

## IMPACT OF LATE PLEISTOCENE-HOLOCENE CLIMATIC FLUCTUATIONS ON THE PHYLOGEOGRAPHIC STRUCTURE AND HISTORICAL DEMOGRAPHY OF *ZAMIA PRASINA* (CYCADALES: ZAMIACEAE)

## IMPACTO DE LAS FLUCTUACIONES CLIMÁTICAS EN EL PLEISTOCENO TARDÍO-HOLOCENO SOBRE LA ESTRUCTURA FILOGEOGRÁFICA Y DEMOGRAFÍA HISTÓRICA DE *ZAMIA PRASINA* (CYCADALES: ZAMIACEAE)

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### Abstract

**Background:** Glacial periods during the Pleistocene have been hypothesized to have greatly influenced geographical patterns of genetic structure and demography of many tropical species. The Glacial Refugium Hypothesis proposes that, during cold, dry glacial periods, populations of moisture-affinities tropical species were restricted to sheltered, humid areas and that, during warmer and more humid interglacial periods, these populations expanded. Some mountain regions in the tropics acted as refugia during the cold, dry periods of the Pleistocene for several temperate forest taxa, which recolonized the humid areas farther north during the interglacial periods.

**Questions:** (1) Did Late Pleistocene-Holocene climate changes affect the historical demography of *Zamia prasina*? (2) Does the historical distribution of *Zamia prasina* agree with the Glacial Refugium Hypothesis?

**Study species:** *Zamia prasina* W.Bull. (Zamiaceae), the only cycad native to the Yucatan Peninsula Biotic Province (YPBP).

**Methods:** Five individuals were collected in 23 populations and characterized using two DNA regions: plastid *atpF-atpH*, and nuclear ITS2. Genetic diversity, phylogeographic structure, historical demography, and potential distributions were assessed.

**Results:** Our results showed moderately high genetic diversity and low, but significant, phylogeographic structure. Two genetic groups were identified, one in the eastern part of the Peninsula, the other in the western. The changes in historical demography suggest that *Z. prasina* experienced a population expansion following the warm conditions of the Holocene.

**Conclusions:** The population dynamics of *Zamia prasina* are in accordance with the Glacial Refugium Hypothesis.

**Key words:** Cycads, demography expansion, diversity and genetic differentiation, glacial refugium hypothesis, Yucatan Peninsula Biotic Province (YPBP).

### Resumen

**Antecedentes:** Los períodos glaciales ocurridos en el Pleistoceno tuvieron gran influencia en la estructura genética y demografía de muchas especies tropicales. La Hipótesis del Refugio Glacial propone que, durante los períodos glaciales secos y fríos, las poblaciones de especies tropicales se restringieron a áreas húmedas y que, durante los períodos interglaciales más cálidos y húmedos, estas poblaciones se expandieron. Algunas regiones montañosas de los trópicos actuaron como refugios durante los períodos fríos y secos del Pleistoceno para varios taxones de bosques templados, que recolonizaron las áreas húmedas más al norte durante los períodos interglaciales.

**Preguntas:** 1) ¿Los cambios climáticos del Pleistoceno tardío-Holoceno afectaron la demografía histórica de *Zamia prasina*? 2) ¿La distribución histórica de *Zamia prasina* responde a la Hipótesis de Refugio Glacial?

**Especies de estudio:** *Zamia prasina* W. Bull. (Zamiaceae), la única cicada nativa de la Provincia Biótica península de Yucatán (PBPY).

**Métodos:** Se colectaron cinco individuos en 23 poblaciones y se caracterizaron utilizando dos regiones de ADN: cloroplasto (*atpF-atpH*) y nuclear (ITS2). Se evaluó la diversidad genética, estructura filogeográfica, demografía histórica y distribución potencial.

**Resultados:** Nuestros resultados mostraron una diversidad genética moderadamente alta y una estructura filogeográfica baja, pero significativa. Se identificaron dos grupos de poblaciones diferenciados genéticamente, uno ubicado en el sureste y el otro al norte de la Península. Los cambios en la demografía histórica sugieren que *Z. prasina* experimentó una expansión poblacional en las condiciones cálidas del Holoceno.

**Conclusiones:** La dinámica poblacional de *Zamia prasina* se ajustan a la Hipótesis de Refugio Glacial.

**Palabras clave:** Cicadas, expansión demográfica, diversidad y diferenciación genética, hipótesis de refugio glacial, Provincia Biótica Península de Yucatán (PBPY).

