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RESEARCH PAPER

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Evolving in isolation: Genetic tests reject recent connections of Amazonian savannas with the central Cerrado

Luciana C. Resende-Moreira[1,2] | L. Lacey Knowles[2] | Andréa T. Thomaz[2] | Joyce R. Prado[3] | Andrea P. Souto[1] | José P. Lemos-Filho[4] | Maria Bernadete Lovato[1]

[1]Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

[2]Department of Ecology and Evolutionary Biology, Museum of Zoology, University of Michigan, Ann Arbor, Michigan

[3]Departamento de Ciências Biológicas, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, Piracicaba, SP, Brazil

[4]Departamento de Botânica, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Correspondence
Maria Bernadete Lovato, Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Caixa Postal 486, 31270-901, Belo Horizonte, Minas Gerais, Brazil.
Email: lovatomb@icb.ufmg.br

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Abstract

Aim: The effects of past climatic shifts remain enigmatic for the Amazon region, especially for islands of savanna within the tropical forest known as "Amazonian savannas" (AS). These disjunct savanna areas share many plant and animal species with the Cerrado biome in central Brazil (the CC), fuelling debate over historical connections. We evaluate hypothesized corridors between the CC and the AS, and specifically investigate whether a history of isolation versus recent connections is supported by genetic tests.

Location: Cerrado and Amazon biomes.

Taxon: Two woody plant species: *Byrsonima coccolobifolia* and *B. crassifolia* (Malpighiaceae).

Methods: Analyses of genomic data (SNPs from more than 4,500 loci) in 28 populations, as well as chloroplast DNA (cpDNA), were used to test for parallel geographic structuring between the CC and AS—an expected structure if putative corridors provided regional connections between different areas of the CC and AS, and divergence times between the CC and AS were estimated using a composite-likelihood method based on the site frequency spectrum.

Results: Genomic data, in contrast with cpDNA, generally show strong, concordant genetic structure between the CC and AS in both species, rather than regional grouping of CC with AS populations. In addition, divergence between the CC and AS predates the Last Glacial Maximum.

Main conclusions: Our results suggest the AS have remained relatively isolated from the CC even though the strong structure of genomic variation is not shared by cpDNA. We note that past evidence of putative corridors between the CC and AS based solely on cpDNA should be interpreted cautiously since the lack of structure may reflect limited genetic resolution rather than gene flow. As such, the uniqueness of AS may be more pronounced than previously thought, highlighting the importance of protecting these highly threatened areas.

KEYWORDS
Amazon, *Byrsonima*, Cerrado, corridor, Malpighiaceae, phylogeography, RAD-seq, relict, savanna

Luciana C. Resende-Moreira and L. Lacey Knowles contributed equally to this work.

1 | INTRODUCTION

Climate change has induced historical shifts in landscapes, including the fragmentation of once widespread biomes into relatively isolated patches. The persistence of such populations and the evolutionary dynamics shaping their current genetic structure are commonly considered in studies of the Northern hemisphere following the glacial retreat of the Pleistocene (Hewitt, 2004; Knowles & Massatti, 2017; Pielou, 1992). However, the impact of past climatic shifts is not unique to these areas. The effects of climatic extremes are worldwide, with documented shifts of biomes leaving behind relict populations (e.g., Bonatelli et al., 2014; Migliore et al., 2013; Ornelas, Ruiz-Sanchez, & Sosa, 2010). However, tropical regions remain critically understudied relative to their northern counterparts. The evolutionary history of many tropical biomes is also enigmatic because of particularly sparse palynological or fossil evidence (e.g., Jaramillo et al., 2010) and limited or inconsistent support for a range of different hypotheses regarding the magnitude of climate-induced distributional shifts.

Such uncertainty is exemplified by debates over the evolutionary history of the central Cerrado (CC) and Amazonian savannas (AS) of Brazil (Figure 1). The CC is a hyper-diverse, yet relatively understudied savanna biome that covers over 2 million km^2. Many plant and animal taxa (including over 70 woody species) are present in the CC and AS, with some AS displaying higher floristic similarity to locations within the CC than to geographically proximate AS (Ratter, Bridgewater, & Ribeiro, 2003), suggesting past connections between the CC and AS (Prance, 1996; Silva, 1995; Silva & Bates, 2002), rather than independent long-distance dispersal events (see Pennington, Lewis, & Ratter, 2006). However, different hypotheses narrate how the retraction of the Cerrado from its former maximum extent might have occurred, which include past connections—that is, corridors—between the Cerrado and areas where AS persist today. Where such corridors might have existed, and which geographic areas they might have connected are still debated. For example, three different corridors between the CC and AS have been proposed: a coastal corridor, a central Amazonian corridor, and an Andes corridor (Haffer, 1967, 1974; Webb, 1991). Depending on the study, support for hypothesized corridors differ, as does the purported timing of past connections between the CC and AS (e.g., Bueno et al., 2017; Quijada-Mascareñas et al., 2007; Savit & Bates, 2015; Vargas-Ramírez, Maran, & Fritz, 2010; Werneck, Nogueira, Colli, Sites, & Costa, 2012). That is, the uncertainty over the geographic location of corridors is paralleled by debate over when such connections might have occurred (e.g., during the Miocene and Pliocene, Pascual & Jaureguizar, 1990; versus the Pleistocene, Haffer, 1969; Prance, 1982; van der Hammen, 1991), including whether such connections might have been forged during the drier climate of the Last Glacial Maximum, LGM, especially given the lack of support for such late Pleistocene expansion based on palynological evidence (Colinvaux, Irion, Rasanen, Bush, & de Mello, 2001; Kastner & Goni, 2003; Mayle, Burn, Power, & Urrego, 2009).

Here, we address the extent to which the AS have evolved in isolation from the CC by quantifying population genetic structure of two widely distributed tree species that are common in both the CC and AS—*Byrsonima coccolobifolia* Kunth and *Byrsonima crassifolia* (L.) Kunth (Ratter et al., 2003). Specifically, we test the degree to which Cerrado populations are genetically distinct from the AS, as opposed to exhibiting parallel geographic structuring of genetic variation within the CC and among AS, as expected if multiple corridors provided regional connections between different areas of the CC and different subsets of AS. We conducted this test using genomic data (i.e., more than 7,000 and 4,500 loci sequenced in 86 and 68 individuals of *B. coccolobifolia* and *B. crassifolia*, respectively), as well as assays of the geographic structure of chloroplast DNA (cpDNA) across an even broader sampling of populations. In addition to the individual histories, we consider the degree to which the taxa show concordant patterns of genetic variation. As ecologically similar, dominant and co-distributed taxa, concordance would lend support to common factors structuring the history of constituent taxa in this diverse biome (Avise, 2004), thereby overriding stochastic processes associated with the biomes dynamic history (Behling & Hooghiemstra, 2001; Ledru, 2002; but see Massatti & Knowles, 2014, 2016). Lastly, we estimate divergence times between the CC and AS to determine how long the AS may have been evolving independently of the CC.

2 | MATERIAL AND METHODS

2.1 | Study species

Byrsonima Rich. Ex Kunth is a common genus, with most of its diversity represented by South American savanna taxa, many of which co-occur (Anderson, Anderson, & Davis, 2006; Ratter et al., 2003). *Byrsonima coccolobifolia* and *B. crassifolia* are the most common species from the genus in the Cerrado and in the Amazonian savannas (Ratter et al., 2003), with the range of *B. crassifolia* extending into the savanna woodlands of Central America and Mexico (Anderson, 1981). Its fleshy fruits are bird-dispersed (Anderson, 1983) and flowers are pollinated by oil-collecting bees, especially *Centris* species (Benezar & Pessoni, 2006; Vinson, Williams, Frankie, & Shrum, 1997).

2.2 | Sampling and DNA extraction

Population sampling of *B. coccolobifolia* and *B. crassifolia* covered both species' ranges across the CC and AS (Figure 1; for details see Table S1.1, Appendix S1 in Supporting Information) and was informed by occurrence data from NeoTropTree (Oliveira-Filho, 2017) and the INCT—Virtual Herbarium of Flora and Fungi (http://inct.splink.org.br/index). A total of 158 individuals across 16 populations of *B. coccolobifolia* and 15 populations of *B. crassifolia* were sequenced using RADseq (described below; see also Table 1 and Figure 1). In addition, cpDNA was sequenced in a larger set of populations (i.e., 46 populations and 218 individuals; for detailed sampling information see Appendix S2). Voucher specimens of all sampled populations were deposited in the Herbarium of Departamento de Botânica, Universidade Federal de Minas Gerais (BHCB) and the Herbarium of Universidade Estadual do Oeste do Paraná (UNOP).

