ORIGINAL ARTICLE

Phylogeography of the dry vegetation endemic species Nephila sexpunctata (Araneae: Araneidae) suggests recent expansion of the Neotropical Dry Diagonal

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ABSTRACT

Aim The Neotropical Dry Diagonal (DD) is a corridor of distinct dry environments in central South America. The main hypotheses suggest that these environments may have expanded during the glacial cycles together with a retraction of rainforests, and then shrank when the climate became wetter and warmer. However, few studies have explicitly tested the hypotheses on DD evolution. We conducted a phylogeographical study on *Nephila sexpunctata*, a spider endemic to Neotropical dry habitats, to enrich the understanding of DD evolutionary history.

Location South-western portion of the DD (Cerrado and Chaco).

Methods We sequenced two mitochondrial regions and one nuclear DNA region of 65 individuals from 10 locations across the species' range. We conducted analyses of genetic structure, variability, demography and inferred divergence times. We used an ecological niche modelling framework to generate hypotheses on the species' distribution along the last glacial cycle and a model-based approach to test the demographical scenarios that might explain the genetic patterns.

Results Both markers presented low genetic diversity. Mitochondrial markers had high genetic structure, with specific geographical lineages within each biome, while the nuclear marker presented low genetic structure. Phylogeographical and demographical events encompass the last glacial cycle, with a strong post-Last Glacial Maximum (LGM) population and spatial expansion. Model testing corroborated the recent demographical expansion of *N. sexpunctata*.

Main Conclusions Our data suggest that Quaternary climate cycles, when moist forests periodically expanded towards the dry vegetation, influenced the diversification of DD organisms by promoting demographical events. These events might explain the genetic structure observed in *N. sexpunctata*; differently from what was expected by the Pleistocene refugia model, some DD species may have experienced post-LGM expansion. Climate and geological events have both affected DD-endemic organisms' diversification.

Keywords

Cerrado, Chaco, dry diagonal, ecological niche modelling, Last Glacial Maximum, Neotropics, *Nephila*, phylogeography, Pleistocene refugia model

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INTRODUCTION

The Neotropics are the most diverse biogeographical region of the planet, presenting a great variety of ecoregions, from tropical rain forests to drier open formations. The largest continuous portion of dry vegetation in this region forms a south-west-north-west corridor that crosses South America, called the Dry Diagonal. It is composed of three biomes: Caatinga, the largest Neotropical nucleus of Seasonally Dry Tropical Forests (SDTFs) in northeastern Brazil; Cerrado, a mosaic of phytophysiognomies predominantly covered by savannas in the central part of the continent; and Chaco, composed mainly of subtropical dry forests and open woodlands in northern Argentina, Paraguay and Bolivia (Fig. 1). Evidence suggests that in the Eocene, when the climate was wetter and warmer than today, this area was covered by wet forests; the Amazon and Atlantic Forest were connected, forming a rain forest belt through most of South America (Morley, 2000). The DD may have been formed during the Oligocene and Miocene periods of cooling and dryness that led to the expansion of grasslands and the origin of DD-endemic lineages (Simon *et al.*, 2009).

Species broadly distributed in the DD frequently present distinct lineages in each biome (Werneck *et al.*, 2012a; Fouquet *et al.*, 2014), indicating putative isolation events. Diversification in this region has been associated with orogenic events along the Neogene, such as the uplift of the Brazilian Plateau with subsidence of Chaco and other lowlands around 5–7 Ma (Silva, 1995; Carvalho *et al.*, 2013) and marine transgressions in the Chacoan region in the Late Miocene (Ruskin *et al.*, 2011). Another hypothesis is that Quaternary climate oscillations promoted isolation within the DD, since independent data suggest that during glacial cycles the rain forests expanded through Chaco and Cerrado, scattering and reducing the range of these drier biomes (Nores, 1992; Sobral-Souza *et al.*, 2015). If these biomes were primarily affected by orogenic events, species and lineage divergence would date to Neogene (Silva, 1995); however, if glacial cycles' climate changes influenced the biomes more drastically, the taxa would show a Quaternary diversification (Silva, 1995).

Even though there are hypotheses that predict the effects of Quaternary climate changes on Neotropical dry-vegetation dwellers, few studies have effectively tested them. The
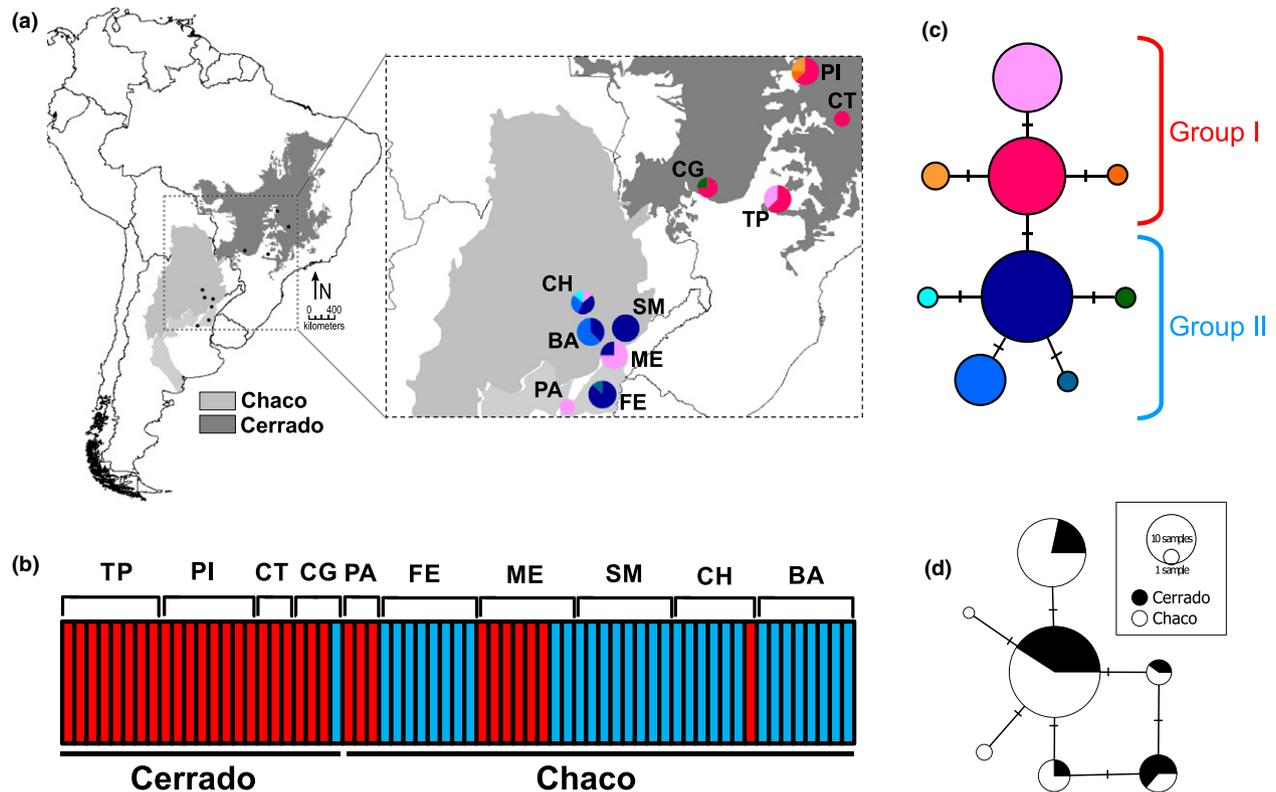