Throughout the Pleistocene ( $\approx 2.5$  Ma), extended blankets of glacial ice covered the highest latitudinal zones of the planet, giving rise to glacial periods, which alternated with interglacial periods, when these areas were partially free of ice (Svensson *et al.* 2005). The glaciation period had a greater impact on the northern hemisphere biota than on the southern hemisphere given that the large land masses very close to the Arctic could channel the glaciers toward the south, whereas an ocean separated the Antarctic from the southern continents (Svensson *et al.* 2005). The glacial periods of the Pleistocene also greatly influenced the geographic distribution, the genetic structure and demography of tropical species (Stewart *et al.* 2010, Ramírez-Barahona & Eguiarte 2013). Some the mountain regions located in the tropics acted as areas of refugium during the cold, dry periods of the Pleistocene for several temperate forest taxa, which recolonized the humid areas farther north during the interglacial periods (Ramírez-Barahona & Eguiarte 2013). These Neotropical refugia might have remained continuously humid, while the savannahs and dry tropical forests expanded, leaving populations featuring high genetic structure in different areas of refugium (Haffer 1969, Graham 1973, Toledo 1982, Myers 1982, Pennington *et al.* 2000). The Holocene was also a period of climatic fluctuations. The Holocene Climate Optimum (9-5 Ka) was a warm period; the global climate was most likely between 0.5-3 °C warmer than it is today. Then the temperatures decreased progressively with cyclic heating/cooling periods until now (Walker *et al.* 2012).

Tropical forests and their biota have a complex evolutionary history (Ornelas *et al.* 2013); for example, *Dioon edule* (Zamiaceae) (González-Astorga *et al.* 2003) and *Zamia paucijuga* (Zamiaceae) (Nolasco-Soto *et al.* 2015) experienced demography changes in their populations influenced by the Pleistocene glaciations. Historic demography changes in plant species could be consistent with either the Glacial Refugium Hypothesis (GRH), which propose that populations contracted to one or more southerly refugia during the cold-dry glacial and expanded out from them in warm-humid interglacials. This hypothesis is widely accepted for temperate species such as subtropical columnar cacti in the mid latitudes of the Northern Hemisphere (Soltis *et al.* 2006). In contrast, the Interglacial Refugium Hypothesis (IRH) suggests that in the intertropical open dry vegetation of South America (Caatinga, Cerrado and Chaco biomes), populations of some species contracted to warm and humid refugia during the interglacials and expanded outward under the cold-dry climate of the Ultimate Glacial Maximum (LGM) ( $\approx 80$  Ka) (Cornejo-Romero *et al.* 2017). Several authors have proposed a refugium theory as the underlying model for glacial and postglacial population dynamics of tropical species during the LGM (Farrera *et al.* 1999, Ramírez-Barahona & Eguiarte 2013, Cornejo-Romero *et al.* 2017).

The degree of aridity in the tropics generated by the reduced precipitation during the LGM and its influence on the

distribution of the Neotropical forests have been controversial topics (Ramírez-Barahona & Eguiarte 2013). In one of the pioneer publications on the impact of the glaciations of the Pleistocene in the Neotropics, Van der Hammen (1961) postulated two opposing hypotheses: (1) During the glaciations, the lower temperatures generated a reduction in precipitation and, consequently, an increase in aridity in the Neotropics. (2) The cold phases of the glaciations were accompanied by an increase in precipitation, and the warm phases were much drier; therefore, the glaciations did not lead to a reduction in the precipitation and did not significantly affect the continuous and stable distribution of the tropical rainforests (Farrera *et al.* 1999). However, the information available to date has been based largely on limited and conflicting paleoecological data (Ramírez-Barahona & Eguiarte 2013). In general, the existing records indicate greater tropical aridity, with low lake levels in regions such as tropical Africa (Caballero *et al.* 1999). These records have been interpreted as an indication that, during the LMG, climates were drier (Kutzbach *et al.* 1993). In the Trans-Mexican Volcanic Belt, several records of the internal basins suggest greater aridity (Lozano-García *et al.* 2005, Caballero *et al.* 2010), while west-central Mexico have been proposed to have had relatively humid conditions (Bush *et al.* 2009, Bradbury 2000).

During the Last Interglacial periods (120 Ka), the climate of the YPBP became warmer and more humid (Metcalfe *et al.* 2000). During most of the LGM, the climate of the YPBP was dry, and the temperature was approximately 6 °C colder than at present. Savannahs and scrublands covered most of the region up to the Early Holocene (10 Ka) (Orellana *et al.* 2003). In an analysis of Holocene fossil pollen samples from the YPBP, to reconstruct the vegetation and to develop a precipitation record for the last 7,900 years, a gradual increase in precipitation and expansion of the tropical forest during the Middle Holocene was shown (Vela-Pelaez *et al.* 2018).