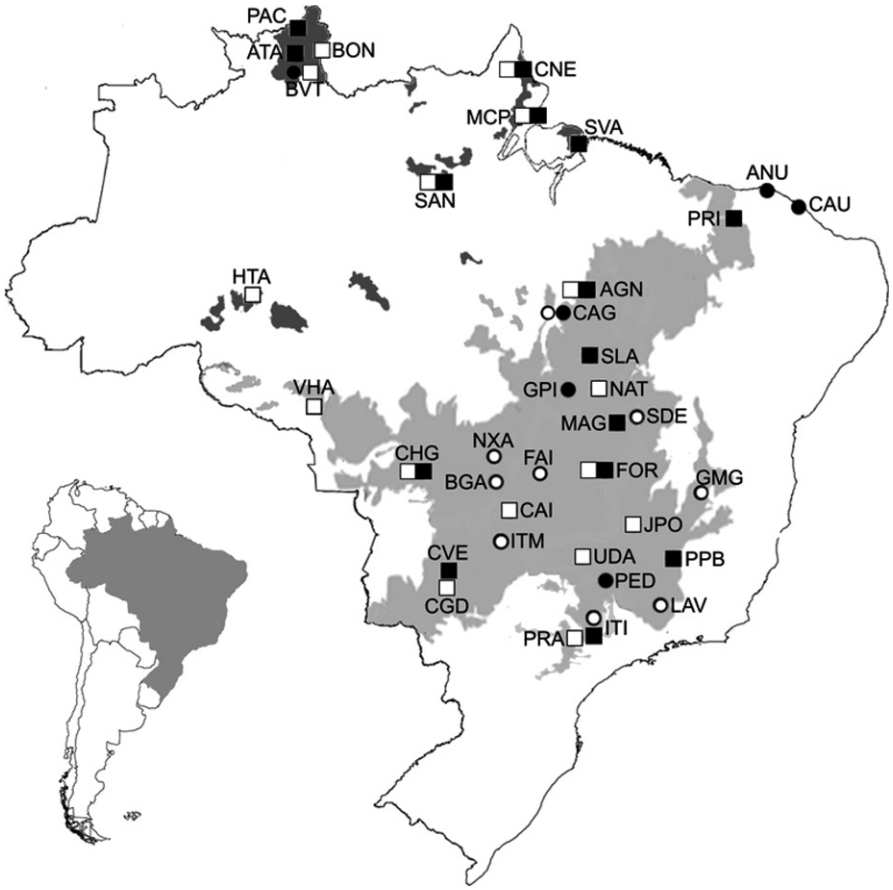

FIGURE 1 Geographic location of sampled *Byrsonima coccolobifolia* and *B. crassifolia* populations (white and black symbols, respectively) across the Cerrado (light grey) and Amazonian savannas (dark grey). Populations with both genomic and cpDNA sequences are marked by squares, whereas those with only cpDNA are marked by circles

DNA was extracted using a CTAB protocol (Novaes, Rodrigues, & Lovato, 2009) from silica-gel dried leaves that were stored at −20°C until DNA extraction. DNA quality was evaluated with Nanodrop® (Thermo Scientific, Walthham, USA) and quantified with Qubit® (Thermo Scientific).

2.3 | Genomic dataset

Two genomic libraries were prepared (one for each species) following the double-digest restriction site-associated DNA sequencing (ddRADseq) protocol of Peterson, Weber, Kay, Fisher, and Hoekstra (2012). Briefly, genomic DNA was digested with the restriction enzymes *Eco*RI and *Mse*I, ligated to adaptors with unique barcodes, pooled and size-selected using Pippin Prep (Sage Science, Beverly, USA), and sequenced on an Illumina HiSeq2500 to generate single-end 50 bp reads at The Centre for Applied Genomics, Toronto, Canada (protocol details given in Appendix S3).

Genomic data for each species were processed separately using the pipeline STACKS 1.35 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). Reads were demultiplexed and filtered using the program PROCESS_RADTAGS, with sequences from each individual assembled de novo in USTACKS to identify putative loci, and a catalogue of consensus loci built in CSTACKS. Individual genotypes were identified with SSTACKS (for details of raw data processing see Appendix S3). Individuals were grouped according to their sampling localities in POPULATIONS (STACKS pipeline), and biallelic loci from a minimum of two populations were used in population genetic analysis (described below). We chose this parameter to maximize the number of loci retained (i.e., for any given minimum of missing data, there is a drop out of loci as the number of individuals increases; Huang & Knowles, 2016). A custom script (available on https://github.com/KnowlesLab; Thomaz, Malabarba, & Knowles, 2017) was used in R 3.2.2 (R Core Team, 2017) to exclude loci with high theta values (located within the upper 95% quantile) and SNPs from the two last nucleotides (Figure S1.1 in Appendix S1) to guard against sequencing and assembly errors. Following this step, the software PLINK 1.07 (Purcell et al., 2007) was used to identify SNPs with a maximum of 20% of missing data and with a minimum stack depth per individual (m) of five for inclusion in the final dataset.

Processed genomic data resulted in 28,487 SNPs for *B. coccolobifolia* and 14,855 SNPs for *B. crassifolia*, and a total of 7,115 and 4,543 loci with one biallelic SNP per locus in each species, respectively; hereafter, we refer to this genetic variation sampled across the genome as "genomic" variation or structure. An average of 81% of reads per individual were retained, with a mean coverage depth per locus of 23.6 ± 8.8× after processing and assembly, which is considered adequate for population genomics inference (see Buerkle & Gompert, 2013 for details). These loci were identified from the 226 million reads for the 183 individuals sequenced on two Illumina lanes (average of 1,236,269.5 ± 684,965.7 reads per individual; 29

TABLE 1 Number of individuals sampled, *N*, and estimates of genetic diversity per population of *Byrsonima coccolobifolia* and *B. crassifolia* (see Figure 1 for distributional map of sampled populations)

Pop	N	H_{OBS}	H_{EXP}	π
Byrsonima coccolobifolia				
A-coBON	4	0.053	0.049	0.058
A-coBVT	5	0.056	0.052	0.059
A-coCNE	6	0.059	0.054	0.059
A-coMCP	6	0.059	0.056	0.063
A-coSAN	5	0.057	0.053	0.060
A-coHTA	5	0.063	0.060	0.067
coCAI	5	0.065	0.064	0.071
coVHA	6	0.064	0.065	0.072
coCHG	5	0.064	0.060	0.068
coCGD	6	0.062	0.062	0.068
coJPO	6	0.067	0.068	0.075
coUDA	6	0.063	0.064	0.070
coPRA	5	0.068	0.062	0.070
Byrsonima crassifolia				
A-crATA	5	0.060	0.055	0.063
A-crPAC	1	0.057	0.029	0.057
A-crCNE	5	0.065	0.067	0.076
A-crMCP	6	0.067	0.070	0.078
A-crSAN	6	0.065	0.068	0.075
A-crSVA	6	0.067	0.071	0.079
crPRI	2	0.065	0.051	0.070
crAGN	5	0.055	0.055	0.062
crSLA	6	0.056	0.055	0.061
crMAG	6	0.059	0.061	0.068
crFOR	5	0.064	0.064	0.072
crCHG	5	0.058	0.059	0.067
crCVE	2	0.059	0.044	0.062
crPPB	2	0.056	0.040	0.060
crITI	6	0.057	0.059	0.066

Amazonian savanna populations are identified with an A preceding the population label. Estimates of genetic diversity per population are based on all polymorphic nucleotide positions of filtered genomic data; H_{OBS}, observed heterozygosity; H_{EXP}, expected heterozygosity; π, nucleotide diversity.

individuals were excluded due to large amounts of missing data—for details see Table S1.2 in Appendix S1).