Figure 1 (a) Map with *Nephila sexpunctata* sampling locations showing four sites in Brazilian Cerrado (dark grey) and six in Argentinean Dry Forests (Chaco and Espinal - lighter greys; Espinal populations are considered in the Chaco dataset). Pie charts for each population represent the proportion of mitochondrial haplotypes (c) in a given population. (b) BAPS chart showing individual assignment to mitochondrial groups represented by different colours – Group I: red, Group II: blue. (c) Mitochondrial network (COI + 16S-L1-ND1) highlighting nine haplotypes divided into two mitochondrial groups inferred by BAPS: Group I occurs predominantly in Cerrado and Group II in Chaco. Circle size is proportional to frequency, and dashes represent mutational events. (d) Nuclear network (CHP2) coloured by the occurrence of each haplotype in Chaco or Cerrado.

Pleistocene refugia model (PRM – Vanzolini & Williams, 1981), which was first proposed to explain the diversity patterns in the Amazon Forest, asserts that Neotropical rainforests have presented range retraction during the glacials with later recovery in interglacials; therefore, open and dry-vegetation biomes would have undergone the opposite process, shrinking during wet and warm periods and reaching their maximum geographical distribution during glacials. Palaeopalynological (Ledru, 2002) and niche modelling (Sobral-Souza *et al.*, 2015) studies have agreed that shifts in biome distribution have allegedly occurred over time, but the effects on the demography and genetic structure of the dry-vegetation dwellers remains uncertain.

In the past few years, phylogeography has become an important tool for exploring processes that drove biodiversity evolution in highly diverse regions. For the Neotropics, this approach has been used to test the biogeographical hypotheses and to make inferences on biome evolution. Despite the recent increase in the phylogeographical studies in South America, surveys on open formations are scarce, and just a few studies have focussed on organisms endemic to dry biomes (Turchetto-Zolet *et al.*, 2013).

Nephila sexpunctata Giebel, 1867 is a spider restricted to south-western South America, mainly Cerrado, Chaco and adjacent dry forests, and is supposed to have speciated on the continent after a long-distance dispersal event from the Old World in the Miocene/Pliocene (Kuntner *et al.*, 2013). It is accepted that *Nephila* species perform aerial dispersal (Lee *et al.*, 2015), which may promote connection among distant populations. Spiders have been successfully used as phylogeographical models (e.g. Su *et al.*, 2007; Kuntner & Agnarsson, 2011), but in the Neotropical region, only two studies have hitherto used this group (Magalhaes *et al.*, 2014; Peres *et al.*, 2015).

We investigated the recent evolutionary history of Cerrado and Chaco through a phylogeographical study of *Nephila sexpunctata*. Given the poor knowledge about this region's diversification, phylogeographical studies with taxa endemic to these biomes may contribute to clarifying unsolved questions concerning its evolutionary history (Werneck, 2011).

We used mitochondrial and nuclear genetic markers in the first phylogeographical study with an arachnid in this region. We conducted population genetic analyses and estimated divergence times among lineages to detect demographical events. We also used palaeodistribution modelling coupled with a model-based approach to test the alternative scenarios for Cerrado and Chaco biogeographical history. Using an approximate Bayesian computation (ABC) framework, we first sought to elucidate when major diversification events took place for *N. sexpunctata*, so we contrasted four feasible scenarios [panmixia (maintenance of gene flow), post-LGM divergence, diversification in Quaternary or an older split in Neogene] to infer what the main factors were that led to the species diversification. Then we aimed to specifically test the predictions made by the PRM, so we contrasted mutually exclusive hypotheses for each scenario: (1) *N. sexpunctata*

presented no demographical variation in its recent evolutionary history; (2) a previously constant-size population underwent exponential growth; (3) following the PRM, *N. sexpunctata* presented a demographical expansion from the Last Interglacial (LIG ~120ka) to the Last Glacial Maximum (LGM ~21ka), a colder and drier period, and then experienced a population bottleneck from LGM to present day; (4) LGM conditions were harsh even for dry-vegetation species, so the species suffered a population decrease during the glacial, showing a recovery with exponential growth when the climate became warmer and wetter after LGM. This framework, associated with our ecological niche models and genetic analyses, can help to elucidate historical aspects of DD evolution. We compare the patterns found for *N. sexpunctata* with previous studies using other *Nephila* species as well as studies with co-occurring species. We then proceed to make inferences on the evolution of Cerrado and Chaco based on our results, pinpointing some events that might have guided the organization of diversity as seen in the present.

MATERIALS AND METHODS

Sample collection; DNA extraction, amplification and sequencing

We collected adults of *N. sexpunctata* in most of the species' range, comprising 23 individuals from four locations in the Brazilian Cerrado and 42 from six locations in the Argentinean Chaco and Espinal, herein referred as 'Chaco' (Fig 1, Table 1). Opisthosomas and palps were stored in absolute ethanol for species confirmation and deposited in the Coleção Científica de Aracnídeos e Miriápodes of the Instituto Butantan (São Paulo, Brazil), Colección Aracnológica del IADIZA (Mendoza, Argentina) and Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina). Genomic DNA was extracted from 1 to 8 legs using the Wizard Genomic

Table 1 List of *Nephila sexpunctata* sampling locations with details on biome and geospatial coordinates.

Location	Biome	Latitude	Longitude
Brazil – Tupã (TP)	Cerrado	21°55′01.8″ S	50°30′04.6″ W
Brazil – Pirenópolis (PI)	Cerrado	15°55′24.2″ S	49°12′50.6″ W
Brazil – Catalão (CT)	Cerrado	18°06′50.0″ S	47°37′16.0″ W
Brazil – Campo Grande (CG)	Cerrado	21°24′13.8″ S	53°40′43.8″ W
Argentina – Paraná (PA)	Espinal	31°43′32.1″ S	60°17′58.1″ W
Argentina – Federal (FE)	Espinal	30°56′01.0″ S	58°43′44.9″ W
Argentina – Mercedes (ME)	Espinal	29°05′44.6″ S	58°19′46.5″ W
Argentina – Santa Maria (SM)	Chaco	28°02′29.6″ S	58°06′02.2″ W
Argentina – Pq. Nacional del Chaco (CH)	Chaco	26°48′31.1″ S	59°36′21.5″ W
Argentina – Basail (BA)	Chaco	27°52′01.7″ S	59°17′22.0″ W

DNA Purification kit (Promega) following the manufacturer's protocol.