*Zamia prasina* W. Bull. (Zamiaceae), distributed in the tropical rainforests of the central and southeast areas of the YPBP, is the only cycad native to this region. According to the International Union for the Conservation of Nature (IUCN), it is severely threatened (Vovides & Nicolalde-Morejón 2010). The populations of *Z. prasina* almost certainly were affected by the climate changes of the Pleistocene, which have been proposed as the driving force of the diversification in cycads (Nagalingum *et al.* 2011). The cycads are a species group with a long evolutionary history originating in the late Permian (Rull 2012). However, molecular data indicate that current species had a more recent origin (Late Miocene,  $\approx 10-5$  Ma) (Nagalingum *et al.* 2011). The almost simultaneous initiation of the diversification of six of the living genera of the cycads (in Australia, Africa, South-east Asia and tropical South America) suggests a single promotor may have been responsible for their diversification and that event may have been global climate change (Nagalingum *et al.* 2011). The climate fluctuations during the Pleistocene

significantly influenced the current distribution of the cycads (González & Vovides 2002) and that, for the cycads of Mexico, the refugium areas of the LGM were fundamental in the definition of their phylogeographic dynamics (Contreras-Medina & Luna-Vega 2002). Consequently, we sought to determine whether the Late Pleistocene-Holocene (80-10 Ka) climate changes affected the historical demography of *Zamia prasina* and whether the historical distribution of *Zamia prasina* fits with the scenario proposed by the glacial refugium hypothesis. Thus, if this species underwent a contraction during the glaciations (LGM), then we would expect a reduction in the environmentally suitable area and in the effective size of the population during the LGM. Conversely, the effective size of the population and suitable area would increase during an interglacial period. To answer these questions, we used a phylogeographic approach, Bayesian analysis, and niche model. This study represents one of the first phylogeographic studies in the YPBP. We addressed the following objectives: (1) to determine the genetic diversity and phylogeographic structure of *Z. prasina*, (2) to evaluate the influence of the climate changes during the Late Pleistocene-Holocene on the historical demography of *Z. prasina* and (3) to determine whether the climate changes of the

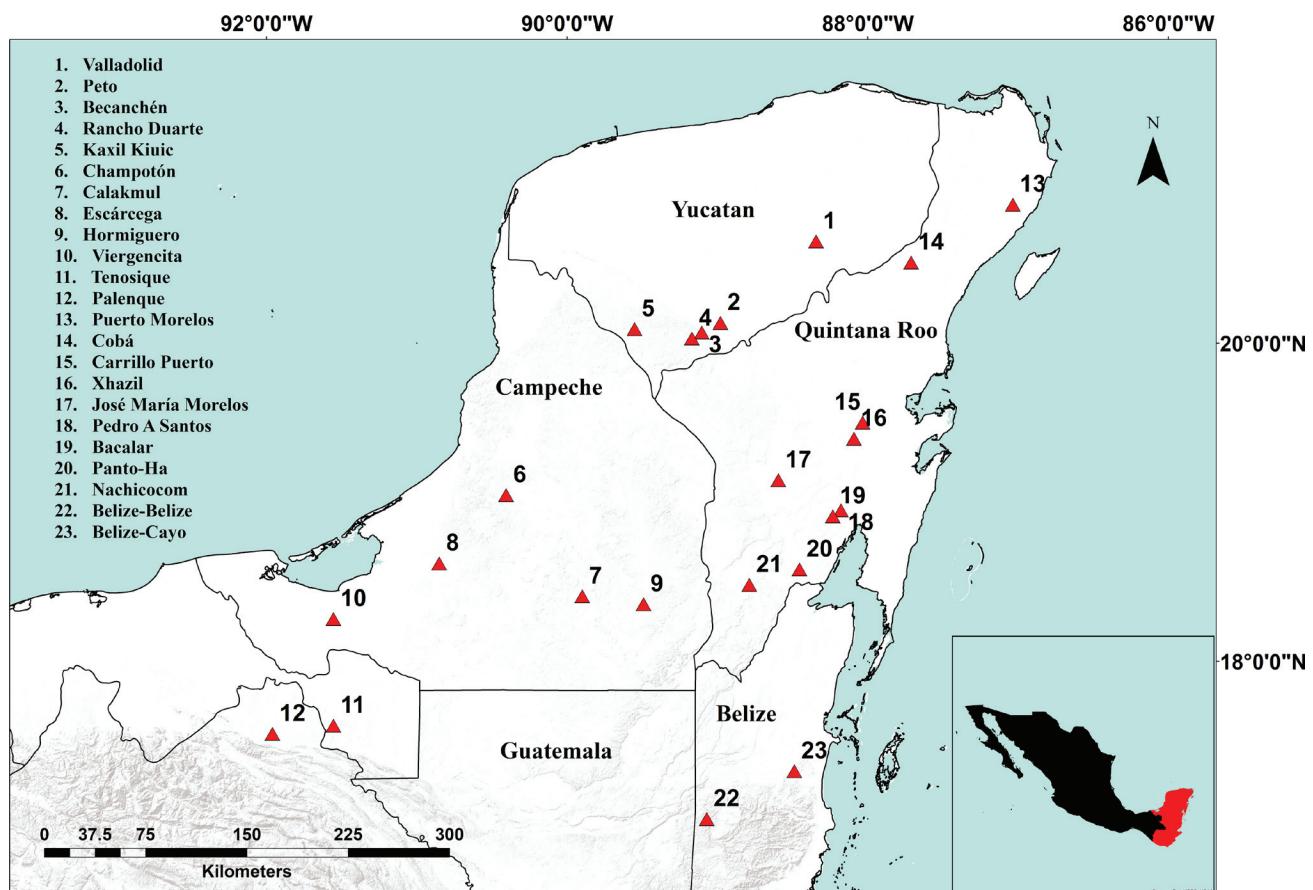
Late Pleistocene-Holocene modified the potential distribution range of *Z. prasina*.