2.4 | Characterizations of genomic variation and structure

Genetic structure was investigated using two different strategies: principal components analysis (PCA), which does not require any assumptions about the underlying genetic model (Jombart, Pontier, & Dufour, 2009), and Bayesian clustering, which applies a coalescent model for inferences about genetic structure. The packages "adegenet" 2.0 (Jombart, 2008; Jombart & Ahmed, 2011) and "ade4" 1.7-2 (Dray & Dufour, 2007) were used to perform a PCA in R; missing data were replaced by the mean frequency of the most frequent allele. The robustness of PCA results was evaluated using datasets with different levels of missing data (5 and 20%; see Huang & Knowles, 2016) and with an additional minimum stack depth per individual of 10. Because these results were qualitatively similar (Figure S3.1 in Appendix S3), the results are not discussed further. Bayesian clustering was performed with the software STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000), with only one SNP per locus. These analyses included admixture among populations and a correlation among allele frequencies with 1 to 10 genetic clusters (*K*) tested. Ten independent runs were performed for each *K*-value, with 100,000 burn-in and 300,000 MCMC iterations (the number of burn-in and MCMC iteration were increased when necessary to reach convergence). The most probable number of cluster was identified with STRUCTURE HARVESTER (Earl & Vonholdt, 2012), and the posterior probability of individual assignment to each cluster was permuted across different runs and visually displayed with CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). A hierarchical analysis with subsets of populations from each inferred genetic cluster was used to test for additional structure within the initial clusters identified by STRUCTURE (e.g. Massatti & Knowles, 2014; Papadopoulou & Knowles, 2015). Hierarchical analyses were performed with the same parameter settings described above, with *K*-values ranging from 1 to the maximum number of populations in each sequential analysis. Note that analyses of genetic structure in *B. coccolobifolia* suggested the presence of a cryptic taxon (i.e., PCA analysis revealed that the individuals were quite divergent, and distinct, from all the other populations; see Figure 2). Because inclusion of these populations (specifically, coAGN, coNAT and coFOR populations) would confound comparisons of CC to AS (e.g., compare PCA with and without these individuals; Figure 2), the populations were removed and are not included in the geographic structure results.

Tests of the association between geography and genetic structure were performed using two approaches in each species. Isolation-by-distance (IBD) was tested by evaluating whether there was a significant correlation between geographic distance and genetic distance ($F_{ST}/(1 - F_{ST})$, Slatkin, 1995) based on 100,000 permutations with the package "vegan" 2.3-1 in R (Oksanen et al., 2017). Additionally, a Procrustes analysis, which retains the relative longitudinal and latitudinal position of populations to test for an association between genetic variation and geography was used (for additional details see Appendix S3), with the significance of the association, t_0, (Wang, Zöllner, & Rosenberg, 2012) evaluated by 10,000 permutations (package "vegan"). The robustness of the association between genes and geography was assessed using a sequential population drop-out procedure (see Knowles & Massatti, 2017). Geographic structuring of genetic variation was also assessed with additional Procrustes analyses conducted on the CC and AS separately.

Lastly, levels of genetic diversity were characterized for each population using the dataset with all SNPs (i.e., not the dataset with only a single SNP per locus). These include estimation of standard population genetics statistics such as nucleotide diversity (π), expected

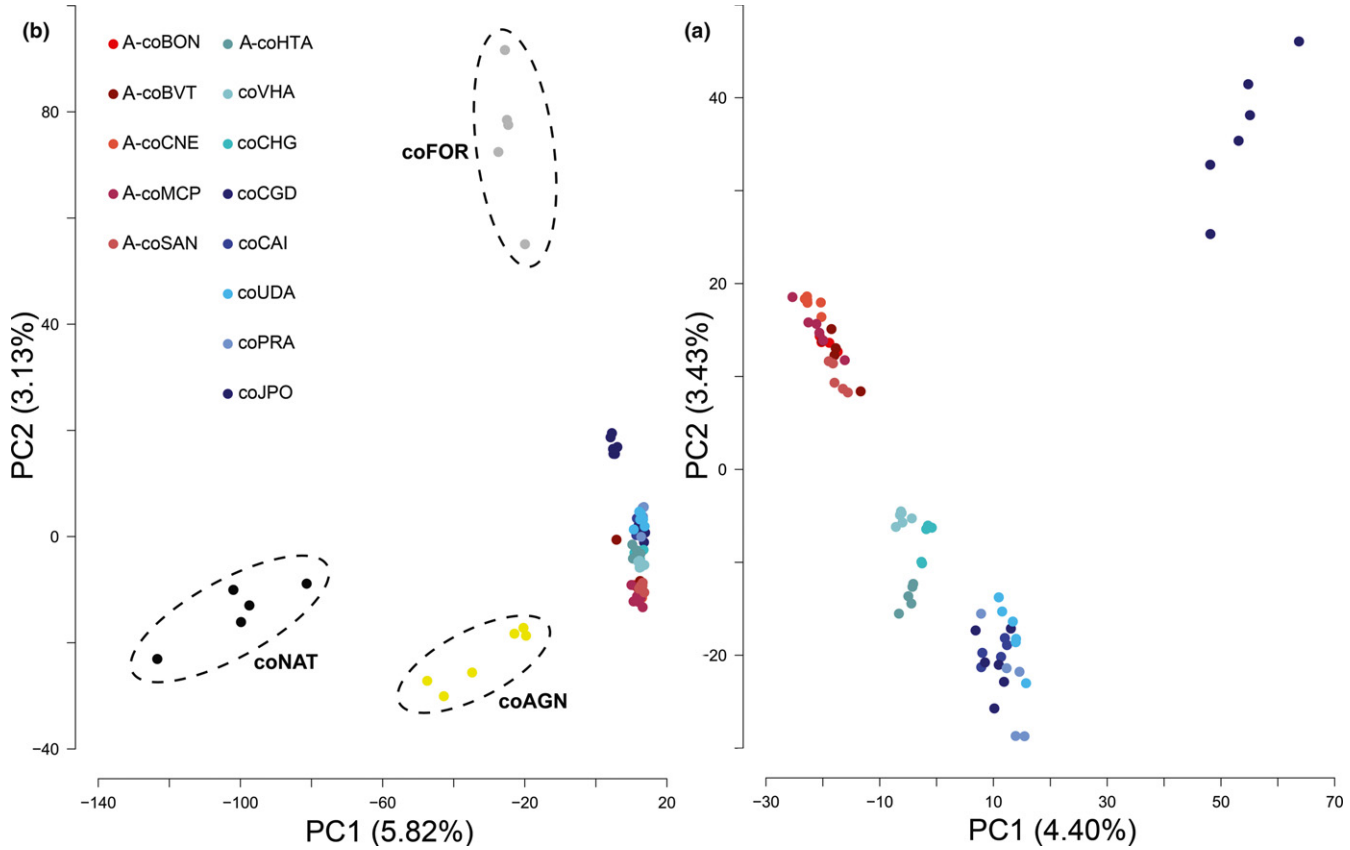

FIGURE 2 Principle Components Analysis (PCA) of *Byrsonima coccolobifolia* including (a) and excluding (b) populations that revealed cryptic genetic diversity indicative of potentially different species (i.e., the three divergent sampled populations: coAGN, coNAT, and coFOR). The amount of variation explained by each axis is given in parentheses and colours indicate population identity

heterozygosity (H_{EXP}), observed heterozygosity (H_{OBS}), and Wright's *F*-statistics (F_{IS} and pairwise F_{ST}), which were calculated using the POPULATIONS module from the STACKS pipeline (Catchen et al., 2013).

2.5 | Estimates of divergence times

Divergence times were estimated between the CC and AS using a composite-likelihood method based on the site frequency spectrum (SFS) and implemented in FASTSIMCOAL2 (Excoffier, Dupanloup, Huerta-Sánchez, Sousa, & Foll, 2013; Excoffier & Foll, 2011). To improve the accuracy of parameter estimates from the SFS (and following the recommendations of the program; see Excoffier & Foll, 2011), we fixed the effective population size of the CC, which was calculated directly from the empirical data, whereas the other parameters were estimated (i.e., the population size of AS, N_{AS}, the ancestral population size, N_{ANC}, and the divergence time, T_{DIV}). Specifically, the population size of CC was calculated from the nucleotide diversity, π, of fixed and variable sites using a nuclear genomic mutation rate of 7×10^{-9} subs/site/generation (Ossowski et al., 2010). This mutation rate was estimated based on spontaneous mutations of *Arabidopsis thaliana*, a herbaceous annual plant, and therefore divergence times estimated here will tend to be relatively more recent than expected if mutation rates in *B. coccolobifolia* and *B. crassifolia* are lower, as suggested for other woody plants (Smith & Donoghue, 2008; Yang et al., 2015).