We amplified two mitochondrial regions: for the cytochrome *c* Oxidase subunit I (COI) we used the universal primers for invertebrates, LCO1490 and HCO2198 (Folmer *et al.*, 1994).The PCR conditions followed were: a denaturation step of 3′ at 94 °C, 30 cycles of 94 °C for 45″, 51 °C for 45″ and 72 °C for 2′, and a final extension step at 72 °C for 3′. The mitochondrial region comprising the large ribosomal subunit gene 16S rRNA, the tRNA-Leu and the NADH-ubiquinone oxidoreductase (16S-L1-ND1) was amplified using the primers 16S-ar (Palumbi *et al.*, 1991) and SPID-ND1 (Hedin, 1997) following the conditions: 94 °C for 5′, 28 cycles of 94 °C for 1′30″, 53 °C for 1′50″ and 72 °C for 2′30″, and an extension step at 72 °C for 10′.

We also explored the intronic regions of different spider genomes to find the candidate markers for phylogeographical studies and developed primers for a novel marker named CHP2 which amplifies a ~300 bp fragment and can be used in other studies on spider phylogeography. Primer sequences were: CHP2F (GTATCGCAAATGCCTTCAGC) and CHP2R (AACAAAGCAGGCTTCATTCG) and PCR conditions were: 95 °C for 1′, 35 cycles of 95 °C for 30″, 56 °C for 30″, 72 °C for 1′ and the final step at 72 °C for 7′. The PCR reactions consisted of 0.5–1 µL of template DNA, 0.2 µL of 5U Taq DNA Polymerase (Thermo-Fisher), 2–2.8 µL of 25 mM MgCl$_2$ (Thermo-Fisher), 2.5 µL of 10× KCl Buffer (Thermo-Fisher), 0.5–1 µL of 10 mM dNTP mix (GE Healthcare), 0.5 µL of each primer 10 µM, and ddH$_2$O to the volume of 25 µL. The amplified products were analysed in an automatic capillary sequencer ABI PRISM 3700 DNA Analyser (Applied Biosystems).

Alignment, genetic diversity and haplotype reconstruction

The sequences were aligned in MEGA 7.0 (Kumar *et al.*, 2016) and were visually inspected; we found the best fitting substitution model according to the AIC on jMODELTEST 2.0 (Darriba *et al.*, 2012) and as both mitochondrial regions had the same best substitution model, they were concatenated for all the analyses. For the nuclear markers, we used DnaSP (Librado & Rozas, 2009) to infer gametic phases, using a minimum posterior probability threshold of 0.9.

Diversity indices were calculated on ARLEQUIN 3.5 (Excoffier & Lischer, 2010); pairwise genetic distances were estimated on MEGA 7. Relationships among haplotypes were visualized on median-joining networks (Bandelt *et al.*, 1999) built on POPART (http://popart.otago.ac.nz).

Population structure and demographical analyses

We assessed the population structure with F_{ST} values calculated on ARLEQUIN 3.5. We also performed an analysis of molecular variance (AMOVA) using Cerrado and Chaco as separate groups, to test the structure between these biomes.

Population structure was also assessed on BAPS 6.0 (Corander *et al.*, 2008), which determines the most likely number of clusters (k) within a given group of sequences. We allowed k to vary between 1 and 20 and performed separate runs for each dataset (mitochondrial and nuclear).

To infer demographical patterns, we performed neutrality tests - Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) - in DnaSP, mismatch distributions analyses in ARLEQUIN, and extended Bayesian skyline plot (EBSP – Heled & Drummond, 2008) in BEAST 1.8.0 (Drummond *et al.*, 2012) for the whole dataset and for each mitochondrial group (see Results). Even though these tests have been widely used for demographical inferences in phylogeographical studies, all of them presume neutrality. In this case, finding similar patterns for mitochondrial and nuclear markers is a stronger evidence for shared demographical changes rather than adaptive selection.

Phylogenetic inferences and divergence times

We conducted a multilocus *BEAST analysis (Heled & Drummond, 2010) in BEAST 1.8.0 to estimate the divergence among major mitochondrial lineages (according to BAPS) taking into account incomplete lineage sorting. For the mtDNA, we used a strict clock (see Results) with a substitution rate of 0.0112 (SD = 0.001) substitutions/site/million years. Bidegaray-Batista & Arnedo (2011) estimated this rate for the Dysderidae family based on the well-resolved geochronology of the Mediterranean basin. Recently, Kuntner *et al.* (2013) found that the substitution rates estimated for the orbicularian families (which include Araneidae) overlapped with those from the Dysderidae, allowing these rates to be implemented to estimate the divergence times for orbicularian taxa. For the nuclear marker, we used the phased sequences under a lognormal relaxed clock and estimated the substitution rate in the analysis. As the outgroup, we chose *Nephila edulis*, an Australasian species, based on the phylogeny of Kuntner *et al.* (2013). COI and 16S-L1-ND1 sequences for this species were obtained from GenBank (accession numbers KC849126 and KC849083, respectively). As no *N. edulis* sequences are available for the newly developed nuclear CHP2 marker, we treated nuclear outgroups sequences as missing data.

A single run was conducted with 200 million generations, sampling every 20,000 generations. Convergence with a stationary distribution was checked on TRACER 1.5 (Rambaut *et al.*, 2014) through values of Effective Sample Sizes > 200 for each prior. We set the burn-in as 10%, discarding the first 1000 trees with TREEANNOTATOR 1.8.0. The resulting species and gene trees were visualized in FIGTREE 1.3.1 (Rambaut, 2012).

Ecological Niche Modelling (ENM)

In the framework applied to model the potential distribution of *N. sexpunctata* under present and past climate scenarios,

we used the 25 known points of present-day occurrence (Table S1) encompassing our sampling points, data is available on the SpeciesLink (http://splink.cria.org.br/) and GBIF (www.gbif.org), and points obtained from Instituto Butantan.

Besides the present time, models were projected to mid-Holocene (~6 ka), Last Glacial Maximum (~21ka) and Last Interglacial (~120ka) scenarios. As CCSM3 is the only source for climate data from the LIG, we used only this climate database for all temporal scenarios.

To infer the potential species distribution, we used five algorithms that represent different modelling techniques (envelope, distance and background) and evaluated the models using the TSS value. We then created a consensus map for each climate scenario (full description of methodology in Appendix S1).

Model testing

The ABC was used for model testing. We performed a hierarchical approach in which we first tested the models within four alternative scenarios that represent different phylogeographical hypotheses (Fig. 2) and then compared the best model for each scenario to obtain the model with highest posterior probability. This approach provides a reliable framework for testing multiple models (as performed by Peres *et al.*, 2015). Scenario 1 (Fig. 2, first line) represents a single panmictic population; scenario 2 (Fig. 2, second line) simulates two populations, one in each biome, with a divergence event dated after the LGM; scenario 3 (Fig. 2, third line) represents the two populations with a split dated in the Quaternary; Scenario 4 (Fig. 2, fourth line) represents the two populations with a Neogene divergence. Within each scenario, four models were tested: (1) constant population (s); (2) a constant-size population that experienced demographical expansion at some point in the past; (3) demographical expansion at LIG with a population bottleneck from LGM to the present; (4) a population bottleneck from LIG to LGM, and then a recent post-glacial expansion. For scenarios 2, 3 and 4, migration was also tested. This approach was performed jointly for both the datasets. More details on the methodology can be assessed in Appendix S1.