## Materials and Methods

**Study area, populations and samples.** This study was conducted in the Yucatan Peninsula Biotic Province (YPBP), which includes the Mexican states of Campeche, Quintana Roo, Yucatan and part of the states of Tabasco and Chiapas, northern districts of Belize (Orange Walk, Corozal, and Belize) and the department of Petén in Guatemala (Carnevali *et al.* 2010). The selection of populations sampled was based on information obtained from Herbarium CICY and from previous studies, Velasco (2015). Herbarium vouchers for each population were deposited at Herbarium CICY. We collected samples from 21 natural populations of *Z. prasina* in the Mexican part of the YPBP and five samples from two populations in Belize (two in Cayo District and three in Belize District) that were donated by Michael Calonje (Montgomery Botanical Center) (Figure 1, Table 1). It was not possible to obtain samples of *Z. prasina* from Guatemala; therefore, any reference to the YPBP here does not include Guatemala.

**Table 1.** Population, Number of individuals for population (*N*), latitude, longitude and respective haplotypes for populations of *Zamia prasina* studied.

Population	<i>N</i>	N. Latitude	W. Longitude	Haplotype <i>atpF-atpH</i>	Haplotype ITS2
1	Valladolid	5	20.633342	-88.343722	A1
2	Peto	4	20.125567	-88.980083	A1, A2
3	Becanchén	5	20.065992	-89.104694	A1, A2
4	Rancho Duarte	4	20.028056	-89.169531	A1, A2
5	Kaxil Kiuic	3	20.086944	-89.551528	A3
6	Champotón	5	19.04675	-90.405556	A1
7	Calakmul	5	18.409306	-89.899444	A1
8	Escárcega	5	18.617028	-90.850889	A1
9	Hormiguero	3	18.359667	-89.491722	A1, A3, A7
10	Virgensita	5	18.266389	-91.554	A3, A4, A5, A6
11	Tenosique	5	17.591472	-91.554	A1
12	Palenque	5	17.539278	-91.959889	A1
13	Puerto Morelos	5	20.862083	-87.035139	A3
14	Cobá	5	20.499972	-87.711806	A1, A3
15	Carrillo Puerto	4	19.501583	-88.034056	A1, A3
16	Xhazil	5	19.400417	-88.092278	A1, A3
17	José María Morelos	5	19.140333	-88.594694	A1, A3
18	Pedro A. Santos	5	18.950778	-88.178833	A3, A8
19	Bacalar	5	18.913278	-88.2325	A3
20	Panto-Ha	5	18.578861	-88.454167	A1, A3
21	Nachicocom	4	18.481778	-88.787833	A1, A3
22	Belize-Belize	2	16.998917	-89.071533	A1, A3
23	Belize-Cayo	3	17.302667	-88.488167	A1, A9



**Figure 1.** Location of the 23 populations of *Zamia prasina* sampled in the Yucatan Peninsula Biotic Province (YPBP). Biotic Province (YPBP). 1: Valladolid, 2: Peto, 3: Becanchén, 4: Rancho Duarte, 5: Kaxil Kiuic, 6: Champotón, 7: Calakmul, 8: Escárcega, 9: Hormiguero, 10: Virgensita, 11: Tenosique, 12: Palenque, 13: Puerto Morelos, 14: Cobá, 15: Carrillo Puerto, 16: Xhazil, 17: José María Morelos, 18: Pedro A Santos, 19: Bacalar, 20: Panto-Ha, 21: Nachicocom, 22: Belize-Belize 23: Belize-Cayo.

