCAT	(AG) ₉	60 °C
mTc-UNICAMP11	JF812978	F: GCTTGTTCTCGACACACAT R: TGGAAGCTAGTTAGGAATCACA	(CA) ₆	60 °C
mTc-UNICAMP12	JF812979	F: GCCATCTCGCTATTCATGTG R: GTCATTGCTAACGTAACCAGAA	(AG) ₁₆	60 °C
mTc-UNICAMP13	JF812980	F: ACTTGTCGCTTGCTTCCTGT R: TTCCTCAACCTCTCCGAATG	(AT) ₆	TD65-55
mTc-UNICAMP14	JF812981	F: ACATGATGCCTGAGCTGTTG R: CTCCAATTTTGACCCCAGAA	(AG) ₁₁	TD65-55
mTc-UNICAMP15	JF812982	F: TGCTTTGAGGCACTTGTC R: TGGCATTGATTGAGAGGTGA	(TG) ₁₂	TD65-55
mTc-UNICAMP16	JF812983	F: CAGGAAGGATACTTCTTAAAGG R: AGTAGAGTCGAGTGGCTTGA	(CT) ₁₂ (CA) ₉ ...(CT) ₄	TD65-55
mTc-UNICAMP17	JF812984	F: CTGCACAGCTTCATGGACTC R: TGATGATCAGGTGGTTTCTCA	(CA) ₉	TD65-55

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Table 2. Characteristics of the product size range in base pairs, number of effectively evaluated genotypes (N), number of alleles (N_A), observed (H_o) and expected (H_E) heterozygosity, polymorphism information content (PIC) and P value of Hardy-Weinberg equilibrium (HWE) for 17 microsatellite loci from *Theobroma cacao* (* - departs significantly from HWE at $P < 0.003$ after the Bonferroni correction, † - significant values for 'null allele' occurrence after the Bonferroni correction).

Locus	Size range	N	N_A	H_o	H_E	PIC	P value
mTc-UNICAMP01	190-208	29	7	0.269	0.286	0.273	0.194
mTc-UNICAMP02	260-290	31	8	0.381	0.746	0.695	0.000*†
mTc-UNICAMP03	282-291	31	5	0.433	0.719	0.661	0.000*
mTc-UNICAMP04	162-186	30	7	0.786	0.742	0.695	0.024
mTc-UNICAMP05	233-242	29	4	0.269	0.544	0.454	0.003†
mTc-UNICAMP06	140-170	32	4	0.107	0.123	0.120	0.032
mTc-UNICAMP07	265-312	28	4	0.240	0.225	0.211	1
mTc-UNICAMP08	278-286	32	5	0.581	0.673	0.605	0.044
mTc-UNICAMP09	148-190	31	6	0.032	0.667	0.596	0.000*†
mTc-UNICAMP10	230-236	32	3	0.094	0.091	0.088	1
mTc-UNICAMP11	290-296	32	2	0.226	0.204	0.180	1
mTc-UNICAMP12	240-290	32	6	0.226	0.369	0.347	0.008†
mTc-UNICAMP13	140-144	30	2	0	0.066	0.064	0.017†
mTc-UNICAMP14	254-258	28	3	0.174	0.166	0.156	1
mTc-UNICAMP15	292-302	32	2	0.067	0.064	0.060	1
mTc-UNICAMP16	244-254	31	4	0.207	0.255	0.240	0.153
mTc-UNICAMP17	296-300	29	3	0.174	0.236	0.213	0.311

each dNTP, 0.5 μ M of each primer and 1 U of *Taq* DNA polymerase (*Invitrogen*, Carlsbad, CA, USA). All primers were first evaluated in a PCR amplification protocol with a 60 °C annealing temperature as follows: 94 °C for 5 min; 34 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 10 min. Later, if necessary, touchdown PCR was performed. The touchdown protocol consisted of 5 steps as follows: a) initial denaturation (94 °C for 5 min); b) 10 cycles of denaturation (94 °C for 1 min), annealing (65 - 1 °C per cycle for 1 min) and extension (72 °C for 1 min); c) step b repeated; d) 14 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 1 min); e) a final extension (72 °C for 10 min). The amplification products were visualized in 0.5× TBE/3 % agarose gels stained with ethidium bromide prior to vertical electrophoresis in 1× TBE/6 % denaturing polyacrylamide silver nitrate-stained gels. Product sizes were determined by comparison with a 10 bp DNA ladder (*Invitrogen*). Statistical analyses were performed with *GENEPOP* on the web (Raymond and Rousset 1995), *PIC Calculator* (Kemp 2002) and *MICRO-CHECKER* (Oosterhout *et al.* 2004).