Point estimates for each parameter were obtained from the run with the highest maximum likelihood from 40 FASTSIMCOAL2 runs with 100,000 to 250,000 simulations per run, and 10 to 40 expectation-conditional maximization (ECM) cycles based upon a stopping criterion of 0.001 as a minimum relative difference between two iterations. Confidence intervals were calculated for each parameter from 100 parametric bootstrap replicates of simulated SFS under a model based on the point estimates. As there is no literature about generation time for those species, divergence time estimates were converted from generations to years assuming a generation time of 3 years, which was observed by a domestication programme to be the age of first fruiting in natural populations (Nascimento W. M. O & Carvalho J. E. U, Embrapa Amazônia Oriental, personal comm.), although we recognize time estimates may be considerably older if generation times of 10–15 years for Cerrado trees were applied (Collevatti, Terribile, Rabelo, & Lima-Ribeiro, 2015; de Lima, Lima-Ribeiro, Tinoco, Terribile, & Collevatti, 2014).

Analyses were run with 15 individuals selected from each of the sampled populations that had the smallest amount of missing data (see Table S3.1 in Appendix S3). Since *B. coccolobifolia* displayed some admixture between the CC and AS, we estimated divergence times

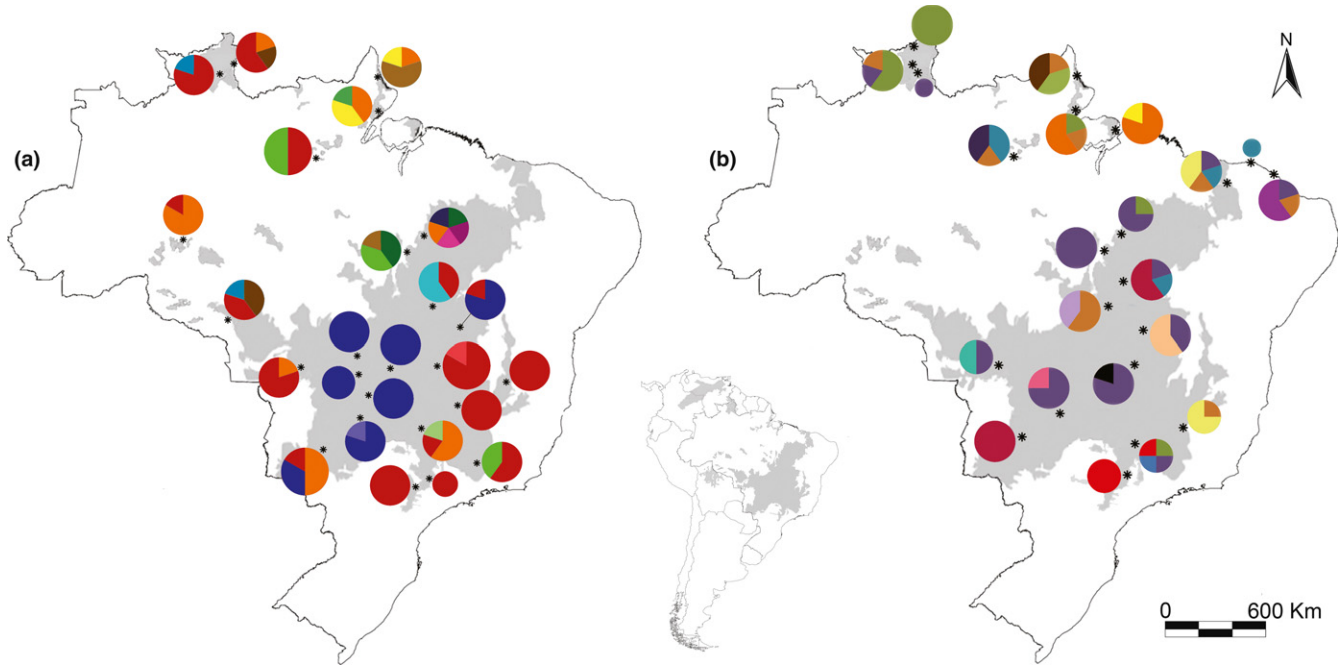

FIGURE 3 Geographic distribution of cpDNA haplotypes (sampling location is marked by small black dot) of *Byrsonima coccolobifolia* (a) and *B. crassifolia* (b), with each distinct haplotype represented by a different colour and the number of individuals sampled in each population indicated by the size of the circles. Shaded areas approximate the distribution of the Cerrado (both the central Cerrado and Amazonian savannas)

with and without the populations that displayed admixture (i.e., populations A-coHTA, coCHG and coVHA). We used a python script to calculate the folded joint SFS based on the vcf file from POPULATIONS (script is available on https://github.com/KnowlesLab; Papadopoulou & Knowles, 2015). Only loci with a minimum coverage of 10 that were present in all selected individuals were used to calculate the SFS. Divergence times were estimated excluding monomorphic sites (i.e., using the "*removeZeroSFS*" option in FASTSIMCOAL2) and assuming no migration between the CC and AS (this assumption is corroborated by other analyses—see below); note that any violation of this assumption would result in underestimated divergence times (i.e., this assumption is conservative with respect to evaluating whether the AS have had a relatively short history of isolation from the CC).

2.6 | Chloroplast DNA data and analysis

The *trnS-trnG* (Hamilton, 1999) and *trnH-trnK* (Demesure, Sodzi, & Petit, 1995) regions of chloroplast DNA were sequenced following protocols described in Resende-Moreira et al. (2017) on the ABI 3730XL DNA Analyser (ThermoFisher Scientific, Walthan, USA). A total of 126 sequences of *B. coccolobifolia* (including 49 sequences from Resende-Moreira et al., 2017) and 116 sequences of *B. crassifolia*, with 1–6 individuals per population (Table S2.1 in Appendix S2), were analysed. Sequences were aligned using the software MUSCLE implemented in MEGA 5.2 (Tamura et al., 2011) and all polymorphisms confirmed by visual inspection. We excluded polymorphisms in microsatellites, which are prone to homoplasy, and indels and inversions were recoded as one mutational step. Haplotypes were identified in DNASP 5.10 (Librado & Rozas, 2009) and their distribution plotted geographically to highlight haplotype diversity across the range of each species, as well as the distribution of widespread versus localized haplotypes. Additional analyses were performed with cpDNA data to calculate diversity indices and to evaluate population structure (see Appendix S2 for details).

3 | RESULTS

Measures of genomic diversity were generally similar across populations in both species (Table 1), whereas cpDNA diversity varied somewhat between taxa and among populations (Table S2.1 in Appendix S2), including the fixation of a single cpDNA haplotype in some populations (Figure 3), which contrasts with genomic diversity estimates (see Table 1). Populations with fixed cpDNA were not disproportionately represented by AS populations (i.e., most AS populations were polymorphic in cpDNA), despite their relatively small size and geographic fragmentation.