**DNA extraction, amplification and sequencing.** DNA was extracted from individuals from each population, using the CTAB protocol (Doyle *et al.* 1987). We amplified the chloroplast intergenic region *atpF-atpH* and nuclear region ITS2 selected by Nicolalde-Morejón *et al.* (2011) using the methods of Lahaye *et al.* (2008) and Baldwin (1992), respectively. These markers were previously used in genetic studies of the genus *Zamia*. The amplified products were visualized in 1 % agarose, and 110 samples for chloroplast and nuclear regions were sent to in Macrogen 2018 for unidirectional sequencing using the forward primer and Sanger sequencing. The sequences were edited in Sequencher v. 5 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned in the software PhyDE® (Müller *et al.* 2010) with the multiple alignment tool Muscle, then they were examined manually. For our ITS2 data set, we confirmed that multiple copies were not present for the following reasons: (1) all sequences had a GC content > 65 %, (2) all BLAST results coincided with the number of base pairs for ITS2 sequences for *Zamia* in GenBank, (3) conserved regions were homologous to ITS2 sequences from *Zamia* in GenBank, and (4) a few

nucleotides differed between our sequences and those in GenBank.

**Genetic diversity and phylogeographic structure.** The number of haplotypes ( $k$ ) and nucleotide and haplotype diversities ( $\pi$  and  $h$ , respectively) were determined using the DnaSP v.5.10 (Librado & Rozas 2009); indels were not considered. The genetic diversity was determined for each DNA region, population, population groups (based on genetic differences) and species (all populations). The genetic structure was determined by means of a non-hierarchical molecular analysis of variance (AMOVA) with 1,000 permutations, using paired genetic differences with the program Arlequin 3.5 (Excoffier *et al.* 2005). To infer the phylogeographic structure, the relationship of  $N_{ST}/G_{ST}$  was calculated. Both are estimators of genetic structure;  $N_{ST}$  considers the nucleotide differences among the haplotypes, whereas  $G_{ST}$  is based on the number of haplotypes and their frequency. The statistical analysis was performed in the PERMUT 2.0 program (Pons & Petit 1996) with 1,000 permutations where, if 5 % of the permuted values are lower than the  $N_{ST}$  observed, then  $N_{ST} > G_{ST}$ , thereby in-

dicating phylogeographic structure. The relationships among the haplotypes were determined through haplotype networks using the TCS network algorithm in the PopArt 1.7 program (Clement *et al.* 2002). This network shows the relationship among the haplotypes and the number of mutational steps separating them. In addition, the geographic distribution of the haplotypes was represented on a map to graphically record the aggregation of the haplotypes.

To confirm the existence of population groups genetically differentiated, an analysis was conducted to detect the geographical location of genetic discontinuities among the populations using Monmonier's maximum difference algorithm implemented in the program BARRIER (Manni *et al.* 2004). From the pairwise genetic distances obtained in Arlequin 3.5 (Excoffier *et al.* 2005), BARRIER identifies the edges where there are greater genetic distances. For obtaining confidence levels for the barriers, 100 replicas of the genetic distance matrix were calculated using Program R ver 4.3.2.

To explore the influence of climatic variables on the genetic structure, a principal component analysis (PCA) was carried out using data for 19 environmental variables, derived from temperature and precipitation, obtained from WorldClim Global Climate Data V. 1.4 (<http://www.worldclim.org/version1>) (Hijmans *et al.* 2005) with a resolution of 1 km<sup>2</sup>. Any environmental variables with a Spearman's rank correlation higher than 0.8 between them were not used. A final set of 11 environmental variables was extracted for the 23 populations of our study (Appendix 1): Mean Diurnal Range (Mean of monthly (max temp - min temp)) (BIO2), Isothermality (BIO3), Temperature Seasonality (BIO4), Max Temperature of Warmest Month (BIO5), Temperature Annual Range (BIO7), Mean Temperature of Coldest Quarter (BIO11), Annual Precipitation (BIO12), Precipitation Seasonality (BIO15), Precipitation of Driest Quarter (BIO17), Precipitation of Warmest Quarter (BIO18) and Precipitation of Coldest Quarter (BIO19). Past software version 3 was used (Hammer *et al.* 2001).