In all, 192 positive colonies were sequenced and 163 microsatellites were obtained. Dinucleotide motifs were the most abundant, followed by tri-, tetra- and penta-nucleotide motifs (with proportions of approximately 94, 3, 2 and 1 %, respectively). Thirty-five primer pairs were designed such that the product size ranged between 100 and 300 bp for accurate genotyping using vertical electrophoresis in 1× TBE/6 % denaturing polyacrylamide gels. Twenty-five of these primer pairs generated consistent patterns of amplification, with fragments matching size expectations based on the sequence information, and these pairs were used for

further characterization. For polymorphism analysis, 32 DNA samples of *T. cacao* were extracted and analyzed from the germplasm collection of *CEPEC/CEPLAC*. To sample greater genetic variation, we selected genotypes collected from the Amazon in addition to Bahia cacao farms. Of these 25 primer pairs evaluated, 17 tagged polymorphic loci whose numbers of alleles varied (Table 1). To measure the frequency of identity between the polymorphic loci presented here and those described in the literature, we used nucleotide *BLAST* (Altschul *et al.* 1990) (using the megablast algorithm). We observed identity (varying from 95 to 97 %) to four sequences (loci: mTc-UNICAMP03, 04, 07, 09). However, these primers had annealing sites different from those described previously. Of these four loci, three (mTc-UNICAMP03, 04 and 07) were similar to microsatellite loci published by Pugh *et al.* (2004) and are included here to evaluate statistical parameters such as polymorphism information content (PIC) and Hardy-Weinberg equilibrium (HWE). No previous paper has described locus mTc-UNICAMP09. The mean number of alleles was 4.41 (range: 2 to 8 alleles), and the PIC varied from 0.060 to 0.695. Genotypic frequencies of the 17 polymorphic microsatellite markers were tested for HWE and linkage disequilibrium (LD) using the Bonferroni correction. The analysis revealed significant deviations from HWE at three microsatellite loci (Table 1), possibly due to the occurrence of null alleles (as observed for mTc-UNICAMP02 and 09) or LD (as observed for mTc-UNICAMP02, 03 and 09). Significant LD was observed among 19 of 136 possible pairs of markers (mTc-UNICAMP01 and 04, 05, 11, 12; mTc-UNICAMP03 and 07, 11; mTc-UNICAMP05 and 14; mTc-UNICAMP12 and 03, 04, 05, 11; mTc-UNICAMP16 and 01, 02, 03, 06, 07, 09, 11, 12).

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The probability of 'stuttering', 'large allele dropout' and 'null alleles' was also evaluated. No microsatellite loci showed a significant probability of 'stuttering' or 'large allele dropout'. Five loci (mTc-UNICAMP02, 05, 09, 12, 13) showed significant values for 'null alleles' according to the Bonferroni correction ($P < 0.003$). Eight

other microsatellite markers were monomorphic for the evaluated population.

Microsatellite markers characterized in this study may be used in future genetic map saturation and genetic diversity studies of cacao genotypes.

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Short Communication

Confirmation of cross-fertilization using molecular markers in ornamental passion flower hybrids

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ABSTRACT. Several interspecific *Passiflora* hybrids are produced in the northern hemisphere for the ornamental plant market. In Brazil, production of passion flower hybrids is limited to the introgression of genes into the main cultivated species, yellow passion fruit, to be used as rootstocks. Confirmation of hybridization in the initial developmental stage is important for breeding perennial and sub-perennial plants, such as passion flowers, reducing time and costs in plant stock maintenance. In order to obtain F₁ hybrids with ornamental potential, four species of *Passiflora* (*P. alata*, *P. gardneri*, *P. gibertii*, and *P. watsoniana*) from the Active Germplasm Bank at UESC were hybridized. Flower buds, in pre-anthesis, of the genitors were previously protected, and the female buds were emasculated. To confirm hybridization, the genomic DNA of the genitor species and the supposed hybrids was extracted and RAPD primers were used

to obtain molecular markers and select passion flower interspecific hybrids. Eight primers were used to confirm hybrids derived from *P. gardneri* with *P. alata*, *P. watsoniana* with *P. alata*, *P. watsoniana* with *P. gardneri*, and *P. gardneri* with *P. gibertii*; 75, 50, 45, and 46% of the informative bands, respectively, confirmed the hybrid nature of these plants. The RAPD technique was effective in the early identification of hybrids; this will be useful for development of hybrid *Passiflora* progeny.