RESULTS

Genetic diversity

The concatenated mitochondrial dataset spanned 1501 bp; HKY was the best fitting model for nucleotide substitution. The dataset presented nine haplotypes structured into two groups (Fig. 1) according to BAPS (see *Genetic structure* session). Cerrado populations presented five haplotypes, with

Figure 2 Demographical scenarios hypothesized for *Nephila sexpunctata* evolution in Cerrado and Chaco. Scenario 1: panmictic population; scenario 2: two populations, one in each biome, with a post-LGM divergence; scenario 3: two populations with a Quaternary divergence; scenario 4: two populations with a Neogene split. Within each scenario, four hypotheses were tested: (1) no demographical changes along time (models 1, 5, 9 and 13); (2) exponential growth of a previous constant-size population (models 2, 6, 10 and 14); (3) glacial expansion followed by the interglacial bottlenecks (as predicted by the PRM, models 3, 7, 11 and 15); (4) glacial bottlenecks with post-glacial recovery (models 4, 8, 12 and 16). The best overall model is highlighted. LGM = Last Glacial Maximum (~21ka); LIG = Last Interglacial (~120ka).
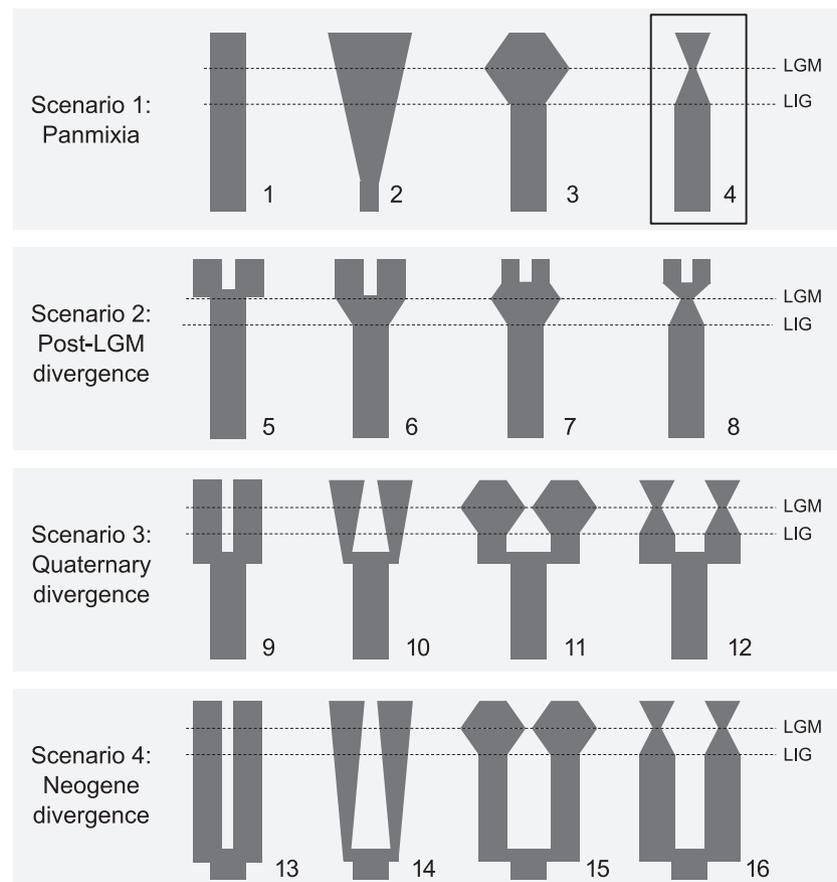
four exclusive; Chaco populations also presented five haplo-types, four exclusive.

Diversity levels were very low (Table 2). Three out of ten populations were monomorphic, one in Cerrado and two in Chaco. The CHP2 fragment comprised 232 bp and we found seven haplotypes. No indels were found in any of the sequences. TN93 + G was the best fitting model for nucleotide substitution. Chaco populations presented all haplotypes whilst five were detected in Cerrado; only two haplotypes were exclusive, both in Chaco. No population was monomorphic. Diversity levels were also low (Table 3). Nuclear nucleotide diversity was about ten times higher than that of the mitochondrial.

Genetic structure

We found no signs of isolation by distance in either dataset ($P = 0.319$ for nuclear marker; $P = 0.038$ for mitochondrial dataset, but correlation premises were violated – see Fig. S1).

The two BAPS mitochondrial groups are separated by only one mutational step. Group I occurs in Cerrado and Group II in Chaco, with some admixture (Fig. 1). The nuclear dataset showed no signs of subdivision in genetic/geographical groups. CHP2 network was highly unstructured (Fig. 1d); five nuclear haplotypes were shared between individuals from both mitochondrial groups.

F_{ST} value was higher for mtDNA (0.59) than for nDNA (0.09), showing a stronger structure. Pairwise values were high in the mitochondrial dataset mainly between populations from different biomes or populations from the same biome belonging to different genetic groups; most of the nuclear comparisons were not significant (Table S4).

AMOVA analyses using biomes as geographical groups (Cerrado × Chaco) were different for each marker; for mtDNA nearly a third of the variation was between groups, evidencing the strong structure between biomes; for the nuclear marker, over 90% of variation is within populations, with no structure pattern (Table 4).

Table 2 Mitochondrial genetic diversity indices and neutrality tests for *Nephila sexpunctata* populations and genetic groups inferred by BAPS. N = number of sequences; h = number of haplotypes; ss = number of segregating sites; Hd = haplotype diversity; π = nucleotide diversity; F_S = Fu's F_S; D = Tajima's D; R2 = R2 statistic; F_{ST} = fixation index, SD = standard deviation. *$P < 0.05$; **$P < 0.02$.

Location	N	h	ss	Hd (SD)	π (SD)	F_S	D	R2	F_{ST}
TP	8	2	1	0.536 (0.123)	0.00036 (0.00037)	0.866	1.166	0.268	–
PI	8	3	2	0.607 (0.164)	0.00045 (0.00044)	−0.478	−0.448	0.197	–
CT	3	1	0	0.000 (0.000)	0.00000 (0.00000)	–	0.000	–	–
CG	4	2	2	0.500 (0.265)	0.00067 (0.00066)	1.099	−0.710	0.433	–
PA	3	1	0	0.000 (0.000)	0.00000 (0.00000)	–	0.000	–	–
FE	8	2	1	0.250 (0.180)	0.00017 (0.00024)	−0.182	−1.055	0.331	–
ME	8	2	2	0.429 (0.169)	0.00057 (0.00051)	1.653	0.414	0.214	–
SM	8	1	0	0.000 (0.000)	0.00000 (0.00000)	–	0.000	–	–
CH	7	4	4	0.809 (0.130)	0.00089 (0.00071)	−0.914	−0.876	0.192	–
BA	8	2	1	0.536 (0.536)	0.00036 (0.00037)	0.866	1.166	0.268	–
Group I	32	4	3	0.599 (0.049)	0.00045 (0.00039)	−0.451	−0.197	0.118	–
Group II	33	5	4	0.481 (0.089)	0.00035 (0.00033)	−2.252	−1.166	0.084	–
Total	65	9	8	0.773 (0.027)	0.00081 (0.00058)	−2.771	−0.732	0.074	0.59

Table 3 Nuclear genetic diversity indices and neutrality tests for *Nephila sexpunctata* populations and genetic groups inferred by BAPS. N = number of sequences; h = number of haplotypes; ss = number of segregating sites; Hd = haplotype diversity; π = nucleotide diversity; F_S = Fu's F_S; D = Tajima's D; R2 = R2 statistic; F_{ST} = fixation index, SD = standard deviation. *$P < 0.05$; **$P < 0.02$.