*Estimate of divergence times.* The time of divergence for intraspecific diversification of *Zamia prasina* and a possible relationship with pre-Pleistocene and Pleistocene events was estimated using Bayesian inference (BI) implemented by the program BEAST 2 (Bouckaert *et al.* 2014). The model of sequence evolution HKY+G was employed for both regions according to the results of the AIC model selection from jMODELTEST (Posada 2008). An uncorrelated relaxed clock Log Normal model (UCLD) and a coalescent model assuming constant size were used to model the tree prior. The tree was calibrated using the 95 % highest posterior density (HPD) age reported by Calonje *et al.* (2019). Prior distributions for all calibrated nodes were conservatively set to uniform using the minimum and maximum age bounds outlined below.

The age intervals for divergence between *Microcycas calocoma* and *Zamia* genus was used for the tree root node (33 to 84.5 Ma) (I), the crown node of *Zamia* (9 to 22.1 Ma) (II). Divergence between *Zamia prasina* and *Zamia variegata* Warsz. (0 to 0.82 Ma) (III) and the divergence

between the clades formed by *Z. prasina* plus *Z. variegata* and the clade *Z. spartea* Ac.DC. plus *Z. furfuracea* Aiton plus *Z. loddigesii* Miq. (0.51 to 1.53 Ma) (III). Twelve species (*i.e.*, *Microcycas calocoma*, *Zamia variegata*, *Z. furfuracea*, *Z. paucijuga*, *Z. loddigesii*, *Z. spartea*, *Z. lacandona*, *Z. pseudoparasitica*, *Z. manicata*, *Z. inermis*, *Z. soconuscensis*, *Z. fischeri*, were chosen as outgroups.

For divergence time estimations, Markov chain Monte Carlo (MCMC) were run for three independent 50 million generations, sampling every 5,000 generations. BI analyses were run using the CIPRES Science Gateway (Miller *et al.* 2010). We combined the log and trees files from each independent run using LOGCOMBINER 1.8.0 (Drummond & Rambaut 2007), then viewed the combined log file in TRACER 1.6 to ensure that effective sample sizes for all priors and the posterior distribution were > 200, making sure that parameter values were fluctuating at stable levels. Based on these results, the first 5,000 trees were discarded as burn-in, and the remaining samples were summarized as a maximum clade credibility tree with mean divergence times and 95 % highest posterior density (HPD) intervals of age estimates in TREEANNOTATOR. Finally, these results were summarized in a single tree visualized in FIGTREE ver 1.3.1 (Rambaut 2009).

*Historical demography.* The demography processes were analyzed at two levels: (1) species and (2) population groups. Three types of analyses were carried out. (1) Tajima's *D* (Tajima 1989) and Fu's *F<sub>s</sub>* (Fu 1997): neutrality tests were used to detect departures from a constant population size under the neutral model. Population growth was indicated by significant negative values ( $p < 0.05$ ) using Arlequin with 10,000 permutations. (2) Mismatch distribution of pairwise nucleotide differences (Rogers & Harpending 1992) was calculated and compared with expected values for an expanding population using the Ramos-Onsins & Rozas (2010)  $R_2$  statistic. This statistic considers the sample size, the number of singleton mutations in a sequence, the average number of nucleotide differences between two sequences, and the total number of segregating sites. Lower values of  $R_2$  ( $< 0.05$ ) are expected under a recent population growth event (Ramos-Onsins & Rozas 2010). A unimodal type graph shifted to the left indicates many comparisons where the differences between pairs of sequences are small, suggesting a recent expansion in the populations this was carried out in the program DnaSP 5.10 (Librado & Rozas 2009) with 10,000 permutations. (3) Bayesian skyline plot (Drummond & Rambaut 2007) to infer the changes of the effective population size over time, allowing the use of mutation models and independent replacement rates for each region of DNA. The model HKY+G was used for both DNA regions, with a strict molecular clock model and coalescent model. This analysis was performed at three levels: 1) each DNA region, 2) species and, 3) populations groups. The number of substitutions per site per year (s/s/y) for *atpF-atpH* was 0.00056 and 0.00181 for ITS2 and were used to date the crown radiation of *Zamia paucijuga* (Nolasco-Soto *et al.* 2015), also published by Nagalingum *et al.* (2011) for the crown-age of

*Zamia* spp. Thirty million permutations (MCMC) were carried out, and trees were collected every 3,000 generations, using the program BEAST ver 1.8.0. Outputs were visualized with TRACER ver 1.6 to assess stationarity of the MCMC (effective sample sizes > 200).