In both species, the CC populations were genetically differentiated from the AS populations. STRUCTURE analyses (Figure 4) identified separate ancestries for the CC and the AS (with the exception of coHTA in *B. coccolobifolia*), which was corroborated by pairwise F_{ST} -values, which were generally higher between populations from the CC and AS than among populations within the respective regions (Tables S3.2 and S3.3 in Appendix S3). Likewise, Procrustes analyses showed genetically distinct clusters separating the CC and AS regions, except for the coHTA in *B. coccolobifolia*, which clustered with individuals from the CC (Figure 4a), with a significant association between genes and geography ($t_0 = 0.770$, $p < 0.0001$ for *B. coccolobifolia* and

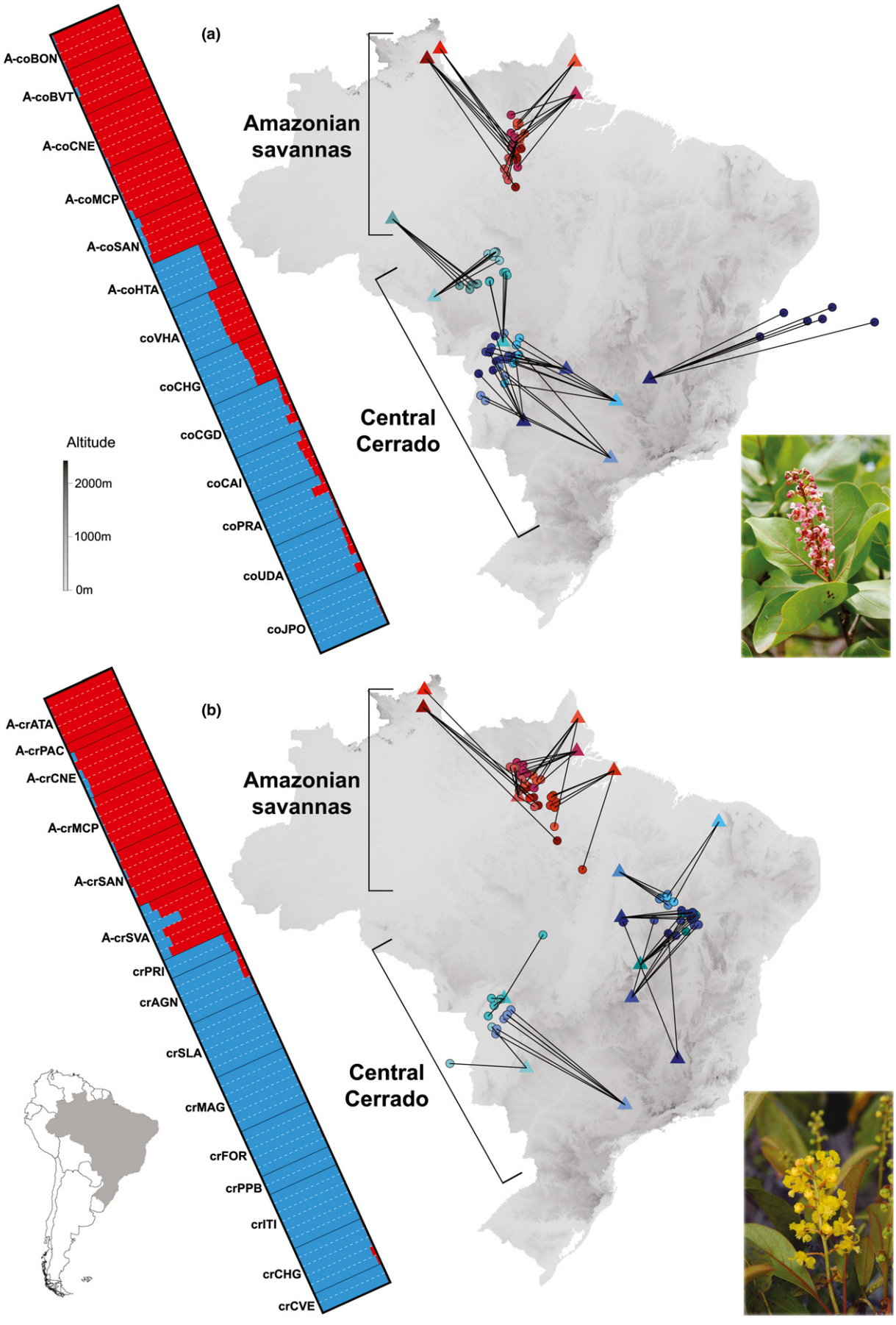
(a)
A-coBON
A-coBVT
A-coCNE
A-coMCP
A-coSAN
A-coHTA
coVHA
coCHG
coCGD
coCAI
coPRA
coUDA
coJPO
Altitude
2000m
1000m
0m
Amazonian savannas
Central Cerrado
(b)
A-crATA
A-crPAC
A-crCNE
A-crMCP
A-crSAN
A-crSVA
crPRI
crAGN
crSLA
crMAG
crFOR
crPPB
crITI
crCHG
crCVE
Amazonian savannas
Central Cerrado

FIGURE 4 Population structure of *Byrsonima coccolobifolia* (a) and *B. crassifolia* (b). Barcharts show the most probable number of groups (*K*) according to STRUCTURE results for each species as different colours along with Procrustes analysis of genetic variation. Each individual in the barchart is demarcated by a white dashed line, and the posterior probability of each individual's ancestry is depicted as the proportion of each colour per individual, whereas populations are labelled and separated by black lines. In each map, the position of an individual in genomic space (shown as circles) relative to sampled locality (show as triangles) is indicated (with individuals colour coded by population). The lines connecting individuals (circles) to localities (triangles) indicate the deviation of an individual genetically from expectations based on their geographic location (i.e., departures from isolation-by-distance). Amazonian savanna populations are identified with an A. Photographs of *B. coccolobifolia* and *B. crassifolia* were provided by Maurício Mercadante and Daniel Nickrent (source: http://www.phytoimages.siu.edu), respectively

t_0 = 0.795, $p < 0.0001$ for *B. crassifolia*). Sequential population drop-out analysis showed the results from the Procrustes analyses are robust (i.e., no single population had a disproportionate effect on the strength of the association between genes and geography, Table S3.4 in Appendix S3). Little admixture between the CC and AS regions was detected in STRUCTURE analyses, with only one AS population of each species (A-coHTA in *B. coccolobifolia* and A-crSVA in *B. crassifolia*) showing any appreciable sign of admixture when all individuals were analysed (Figure 4). Significant IBD was also detected in a correlation analysis between genetic differentiation (measured by F_{ST}) and geographic distances among populations in both species (r = 0.64 and r = 0.61, $p < 0.001$, in *B. coccolobifolia* and *B. crassifolia*, respectively).

Within the CC and AS regions, both species showed significant geographic structure. Within the CC, this local substructure was evident in both the hierarchical STRUCTURE analysis (Figure 5), and the separate Procrustes analyses (Figure 6c,d), which showed three genetic clusters in both species. Note that for *B. coccolobifolia* one of the groups is based on only one population because this is the area where the other three sampled populations appear to belong to a previously unrecognized species (see Figure 2). Separate analyses of the AS populations also detected substructure, with three genetic clusters in STRUCTURE analysis (Figure 5) and the Procrustes analyses (Figure 6a,b). With the primary axes of genetic variation from the Principle Components Analyses separating the CC and AS regions in both species (Figure 4; as well as hierarchical structure detected in the sequential STRUCTURE analyses; Figure 5), the substructure observed within the AS and within the CC clearly accumulated after the separation of AS populations from the CC.

Comparing geographic structuring of genomic data with cpDNA, the genomic variation in *B. coccolobifolia* was generally congruent with the cpDNA (Figure S2.1 and Tables S2.2, S2.4 in Appendix S2), even with broader sampling of the cpDNA dataset (Figure 1). In contrast, cpDNA results for *B. crassifolia* differed from the genomic results and showed a lack of regional or local geographic structure (Figs. 3 and S2.1, and Tables S2.3 and S2.5 in Appendix S2).