Location	N	h	ss	Hd (SD)	π (SD)	F_S	D	R2	F_{ST}
TP	16	4	3	0.517 (0.132)	0.00295 (0.0026)	−1.098	−0.708	0.123	–
PI	16	5	3	0.608 (0.130)	0.00366 (0.0031)	−1.845	−0.173	0.145	–
CT	6	3	3	0.733 (0.155)	0.00546 (0.0046)	0.209	−0.185	0.268	–
CG	8	3	3	0.678 (0.122)	0.00570 (0.0045)	0.723	−0.585	0.210	–
PA	6	2	2	0.333 (0.215)	0.00287 (0.0029)	0.952	−1.132	0.373	–
FE	16	4	3	0.617 (0.096)	0.00305 (0.0027)	−1.019	−0.628	0.134	–
ME	16	5	4	0.808 (0.064)	0.00578 (0.0042)	−0.689	0.353	0.168	–
SM	16	4	3	0.642 (0.081)	0.00323 (0.0028)	−0.893	−0.494	0.138	–
CH	14	5	3	0.670 (0.126)	0.00384 (0.0032)	−1.933**	−0.173	0.148	–
BA	16	2	1	0.533 (0.046)	0.00230 (0.0023)	1.362	1.529	0.267	–
Group I	64	6	4	0.696 (0.044)	0.00467 (0.0034)	−0.542	0.603	0.135	–
Group II	66	6	4	0.602 (0.040)	0.00311 (0.0026)	−1.754	−0.300	0.092	–
Total	130	7	5	0.653 (0.031)	0.00399 (0.0031)	−1.217	0.014	0.093	0.09

Table 4 Results of the analysis of molecular variance (AMOVA) for mitochondrial and nuclear datasets for *Nephila sexpunctata* considering biomes (Cerrado and Chaco) as groups. d.f. = degrees of freedom.

Source of variation	d.f.	Variation (%)	*P*-value
mtDNA			
Among biomes	1	29.07	0.047
Among populations within biomes	8	33.14	< 0.001
Within populations	55	37.79	< 0.001
nDNA			
Among biomes	1	2.13	0.171
Among populations within biomes	8	6.36	0.011
Within populations	120	91.52	0.006

Demographical analyses

Neutrality tests presented non-significant values for mtDNA and nDNA datasets (Tables 2 & 3). The only significant value was Fu's F_S for the population of Parque Nacional del Chaco in the nuclear dataset. Mismatch distribution analyses exhibited contrasting patterns between markers. For the mtDNA, we detected signs of expansion for the whole dataset and for Group II, as evidenced by the non-significant raggedness indexes and unimodal curves of pairwise differences (Fig. 3). The pattern for the nDNA was different, with Group I being the only one presenting evidence of recent expansion (Fig. 3). Multilocus EBSPs pointed to strong and very recent demographical expansion in all datasets. Given that the generation time for the species is 1 year, all expansion times were inferred to have taken place in the Holocene, around 10 ka (Fig. 4).

Phylogenetic inferences and divergence times

The maximum likelihood test for molecular clocks implemented in MEGA7 did not reject the strict clock hypothesis

for the mitochondrial dataset ($P > 0.999$), nor did the path sampling and stepping-stone sampling (Baele *et al.*, 2012, 2013) implemented in BEAST 1.8.0 (lnBF = 1.92 and 2.58 respectively). The gene trees obtained from the *BEAST analysis (Fig. 5) presented different patterns as the mitochondrial gene tree recovered the same groups inferred by BAPS and the nuclear gene tree presented a less structured, admixed topology. Nuclear diversification was inferred to be older than mitochondrial (0.157 and 0.045 Ma, respectively) but confidence intervals overlapped. The joint species tree analysis estimated the divergence between *N. sexpunctata* and the outgroup (*N. edulis*) around 4.13 Ma [95% of the highest posterior density (HPD) = 3.43–4.90 Ma]. We found high statistical support for the split of the two clusters inferred by BAPS and the divergence between these groups was very recent, estimated around 0.0117 Ma (95% HPD = 0.003–0.028).

Ecological Niche Modelling

As TSS values for all algorithms were equal or higher than 0.5 (Table S3), we assumed that they generated reliable predictions. For the LIG scenario there was a very small suitable area for the species' occurrence in the region today covered by Chaco. In the LGM, there were suitable areas in Cerrado, with a distribution disjointed from those in Chaco. Around 6 ka there was a strong expansion of suitable areas in both biomes with the formation of a single joined area of distribution. The current scenario shows an even larger distribution, suggesting recent expansion (Fig. 6).

Model testing

For scenario 1, the most probable model was model 4, which includes a post-LGM expansion after a glacial bottleneck. For scenarios 2 and 3, the best models where those that considered constant populations that experienced exponential
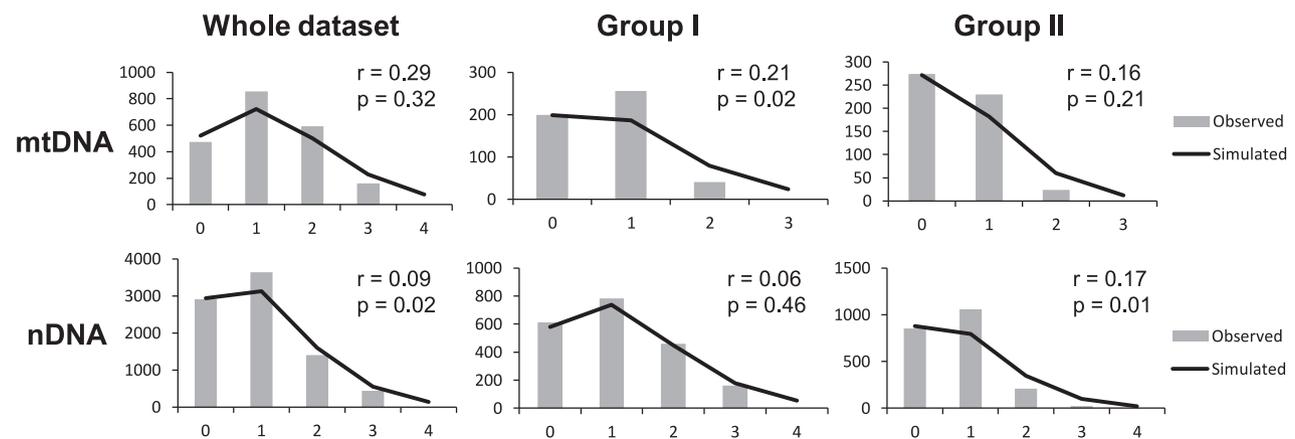