**Potential distribution.** The ecological niche of *Zamia prasina* was modeled based on 80 occurrences obtained from the Global Biodiversity Information Facility (GBIF, <http://data.gbif.org/species/browse/taxon/>) and 103 occurrences compiled from fieldwork. Duplicates occurrences and occurrences with a distance < 20 km among them were removed using the package spThin (Lammens *et al.* 2015) to reduce overfitting promoted by spatial aggregation of the occurrence. A total of 43 records were used. The same 11 environmental variables that were used for the PCA were used for the Ecological Niche Modelling (ENM). The 11 bioclimatic variables were masked to extend of ecoregions (WWF 2006) were *Z. prasina* occurrence exist as hypothesis of the accessible area (M).

The niche of *Z. prasina* was modeled using the maximum entropy algorithm in MAXENT v. 3.3.3 (Phillips *et al.* 2006) with 100 replicates using bootstrapping as a resampling method with no extrapolation and no clamping. For model evaluation 20 % of the total records were used. Logistic output was selected to obtain maps with suitability values. The ENM was projected in the YPBP and Central America, a center of diversity for the genera *Zamia*. The minimum training presence was used as threshold to obtain a binary map of presence-absence. Finally, the ENM obtained was transferred into three past climatic scenarios: the Last Interglacial (LIG  $\approx$  120 ka), the Last Glacial Maximum (LGM  $\approx$  21 ka) and the Middle Holocene (MH  $\approx$  6 ka). Two general models of global circulation (GCM, <http://www.worldclim.org/paleoclimate1>) were employed to transfer the ENM into the LGM and MH scenarios: the Community Climate System Model (CCSM, Collins 2004) and the Model of Interdisciplinary Research on Climate (MIROC, Hasumi & Emori 2004). Both models simulate the climatic conditions in the LGM and MH, with a stronger reduction in the temperature assumed in the CCSM model in comparison with the MIROC model (Otto-Bliesner *et al.* 2007).

## Results

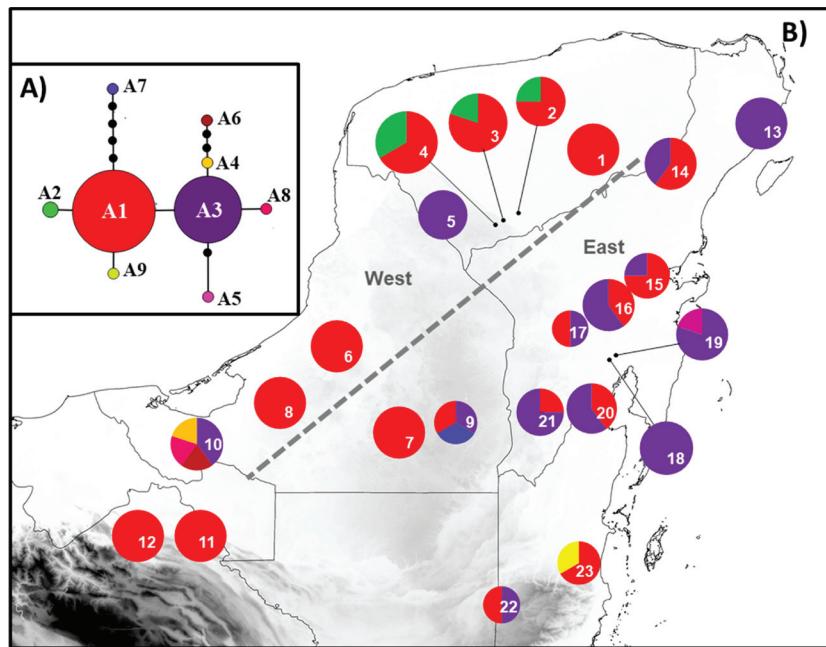
We analyzed 102 sequences of the *atpF-atpH* region, with a length of 462 bp and 90 sequences of the ITS2 region, with a length of 301 bp. For *atpF-atpH*, 23 populations were analyzed. For ITS2 one population was excluded because its sequences coincided with an endophytic fungus.