Divergence time estimates between the CC and AS were 119,379 and 290,541 years for *B. coccolobifolia* and *B. crassifolia*, respectively. These results clearly do not support a LGM divergence even considering that the shortest possible generation time of 3 years was used. We also note the confidence intervals surrounding the parameter estimate for t do not overlap with the LGM. This conclusion is also robust to inclusion of admixed populations of *B. coccolobifolia* (Table 2). Even

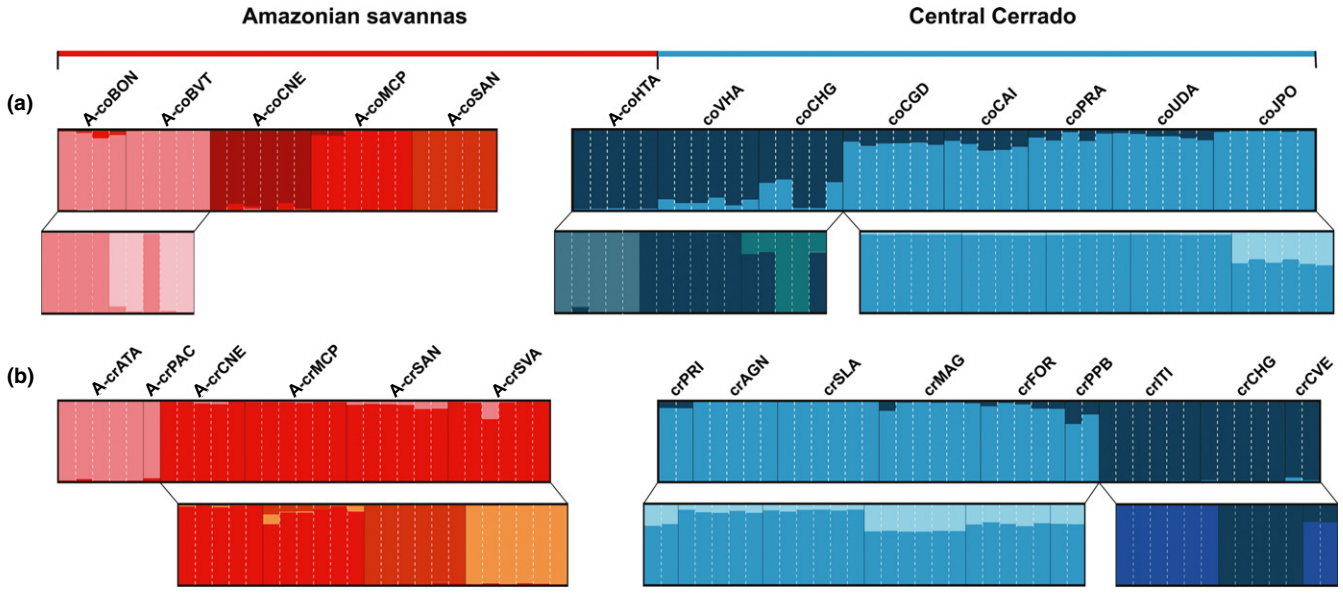

FIGURE 5 Hierarchical population structure of *Byrsonima coccolobifolia* (a) and *B. crassifolia* (b) based on sequential, and separate, STRUCTURE analyses of Amazonian savanna and central Cerrado datasets (i.e., data subsets identified from global analyses; see Figure 4). The most probable number of groups (*K*) is displayed as different colours in each plot, with populations marked by thin black lines, and white lines demarcating sampled individuals with the posterior probability of belonging to each cluster depicted as the proportion of each colour in the bar. Amazonian savanna populations are identified with an A

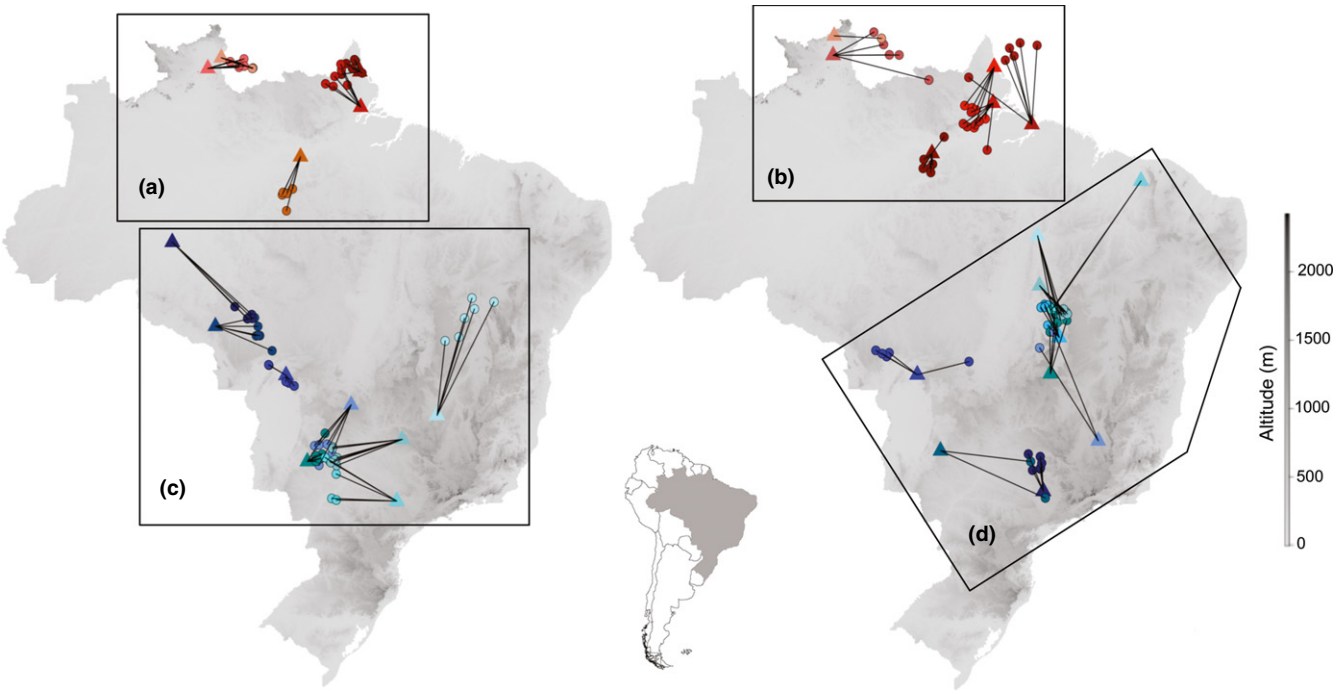

FIGURE 6 Plots of Procrustes analyses carried out separately on regional datasets of the Amazonian savanna (a and b) and central Cerrado populations (c and d) of *Byrsonima coccolobifolia* and *B. crassifolia*, respectively. The lines connect individuals (shown as circles) to sampling location (shown as triangles) indicate deviations from the expected pattern of genetic variation based on isolation-by-distance, where longer lines indicate greater departures from expectations based on where an individual was sampled geographically. Colours indicate population identity

with considering potential errors in the mutation rate, the mutation rate would have to be six to twelve times faster than the one applied here to accommodate a divergence time consistent with the LGM. However, as noted in the methods, mutation rates in woody plants are thought to be slower—not faster—than the one applied here, so a LGM divergence is extremely unlikely.

4 | DISCUSSION

The genomic distinctiveness of the CC populations from the disjunct AS and lack of any regional structure that group populations from the two regions indicate the Amazonian populations have evolved independently (for the most part) from the Cerrado. As such, it is isolation and not recent connections (either via long-distance dispersal or expansion/retraction via corridors) that dominates in this tropical biome; substantial admixture was limited to a single population that borders the Cerrado (see also Buzatti, Lemos, Bueno, & Lovato, 2017 for localized study of another plant species from this focal area). The extent to which these results are generalizable to other taxa from the Cerrado and Amazonian savannas is discussed below, as is what our results suggest about the evolutionary dynamics of relictual populations in tropical systems. In both discussions, we advocate for a more nuanced approach to tests of the relative isolation versus connections of AS populations. In addition, with reference to our own results, we highlight how some refined hypotheses might provide more insight about why one process might predominate over the other in particular taxa or geographic regions. Lastly,

TABLE 2 Divergence time estimates (assuming a minimum of a 3 year generation time; see methods for details) and other demographic parameters for each *Byrsonima* species based on the model of divergence between the Amazonian savannas (AS) and central Cerrado (CC) regions using FASTSIMCOAL2. Specifically, we show results for divergence time, T_{DIV}, ancestral effective population size, N_{ANC}, effective population size for AS, N_{AS}, and number of loci used to calculate the folded joint site frequency spectrum (SFS). Confidence intervals based on 100 parametric boostraps are shown in parentheses. Note that effective population size of the CC (N_{CC}) was calculated directly from the empirical data (i.e., was a fixed parameter in the model) to improve the accuracy of the other parameters estimated from the SFS (following the recommendations for the program; see Excoffier & Foll, 2011)

Species	Loci	T_{DIV} (years)	N_{ANC}	N_{CC}	N_{AS}
Byrsonima coccolobifolia (all populations)	2285	109,611 (87,432–143,886)	100,400 (60,069–176,369)	978,571	343,875 (263,662–502,760)
Byrsonima coccolobifolia (excluding admixed populations A-coHTA, coCHG and coVHA)	1945	119,379 (96,195–169,311)	56,282 (31,697–102,681)	992,857	210,635 (162,674–322,062)
Byrsonima crassifolia	1032	290,541 (240,696–355,920)	117,638 (80,184–166,741)	1,000,000	399,628 (331,352–498,123)

given the general lack of phylogeographic studies of the AS, we reflect on the relevance of our results on the processes contributing to savanna species diversity, as well as to future conservation efforts.