Figure 3 Results of mismatch distribution analyses performed for *Nephila sexpunctata* sequences. mtDNA (above) and nDNA (below) were analysed separately. Results were obtained for all sequences for each marker, and for each mitochondrial group separately. Columns represent the observed pairwise comparisons between individuals, and the continuous line represents the expected comparison in a scenario of sudden expansion. r = Harpending's Raggedness index; *P* = *P*-value.
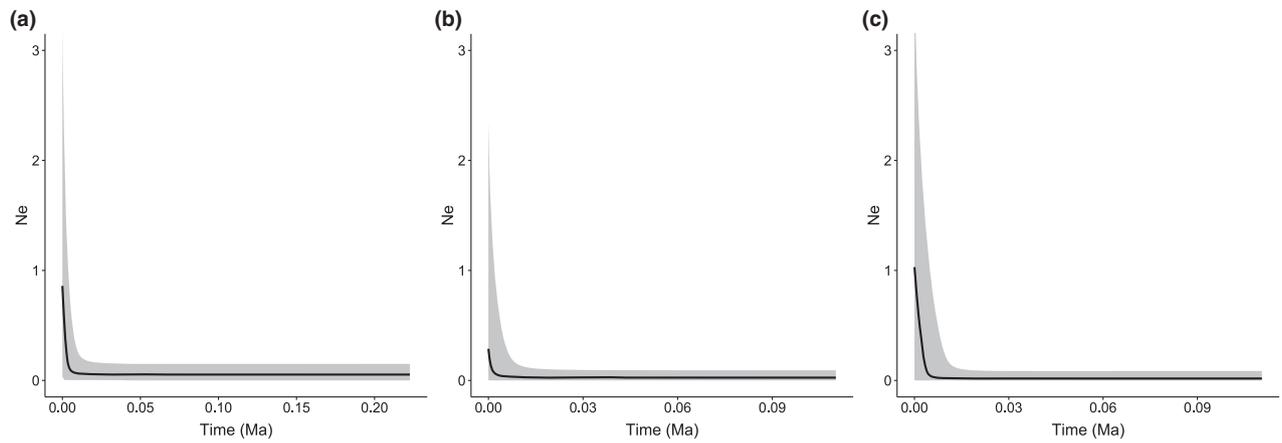
Figure 4 Results of Extended Bayesian Skyline Plots performed for *Nephila sexpunctata*. Analyses were performed using both markers jointly. Continuous line represents the median, and the grey area represents 95% HPD. (a) Results for the whole dataset, (b) Result obtained only for Group I individuals. (c) Result obtained only for Group II individuals.
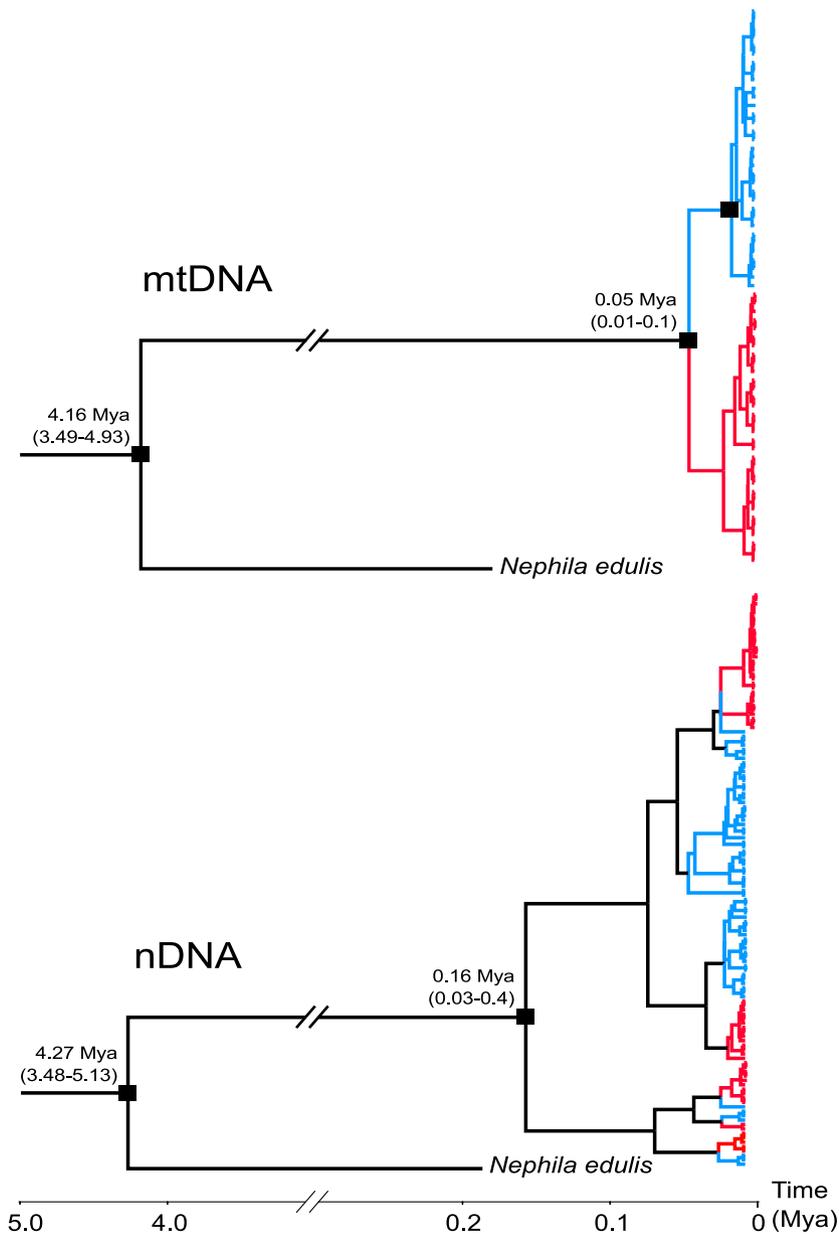