Key words: *Passiflora* L.; Interspecific hybridization; RAPD; Paternity test

INTRODUCTION

Hybridizations between species of the genus *Passiflora* have been carried out in North American and European countries to create and trade several hybrids in the ornamental plant market (Vanderplank, 2000; Ulmer and MacDougal, 2004). Due to the aesthetic attributes of exotic flowers and leaves, and to the great inter- and intraspecific variability, wild passion flowers have been used to decorate gardens and greenhouses since their introduction in the Old World, around 1625 (Ulmer and MacDougal, 2004; Peixoto, 2005). Hybrid passion flowers were first reported in 1819, when Thomas Milne crossed *P. racemosa* Brot. x *P. caerulea* L. and produced the hybrid *P. violacea* (Vanderplank, 2000). In the beginning of the 20th century, there was a considerable increase in hybrid production with ornamental potential, including *P. 'Amethyst'* Vanderplank (unknown genitors); *P. 'Asterix'* Vecchia (*P. 'Barbora'* x *P. caerulea* 'Constance Elliott'); *P. 'Colet'* Sevcik (*P. racemosa* x *P. naviculata*); *P. 'Lady Margaret'* Skimina (*P. coccinea* x *P. caerulea* 'Constance Elliott') (Vanderplank, 2002). There are more than 600 registered hybrids in the International *Passiflora* Society (www.passionflow.co.uk/reg.htm). The largest production center of hybrids is in Germany, controlled by the breeder Dr. Roland Fischer, who bases his work on the induction of polyploidy in selected plants for cultivation in vases (Peixoto, 2005).

In Brazil, the potential of passion flowers as ornamental plants is almost non-explored. In 2007, the first ornamental cultivars of passion flower were released: Roseflora (F_1 *P. coccinea* Aubl. x *P. setacea* DC. x *P. setacea* DC.), Rubiflora (F_1 *P. coccinea* Aubl. x *P. setacea* DC. x *P. coccinea* Aubl.) and Estrela do Cerrado (*P. coccinea* Aubl. x *P. setacea* DC.) (Faleiro et al., 2009). Hybrids have also been produced in Brazil's northeast region for ornamental use, including *P. 'Alva'*, *P. 'Priscilla'* and *P. 'Aninha'* (Santos, 2008), originating from the crossing of *P. palmeri* var. *lanceolata* x *P. foetida* var. *foetida*. The sustainable use of biodiversity and genetic variability in the genus *Passiflora* may offer a diversification alternative for agrobusiness or family agriculture (Abreu et al., 2009).

The use of molecular techniques provides a fast and accurate assessment of paternity at relatively low cost. Random amplified polymorphic DNA (RAPD) markers have been successfully used to confirm hybrids in cross-fertilization (Junqueira et al., 2008). Confirmation strategy based on DNA eliminates the possibility of developing double haploids (Nimura et al., 2003) and of contamination (Faleiro et al., 2003; Junqueira et al., 2008). Moreover, the confirmation of hybrid plants in the initial development stage is important for reducing time and costs in the maintenance of the plants. Therefore, in addition to the time gain and savings of resources from the use of RAPD markers, higher efficiency and dependability in hybrid confirmation may lead to better results for breeding programs. This study aimed at confirming the hybrid nature

of plants originating from crossings of passion flower wild species, through the use of RAPD markers, focusing on the selection of genotypes with ornamental potential.

MATERIAL AND METHODS

The study was carried out with the species *P. alata* Curtis, *P. gardneri* Mast., *P. gibertii* N.E. Brow, and *P. watsoniana* Mast. The plants were kept in the Bank of Active Germplasm at UESC (BAG-Passifloras), located at the campus of Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil (39° 10' W, 14° 39' S; alt. 78 m). For the hybridization procedure, the flower buds were protected with paper bags. The flowers were emasculated, pollinated and protected again. Between 40 and 60 days after the pollination, the fruits were collected. The seeds were washed, dried at room temperature and stored in paper bags at + 5°C. For germination, the seeds were treated in sodium hypochlorite (2.5% of active chloride) for 2 min and rinsed five times. The seeds were scarified with partial removal of the tegument and afterward placed on filter paper (three layers); they were watered daily with a solution containing 100 mg/L gibberellic acid (Ferreira et al., 2005). Around 20 days after germination, the seedlings were transferred to polystyrene trays containing black soil substrate until they reached the proper stage for transplanting.