4.1 | Past connections versus isolation of Amazonian savannas

To explain the similarity between CC and AS, three regional connections or corridors were proposed: the coastal, the central Amazonian, and the Andean corridor (Haffer, 1967, 1974; Silva & Bates, 2002; Webb, 1991). These corridors are hypothesized to have connected the CC and AS during waves of Pleistocene savanna expansions (Haffer, 1969; Silva & Bates, 2002), possibly as recent as the Holocene (see de Freitas et al., 2001). However, our genomic data did not provide strong support for the existence of such corridors in either species. Instead, analyses suggest a history of restricted gene flow between the CC and AS (Figure 4), with the AS evolving in relative isolation from the CC over a history of divergence that predates the LGM (Table 2). This is corroborated by palynological evidence that draws into question any recent large savanna expansions that might have served as connections between the CC and AS (Colinvaux, Oliveira, Moreno, Miller, & Bush, 1996; Colinvaux et al., 2001; Kastner & Goni, 2003; Mayle et al., 2009). The only exception is the admixture detected in one south-western Amazon population (see Figure 4), which is a region where joint analyses of ENMs and cpDNA data in an unrelated plant also found evidence of a connection to the CC (see Buzatti et al., 2017; note this study focused only on this single site so it is not possible to determine if other AS populations in the species remained isolated from the CC).

The independent evolutionary history of CC and AS has important implications for questions beyond those focused on genetic structure per se. Although *Byrsonima* species are suggested to display effective long-distance dispersal (Willis, Franzone, Xi, & Davis, 2014), there is no clear evidence of recent gene flow in *B. coccolobifolia* and *B. crassifolia*, indicating that long-distance dispersal is not common. The genetic isolation of CC from AS (Figure 5), in addition to the compositional similarities in their constituent plant communities (with over 70 woody species in common; Ratter et al., 2003) suggests a more ancient common history, rather than the maintenance by corridors per se. Moreover, it implies that the differences in species composition between the AS and CC might reflect the cumulative loss of species in the AS (community relaxation—Connor & McCoy, 1979), rather than differences in the maintenance of diversity through successful/unsuccessful utilization of corridors. Additional circumstantial evidence of localized extinctions rests in the observation that few Cerrado taxa are found across all AS populations (Ratter et al., 2003). Alternatively, with many taxa restricted in distribution to the CC, there might have been historical restrictions to expansion for many taxa such that they were never part of the AS, even when Cerrado reached its broadest historical distribution. Additional tests will be needed to evaluate this hypothesis. These might include testing for evidence of environmental filtering or differences in the dispersal capabilities of exclusively CC taxa compared with those distributed across the AS, although no significant difference in seed dispersal syndromes for species present in CC and AS has been suggested in past studies (see Vieira, Aquino, Brito, Fernandes-Bulhão, & Henriques, 2002).

4.2 | Conflicting support for connections of the CC and AS

When comparing our results to past studies purported to support hypothesized connections between the CC and AS, several non-mutually exclusive explanations might account for such contrasting support of the corridor hypothesis. These include: (a) differences in the resolution of genetic markers, (b) relying solely upon applications of distributional or ecological-niche models, and (c) differences among taxa in access to the corridors due to historical contingencies or differences in the taxa themselves (i.e., species-specific traits). Below, we consider each of these explanations in turn with reference to results from our analyses of *B. coccolobifolia* and *B. crassifolia*.

The genetic marker applied to test a phylogeographic hypothesis can impact the likelihood that a study might find support for or refute a particular hypothesis (Knowles, 2009). In particular, tests that rely upon genetic structure as evidence of isolation (e.g., when support for putative corridors is based on the lack of genetic differentiation between CC and AS regions; Savit & Bates, 2015) may be obscured by limited genetic resolution, including an insufficient time for the sorting of ancestral polymorphism or the lack of mutation variation for detecting historical divisions (Ball, Neigel, & Avise, 1990; Papadopoulou & Knowles, 2015; Thomaz et al., 2017). For example, sharing of chloroplast haplotypes between CC and AS populations in *B. coccolobifolia* and *B. crassifolia* (Figure 3) could suggest recent gene flow or connections, as might the lack of regional cpDNA clades separating the CC and AS regions (Figure S2.1 in Appendix S2). However, analysis of genomic data clearly shows that CC and AS regions are genetically distinct in both species (Figure 4). In other words, if chloroplast data by itself are going to be used to refute a hypothesis of isolation, it is important to test whether the data may be consistent with a history of isolation, which can be evaluated using computer simulations (see Knowles & Maddison, 2002). Alternatively, and as we apply here, additional markers can be used to model the divergence history of the species (as opposed to the history of a single locus; see Knowles, 2009). Here, the parameterized divergence models support a long history of isolation between CC and AS regions that predate the LGM (Table 2).

For the Cerrado, evidence for the existence of corridors connecting areas north and south of Amazon comes primarily from distributional data (e.g., Ávila-Pires, 1995; Nogueira & Rodrigues, 2006; Silva & Bates, 2002), with some support for distinct routes of movement suggested by a few phylogeographic studies (e.g., Buzatti et al., 2017; Quijada-Mascareñas et al., 2007; Savit & Bates, 2015). As with concerns regarding inferences based on a single locus, inferences based on ENMs alone might also be misleading (as opposed to

considering ENMs jointly with molecular data; reviewed in Alvarado-Serrano & Knowles, 2014). Specifically, although ENMs might be used to identify possible connections, without genetic data, it is not possible to test whether species actually utilized purported corridors (i.e., gene flow might not have been associated with the corridors inferred from ENMs for many of the different reasons discussed above). Here, we advocate that, as with the interpretation of single locus data, extreme caution is needed. More specifically, studies based solely on ENMs should be used to generate hypotheses, but do not (by themselves) constitute evidence for supporting the corridors hypothesis.

Assuming that such corridors existed, it is possible that some species just by chance, found themselves in the right place at the right time to have access to a corridor, whereas others did not. On the other hand, the lack of consistent support for corridors could also reflect deterministic processes related to species-specific differences (Massatti & Knowles, 2014, 2016; Papadopoulou & Knowles, 2016). Indeed, the organisms investigated in the phylogeographic studies that sampled broadly the AS include different taxonomic groups (e.g., birds, snakes, and this study with plants) with distinct dispersal abilities and the distribution of some investigated taxa is not restricted to the savannas (e.g., Savit & Bates, 2015). Although it is possible that *B. coccolobifolia* and *B. crassifolia* differ from taxa for which future genomic analyses might show corridors between the CC and AS regions, it is not obvious why *B. coccolobifolia* and *B. crassifolia* would not have utilized corridors (if they existed). First, they are very common species and widely distributed (Figure 1), so unlike rare or patchily distributed species, they most probably would have had access to any putative corridor. Second, these attributes also make it less likely that any species-specific traits would have restricted their movement (i.e., they obviously can readily disperse to occupy vast areas of the Cerrado biome).

4.3 | Scale-specific effects of climate-induced distributional shifts

As possible remnants of a dynamic historical past, the tropical Amazonian savannas are similar to relict populations in northern latitudes (Pielou, 1991). However, this dynamic history, with cycles of climate-induced distributional shifts, contributes to the enigmatic nature of tropical relicts and debate over their role as drivers of divergence (e.g., Capurucho et al., 2013). By rejecting hypothesized periods of connectivity between CC and AS through putative expansions during glacial–interglacial periods (Prance, 1996; Silva & Bates, 2002), our study raises some intriguing questions about divergence of Cerrado species. Here, we make the argument that connections forged during cycles of expansion, while not extensive enough to support corridors between the CC and AS, may have played an important role in divergence within the CC and within the AS. In other words, geographic scale determines whether climatic oscillations promote connections. Likewise, we note that the existence of regional structure itself within both the CC and AS, suggests a limit on the level of connectiveness across populations in the past (otherwise, the regional structure would have been lost, and the only structure would be the population level structure that was also observed; see Figure 5).