Figure 5 Bayesian gene trees inferred for *Nephila sexpunctata* mitochondrial (above) and nuclear (below) datasets. Branch colours represent the two mitochondrial groups inferred by BAPS. Black squares represent nodes with posterior probability > 0.95.
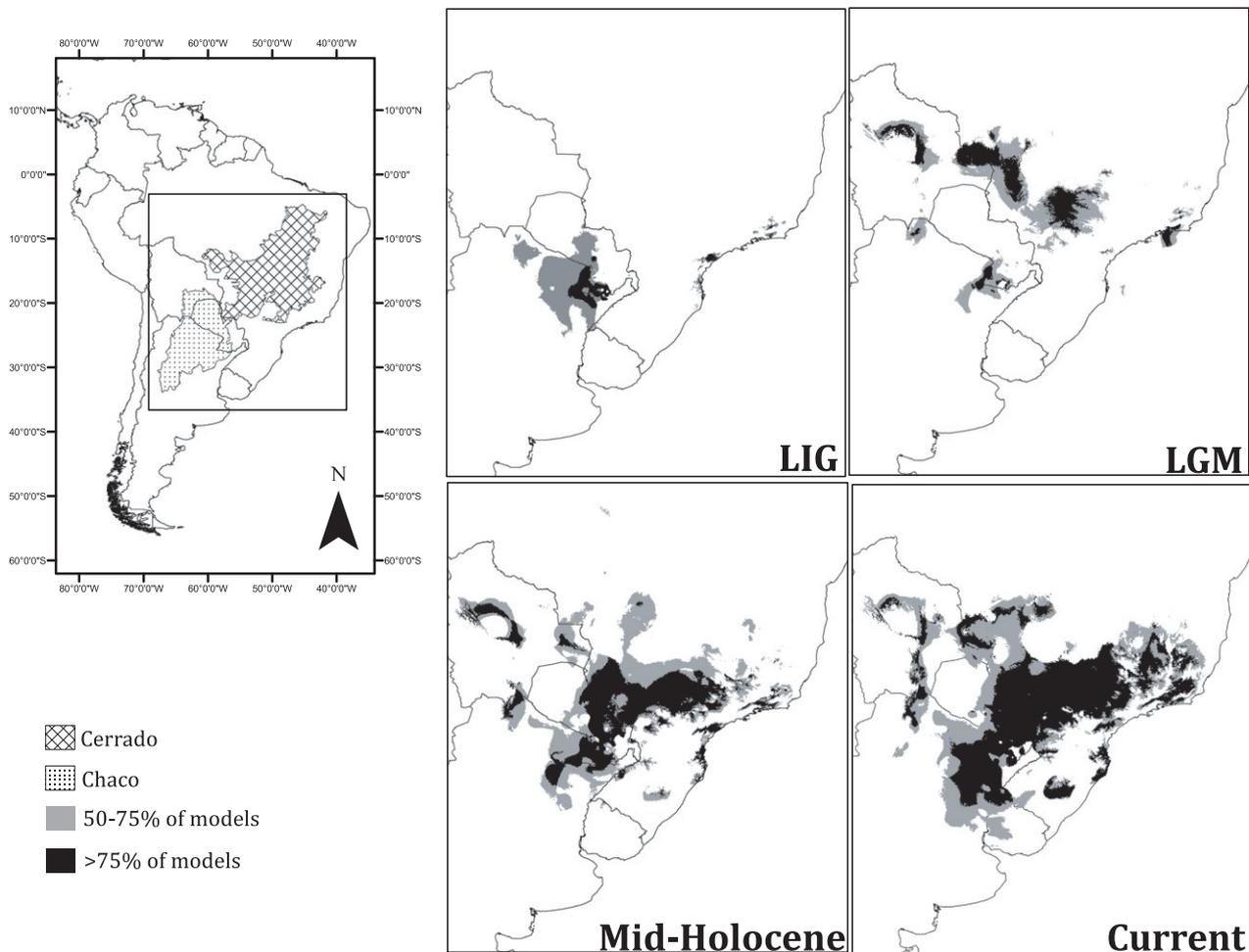
Figure 6 Ecological Niche Modelling showing suitable areas for *Nephila sexpunctata* occurrence over the last glacial cycle. Top left – 120ka; Top right – 21ka; Bottom left – 6ka; Bottom right – present day.

growth (models 6 and 10). For scenario 4, the best fit was model 15 (a glacial expansion with a post-LGM bottleneck). For scenarios 3 and 4, models that considered migration were strongly supported (Table 5).

When the analysis was performed among scenarios to find the best overall model, the panmixia (model 4) was preferred, but the post-LGM divergence (model 6) also had a high posterior probability (Table 5). Quaternary and Neogene divergence were least supported. Parameter estimation for models 4 and 6 can be assessed in Appendix S2 (Table S5). Therefore, the results endorse a recent demographical expansion.

DISCUSSION

Phylogeographical patterns of *N. sexpunctata*

The ABC results showed as the best overall model one single population that experienced a bottleneck during the glacial with a post-LGM recovery, opposite to what was expected by the PRM. The fact that the panmixia model was the preferred is against our assumption that different lineages were isolated in each biome, as found for lizards and frogs (Werneck et al., 2012a; Recoder et al., 2014), but we could not discard a post-LGM divergence as model 6 also showed a high posterior probability. The significant genetic structure among *N. sexpunctata* populations coupled with the low genetic divergence among haplotypes and the strong dispersal in the species may have generated genetic patterns that fit similarly the two scenarios. In both cases, the results endorsed a scenario of recent demographical expansion, and against what was expected by the PRM.

The low genetic diversity of *N. sexpunctata* contrasts with the values reported for other *Nephila* species (Su et al., 2007, 2011). Low levels of genetic diversity are common for Cerrado and Chaco species (Babb et al., 2011; Brito et al., 2013) and are frequently associated with bottlenecks because of the reduction of the suitable habitats of species adapted to dry conditions during the Quaternary (e.g. Bonatelli et al., 2014).

Discrepancies between nuclear and mitochondrial structuring patterns have been recurrently reported (Turchetto-Zolet et al., 2013) and are a likely product of multiple factors such

Table 5 Results of the model selection performed for both datasets. For every scenario posterior probabilities (PP) of each model are displayed, including the most recurrently recovered model (in bold). The analyses were performed within and among scenarios. Details on each model can be found in the Appendix S1.

Scenario	Within scenarios		Among scenarios	
	Model	PP	Migration (Y/N) – PP*	PP
1 (panmixia)	1	0.217	Not tested	–
	2	0.308		–
	3	0.050		–
	4	**0.428**		**0.489**
2 (post-LGM divergence)	5	0.307	(N) 0.503	–
	6	**0.499**		0.412
	7	0.046		–
	8	0.148		–
3 (Quaternary divergence)	9	0.300	(Y) 0.663	–
	10	**0.571**		0.096
	11	0.107		–
	12	0.022		–
4 (Neogene divergence)	13	0.006	(Y) 0.999	–
	14	0.002		–
	15	**0.973**		0.003
	16	0.019		–

*For each scenario, we chose the best model and then contrasted it with a similar one that considered bidirectional migration, and performed a new selection between these two models. In this column, we show if the model with (Y) or without (N) migration was preferred, and the PP of this model. Panmixia models were not tested for migration as they assume a single population with random mating.

as mtDNA introgression, incomplete lineage sorting, demographical asymmetries (Toews & Brelsford, 2012) and potential selection on the mitochondrial genome (Bazin et al., 2006). A slower rate of evolution, coupled with an effective population size four times higher for the nuclear genome compared with the mitochondrial genome, may also lead to the distinct structuring patterns observed. In N. sexpunctata, as for many other terrestrial invertebrates, these factors might help explaining the higher structure found on the mitochondrial markers.