DNA was extracted from seedlings between 40 and 60 days after germination and used for amplifications by the polymerase chain reaction (PCR). The DNA extraction method used was that described by Doyle and Doyle (1990). The amplification reactions were carried out in duplicate using a total volume of 25 µL containing 10 mM Tris-HCl, pH 8.3, 2 mM MgCl₂, 100 µM of each desoxyribonucleotide (dATP, dTTP, dGTP, and dCTP), 0.4 µM primer, 1 U Taq polymerase and around 30 ng DNA. Four to eight RAPD primers were tested, which were from an Operon kit and from the University of British Columbia (Table 1). In each PCR, DNA samples of male and female genitors were used, in addition to one sample of each hybrid plant per crossing. The amplifications were carried out in a thermal cycler programmed for 40 cycles (1 min at 94°C, 1 min at 32°C and 2 min at 72°C). After 40 cycles, a final extension phase of 7 min at 72°C was carried out, and finally, the temperature was reduced to 15°C. The separation of RAPD fragments was carried out on a 1.6% (w/v) agarose gel stained with ethidium bromide, and the results were photodocumented under a UV transilluminator. The molecular markers generated by the different RAPD primers were analyzed in terms of presence or absence of informative bands to confirm cross-fertilization. Only the informative bands with high reproducibility and definition were considered.

Table 1. Sequences of the eight RAPD decamer primers used to confirm cross-fertilization.

Primer	Sequence 5'-3'
OPD01	(5'-ACCGCGAAGG-3')
UBC03	(5'-CCTGGGTCCA-3')
UBC04	(5'-CCTGGGTGGA-3')
UBC06	(5'-GCCCCGGTTA-3')
UBC11	(5'-CCGGCCTTAC-3')
UBC17	(5'-CTTACCCGTGC-3')
UBC23	(5'-GTCCACACGG-3')
UBC25	(5'-ACCCCCGCCG-3')

Primers from University of British Columbia (UBC) and Operon (OPD).

RESULTS

From the crossings carried out between *Passiflora* species, 22, 37, 51, and 31 seedlings were obtained from the following hybridizations *P. gardneri* vs *P. alata*, *P. watsoniana* vs *P. alata*; *P. watsoniana* vs *P. gardneri*, and *P. gardneri* vs *P. gibertii*, respectively. All four crossings had their hybrids confirmed by the presence of at least four informative bands (Figure 1; Table 2). The highest percentage of positive informative bands (present in the hybrids) was obtained in crosses between *P. gardneri* and *P. alata* (75%). However, only six were considered to be markers of four primers. We used the largest number of primers in the cross *P. gardneri* vs *P. gibertii*, but only 46% of the bands were considered to be informative, confirming the cross-fertilization. The crossing between *P. watsoniana* vs *P. alata* indicates that half of the primers used did not produce informative bands, but four bands did confirm the hybrids.

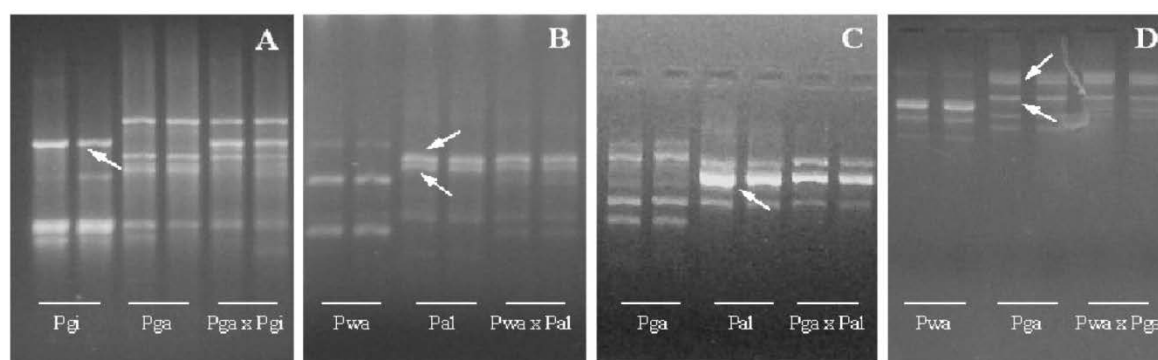


Figure 1. Standard amplification of samples of genomic DNA of the genitors and interspecific hybrids. **A.** *Passiflora gardneri* (Pga) vs *P. gibertii* (Pgi), with UBC23 primer. **B.** *P. watsoniana* (Pwa) vs *P. alata* (Pal) with UBC11 primer. **C.** *P. gardneri* vs *P. alata* with OPD01 primer. **D.** *P. watsoniana* vs *P. gardneri* with UBC04 primer. Arrows indicate informative bands.