What might limit the role of climate-induced distributional shifts at the larger scale—that is, why were distributional shifts not associated with connections between the CC and the AS? The most obvious answer is that the extent of savanna expansion (or conversely forest contraction) may have been more limited than previous proposals. For example, suggestions of a fairly stable forest during the LGM, especially for the western part of Amazon (e.g., Bush, Silman, & Urrego, 2004; Cheng et al., 2013; Colinvaux, Oliveira, & Bush, 2000), offer an alternative to Haffer's (1969) scenario of forest fragmentation during glacial periods. In addition, recent isotopic data sampled from the Amazon dry corridor (i.e., an area of current lower precipitation within the Amazon, Haffer, 1969) suggest forest physiognomies during the LGM consistent with the maintenance of rain forest (Wang et al., 2017), and/or its replacement by dry-forest habitats, instead of savanna (Bush, 2017; Pennington, Prado, & Pendry, 2000).

Within the CC, past phylogeographic studies of plants have documented an east–west split that is generally concordant with *Byrsonima* (Figure 6c,d) (reviewed in Leal, Palma-Silva, & Pinheiro, 2016). Likewise, a regional genetic structuring of individuals sampled in southern, central-northern and north-western portions of Cerrado has been observed in other organisms, including frogs and lizards (Prado, Haddad, & Zamudio, 2012; Santos, Nogueira, Giugliano, & Colli, 2014). This spatial concordance across studies highlights how these CC communities may be shaped by similar historical processes. Similarly, among the AS populations, regional divergence is clear, as is differentiation among individual populations (Figures 5 and 6). However, it is not clear what accounts for the observed regional structure of AS populations of both *Byrsonima* species because unfortunately, unlike the CC, there are extremely limited data in terms of genetic analyses of broadly sampled AS populations (in fact, we are not aware of any other studies besides ours). We note that in other types of open habitat (e.g., birds inhabiting white sand vegetation), genetic data provide evidence of recent population expansion during the late Pleistocene (Capurucho et al., 2013; Matos et al., 2016), suggesting that the connections we propose among AS populations based on regional structuring of genetic variation may not be an anomaly. It is clear that future research will be charting new directions about the drivers of divergence within the CC and AS as the focus shifts from one built on a history of corridors connections, to the independent evolutionary trajectories of the CC and AS.

4.4 | Conservation of Cerrado and Amazonian savannas

Despite the high endemism and species diversity, the Cerrado is rapidly being loss (less than 20% remains undisturbed; Strassburg et al., 2017), especially with the expansion of agriculture, cattle ranching, and charcoal production, and conservation of the Cerrado biome has received little attention. Although rates of loss have decreased over the last several years (i.e., since 2010), we are nevertheless loosing Cerrado faster than Amazon Forest (Françoso et al., 2015).

Given the extent of the biome, covering 2 million km^2, assessments of genetic diversity and population structure arguably could provide important guidance in conservation efforts. Yet, with relatively sparse geographic sampling, and limited genomic study, such information is rarely considered in conserving this highly threatened biome. Analyses of broadly distributed taxa in particular, like *B. coccolobifolia* and *B. crassifolia*, could be used to devise conservation strategies that protect not only the constituent taxa, but also preserve diversity generating processes (see Moritz, 2002). For example, our study revealed an unexpected cryptic species in *B. coccolobifolia* from the central and northern areas of the CC, an area reportedly of high species richness (Ratter et al., 2003). Other phylogeographic studies on Cerrado trees suggest the highest genetic diversity occurs in central areas of the Cerrado as well (e.g., Collevatti, Castro, Lima, & Telles, 2012; Novaes, Lemos-Filho, Ribeiro, & Lovato, 2010; Souza, Collevatti, Lima-Ribeiro, Lemos-Filho, & Lovato, 2017); however, many of these have limited sampling of northern areas. Our results, as those with more extensive sampling (Collevatti, Terribile, Diniz, & Lima-Ribeiro, 2015; Ribeiro, Lemos, Buzatti, Lovato, & Heuertz, 2016) have revealed high genetic diversity in north-eastern plant populations, highlighting the importance of these areas to conservation efforts of the Cerrado, especially since these areas are part of an expanding agricultural frontier.

Perhaps most importantly, our broad sampling of plant species from the AS identifies a number of factors relevant to developing conservation priorities for the AS. First, we show that these populations display levels of genetic diversity similar to CC populations, which is somewhat reassuring about their general health from a genetic prospective (i.e., they do not show disproportionately depressed levels of diversity; see Figure 3 and Table 1). However, their apparent genetic isolation does place them at substantial risk (Figure 4). Moreover, these populations arguably should be considered as unique Cerrado environments in conservation efforts of the biome, given their relatively long isolated history from the CC (see Table 2). Even though most AS display much less species diversity than the CC (but see Ratter et al., 2003 for exceptions), some AS contain more than 250 plant taxa (Miranda, Absy, & Rebelo, 2003; Sanaiotti, 1997), in addition to vulnerable and endemic species of birds, reptiles, amphibians, and plants (Barbosa, Campos, Pinto, & Fearnside, 2007; Carvalho, 1997; França, Mesquita, & Colli, 2006; Rocha & Miranda, 2014). It is also important to note that the number of species in the AS most likely is larger considering that these areas are highly understudied (Carvalho & Mustin, 2017). Lastly, AS are under particularly high anthropogenic disturbance because they are misleadingly considered as natural pastures in an environment largely dominated by forest (Miranda et al., 2003), making immediate attention as conservation units an imperative (Carvalho & Mustin, 2017).

5 | CONCLUSIONS

Our results show independent evolution of the CC and AS populations of both broadly distributed tree species studied here (*B. coccolobifolia* and *B. crassifolia*), casting doubt on the importance of corridors in structuring Cerrado plant communities. In the context of understanding the evolutionary history of AS populations in particular, it is possible that climatic change in the tropics, and/or differences in the traits of the species themselves, might make certain corridors more or less accessible during different geologic periods (Silva & Bates, 2002; Wüster et al., 2005), but careful consideration of this hypothesis will require expanding the dataset to other broadly distributed taxa. Specifically, our genomic data suggest a relatively long history of isolation between the CC and AS regions that predates the LGM, as well as population structuring of genetic variation within regions in both species. The contrast between genetic structure of genomic versus chloroplast datasets also highlights the need for cautious interpretation of what constitutes evidence for the corridor hypothesis. Our findings suggest that methodology, not biology, may contribute to some of the differences in support for the corridor hypothesis reported across studies. Lastly, as a biodiversity hotspot, these results have direct implications for diversification in the Cerrado, as well as its conservation, especially given extensive and ongoing habitat destruction (Carvalho & Mustin, 2017; Mittermeier et al., 2004).

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ORCID

Luciana C. Resende-Moreira http://orcid.org/0000-0003-2977-8433
Andréa T. Thomaz https://orcid.org/0000-0002-9755-2674
Joyce R. Prado http://orcid.org/0000-0002-2025-5479

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BIOSKETCH

The authors are broadly interested in biogeographical and evolutionary history of the Neotropics. Together the authors bring different expertise, forming a complementary team that spans from sampling and data collection to hypothesis generation and testing about the Cerrado evolutionary history. This work was part of a collaboration between UFMG and the University of Michigan.

Author contributions: L.C.R.M., M.B.L., J.P.L.F., and L.L.K. designed the study; L.C.R.M., M.B.L., and J.P.L.F. collected samples; L.C.R.M., A.T.T., J.R.P., and A.P.S. generated data and performed data analysis; L.C.R.M., L.L.K., and M.B.L. wrote the paper and all authors contributed with comments.

DATA ACCESSIBILITY

Chloroplast DNA sequences are available on GenBank (accession numbers MK120204 - MK120271) and Illumina raw data were deposited at NCBI Sequence Read Archive (SRP158434).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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