The mitochondrial dataset presented two main lineages almost entirely related to each biome (Cerrado/Chaco, Fig. 1), and indeed a significant part of the species' diversity is organized between biomes (Table 4). As BAPS assigns groups for both genetic and geographical similarities, geography may play an important role in the characterization of the two clusters, since just one mutational step separated them. The admixture observed - represented by the occurrence of both mitochondrial lineages in some populations - may be because of the high dispersal ability of Nephila species (Kuntner & Agnarsson, 2011).

The signs of recent demographical expansion in N. sexpunctata after LGM (Figs 3 & 4) are congruent with the post-glacial habitat expansion inferred by ENMs and the

results from ABC. Lack of significance for neutrality tests may be because of the very low genetic variability in our sample, leading to analyses' low statistical power (Excoffier et al., 2009). Recent population and range expansions have been found for several DD species (e.g. Prado et al., 2012; Novaes et al., 2013), and are related to habitat expansion after the retraction of Cerrado vegetation during the LGM (Ledru et al., 1996) because of the extreme arid and cold conditions. Hence, the N. sexpunctata range would be reduced in LGM, expanding in the Holocene as climate conditions in southern Cerrado became warmer and wetter (Ledru, 2002), more suitable for the species' occurrence. These results do not support the idea of interglacial refuges for dry-habitat adapted organisms as suggested for cactus species (Bonatelli et al., 2014) and indicate that species with different ecological requirements may respond differently to climate fluctuations, with distinct factors shaping each species distribution (Prado et al., 2012).

N. sexpunctata divergence from outgroup N. edulis was estimated on Pliocene, supporting speciation after a recent dispersal event of an Australasian Nephila lineage to the Neotropics, as suggested by Kuntner et al. (2013). Studies using molecular dating have shown that dispersal events between the Palaeotropics and the Neotropics were frequent in the past few million years and are a good explanation for diversity patterns observed in many plants and animals (e.g. Blaimer, 2012; Christenhusz & Chase, 2013). Therefore, an open vegetation-adapted Nephila lineage, ancestral of N. sexpunctata, would have arrived in the Neotropics after the settlement of the DD, where it established itself. Diversification within the species, on the other hand, is very recent; the coalescence of all N. sexpunctata sequences was estimated around 11.7 ka, indicating that the extant diversity radiated during the Quaternary. This pattern is consistent with the simulations for Cerrado range during the Quaternary, which suggests unstable distribution throughout the last glacial cycle (Terribile et al., 2012; Werneck et al., 2012b); these data combined support the hypothesis that climate shifts may have played an important role in the diversification of Neotropical dry biomes (Silva, 1995).

Effects of Quaternary climate fluctuations on DD diversification

Despite the growing interest in Neotropical open formations, there is still a scarcity of testable hypotheses concerning DD evolution. Whereas a substantial amount of work concerning rain forests has indicated that geomorphological (Hoorn et al., 2010; Thomé et al., 2014) and climate (Cheng et al., 2013; Sobral-Souza et al., 2015) events may have worked together to shape biodiversity evolution in the Amazon and Atlantic Forest, the evolution of DD organisms is frequently explained by geological events such as the uplift of the Brazilian plateau (Werneck, 2011; Carvalho et al., 2013). Indeed, recent works have highlighted the role of Pleistocene climate oscillations in shaping current genetic variation,

mainly through shifts in Cerrado range that caused changes in the population size and bottlenecks (Diniz-Filho *et al.*, 2016).

Ecological Niche Modelling for different DD biomes (Werneck *et al.*, 2011, 2012b; Terribile *et al.*, 2012) does not support the predictions of the PRM, which include range expansion of open formations towards rain forests during the glacial periods. The models have shown that conditions were probably too cold and dry for large extents of these biomes during the LGM, and they presumably presented smaller ranges in this period with later expansion in the Holocene/present-day. Genetic and palaeodistributional data for *N. sexpunctata* show a post-LGM demographical and spatial expansion pattern that fits a scenario of post-glacial expansion of South American dry biomes. During LGM, the retraction of Chaco and Cerrado could have decreased habitat suitability for *N. sexpunctata* occurrence, leading to population bottlenecks (as reinforced by ABC results), causing the low levels of genetic diversity observed in the species nowadays. In a phylogeographical study with *Sicarius cariri*, a spider endemic to another counterpart of the DD (Caatinga), Magalhaes *et al.* (2014) found evidence of demographical retraction during glacials with a Holocene recovery, in a pattern similar to what was found for *N. sexpunctata* and against that predicted by the PRM. This finding suggests that glaciations might have affected some DD species in a different way from what was expected by the PRM, possibly because of common mechanisms related to habitat retraction because of harsh climate conditions.

Even though the effects of savanna expansion towards moist forests have long been acknowledged (Brown & Ab'Saber, 1979), the role of rain forest invasions in the diversification of DD organisms has only recently been recognized. Expansion of the Amazon and Atlantic Forest's spatial ranges seems to have shaped the genetic diversity in *Pleurodema*, a genus of Caatinga-endemic frogs, by creating intermittent forest corridors that promoted recurrent isolation among populations (Thomé *et al.*, 2016). Likewise, expansion of the rain forests towards Cerrado and Chaco (Nores, 1992; Sobral-Souza *et al.*, 2015) may have contributed to the structuring observed mainly in the mitochondrial dataset, the low genetic diversity indices and the demographical changes inferred.

In conclusion, our work reinforces the complexity of the diversification process in the Neotropical region. We demonstrated that Quaternary climate oscillations have affected Cerrado and Chaco, and consequently the diversification of DD organisms. This has to be taken into account, besides the frequently invoked main geological events. Our results also show that, unlike what was predicted by the PRM, some open-vegetation organisms have presented recent expansion, indicating that this period was too cold and dry even for their occurrence. The extent to which organisms from other Neotropical dry biomes were affected in a similar manner remains to be elucidated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Details on methodology.
Appendix S2 Supplementary tables.
Appendix S3 Supplementary figures.

DATA ACCESSIBILITY

All sequences are available on GenBank (accession numbers – COI: KX028886 - KX028950; 16S-L1-ND1: KX028951 - KX029015; CHP2: KX029016 - KX029080). Xml input files used in the Bayesian analyses and python scripts for coalescent simulations are available on GitHub (https://github.com/luizbartoleti/Nsexpunctata).

BIOSKETCH

Luiz Filipe de Macedo Bartoleti is interested in the phylogeography of Neotropical organisms, with emphasis on the evolutionary history of Neotropical biomes revealed by widely distributed species. All authors are interested in evolutionary biology and biogeography.

Author Contributions: LFMB and VNS conceived and designed the study. LFMB, EAP and FVHMF carried out fieldwork. LFMB and MJS performed the experiments. MJS and VNS contributed with reagents and material tools. LFMB, EAP, TSS, FVHMF and VNS analysed the data. LFMB, EAP, TSS and VNS wrote the paper. All authors have read and approved the final version submitted.

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