Table 2. Number of RAPD informative bands amplified from DNA of the male genitor and the interspecific hybrids of passion flowers using eight primers.

Primer	Crossings			
	<i>P. gardneri</i> vs <i>P. gibertii</i>	<i>P. gardneri</i> vs <i>P. alata</i>	<i>P. watsoniana</i> vs <i>P. alata</i>	<i>P. watsoniana</i> vs <i>P. gardneri</i>
UBC03	1	-	0	-
UBC04	1	2	4	4
UBC06	3	1	-	-
UBC11	4	-	2	-
UBC17	0	-	2	-
UBC23	2	-	0	4
UBC25	2	1	0	0
OPD01	-	2	-	3
Bands	13	6	8	11
Bands +	6	4	4	5
%	46	75	50	45

Bands + confirm hybrids.

DISCUSSION

The presence of one or more informative bands with a small number of primers can provide the confirmation of cross-fertilizations (Faleiro et al., 2003). Similar results were obtained in hybrids between *P. palmeri* var. *sublanceolata* vs *P. foetida* var. *foetida*, using almost the same RAPD primers used in this study, and interspecific hybridizations were confirmed (Santos, 2008). The absence of informative bands in some primers may be explained by low reproducibility, weak fragment amplification or even their low monomorphic features among the parents. Junqueira et al. (2008) confirmed 17 interspecific hybrids between *Passiflora* species, by the presence of informative bands, using RAPD markers. The technique also proved to be efficient in proving that plants obtained from a fruit of *Theobroma cacao*, after crossing with *T. grandiflorum*, were not obtained by cross-fertilization, but rather by self-fertilization (Faleiro et al., 2003).

The species involved in the crossings show important characteristics for ornamental uses, considering aspects related to floescence and features of flowers and leaves. *P. alata* has 10- to 12-cm shiny pendulous flowers, 10-12 cm in diameter, with magenta petals and coronal filaments with several shades of red, white and purple (Nunes and Queiroz, 2006). On the other hand, *P. watsoniana* is smaller than *P. alata*, which is important for the reduction of such features and selection of indoor plants. The glabrous leaves, with purple abaxial face of *P. watsoniana*, are also attractive in terms of leaves.

Development and growth studies indicate that *P. gibertii* is precocious when compared to *P. edulis* f. *flavicarpa* and *P. cincinnata*. The precocity of the presence of reproductive organs in *P. gibertii* has already been observed, evident in the main branch and in the secondary branches, compared to other species, at which time, are still in the vegetative phase (Silva et al., 2005a). The time of flower opening for *P. gibertii* is another attractive feature for its use in hybridizations: its flowers open at 11 am and remain open until 2 pm on the following day, totaling up to 18 h of opening (Silva et al., 2005b).

P. gardneri has flowers that are interesting for decoration, which vary from 6 to 8 cm in diameter, with violet petals and corona filaments. One important aspect concerning *P. gardneri* is the easy compatibility in interspecific crossings. Attempts to obtain hybrids of *P. watsoniana* and *P. alata* with the species *P. gibertii* failed (data not shown). Thus, indirect crossings would be a possible alternative, aiming at the transfer of features of *P. gibertii* in triple hybrids, involving F1 (*P. gardneri* vs *P. gibertii*) and the species *P. watsoniana* or *P. alata*.

The RAPD technique is effective in the early identification of hybrids. The selection of individuals in the hybrid progeny, with recombinant traits of the parents involved in crosses, is promising in obtaining new ornamental hybrids.

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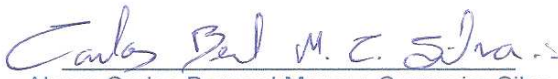
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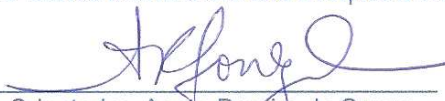
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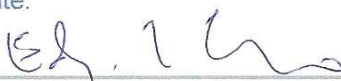
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