**Genetic diversity.** The genealogical relationships indicated that, for *atpF-atpH*, the most frequent haplotypes were A1 and A3, which are thus candidates as ancestral haplotypes given their internal position in the network and their higher number of connections with the other less-frequent haplotypes. Most haplotypes were differentiated in a single mutational step, except for A6 and A7 (Figure 2-A). For ITS2, haplotypes H1,

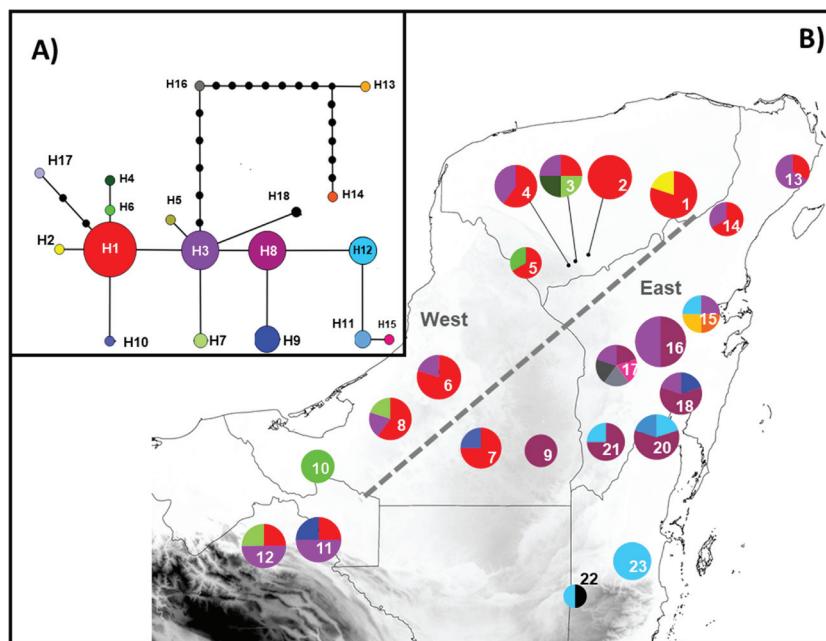
H3 and H8 were the most frequent and thus possible ancestral haplotypes, given their internal position in the network. Most haplotypes were differentiated in only one mutational step. The exceptions were H13, H14, H16 and H17 (Figure 3-A). For *atpF-atpH*, nine haplotypes were obtained (Table 1). The overall haplotype diversity (*h*) was moderate, with a mean value of *h* = 0.564; the nucleotide diversity ( $\pi$ ) was low, with a mean value of  $\pi$  = 0.00186. The populations of Valladolid, Kaxil Kiuic, Champotón, Escarcega, Calakmul, Palenque, Tenosique, Puerto Morelos y Bacalar had only one haplotype; so, *h* and  $\pi$  were equal to zero (Appendix 2). Four populations had exclusive haplotypes: Virgencita (A4, A5 and A6), Hormiguero (A7), Belize-Cayo (A9) and Pedro A. Santos (A8) (Figure 2-B). For ITS2, 18 haplotypes were obtained (Table 1). Haplotype diversity (*h* = 0.827) and nucleotide ( $\pi$  = 0.00852) values were higher than those obtained for *atpF-atpH*. The populations of Peto, Virgencita, Hormiguero and Belize-Cayo presented only one haplotype; therefore, *h* and  $\pi$  were equal to zero (Appendix 3). Five populations had exclusive haplotypes: Becanchén (H4 and H5), Calakmul (H10), Carrillo Puerto (H13 and H14) and José María Morelos (H15 and H16) (Figure 3-B). List of GenBank accession for haplotypes are in Appendix 4.

**Phylogeographic structure.** The non-hierarchical AMOVA showed similar results for *atpF-atpH* and ITS2 regions: 33.85 and 37.23 %, respectively, of the total variation found among populations. For chloroplast and nuclear regions, the values of  $F_{ST}$  were statistically significant ( $F_{ST} = 0.338$ ,  $p < 0.001$ ;  $F_{ST} = 0.372$ ,  $p < 0.001$ ; respectively), indicating genetic differentiation among the populations studied (Table 2). For both regions, the greatest diversity is found within the populations, 66.15 % for *atpF-atpH* and 62.77 % for ITS2. For *atpF-atpH*, the  $N_{ST}$  value observed was statistically higher in comparison with the value of  $G_{ST}$  (0.433 and 0.370, respectively; 96.1 % of permuted values of  $N_{ST}$  were lower than observed  $N_{ST}$ ). For ITS2, the observed value of  $N_{ST}$  was significantly higher than the value of  $G_{ST}$  (0.362 and 0.257, respectively; 86.8 % of permuted values of  $N_{ST}$  were less than the observed  $N_{ST}$ ). Both results indicate signatures of phylogeographic structure.

The chloroplast and nuclear regions of DNA analyzed, the geographical distribution of the haplotypes suggested the existence of two groups, one in the eastern part of YPBP and the other in the western part (Figures 2-B and 3-B). For *atpF-atpH*, haplotype A1 was the most represented in the western region of the YPBP. On the other hand, haplotype A3 was more abundant in the populations of the eastern section of the YPBP. For ITS2, the haplotype H1 was the most represented in the populations of western YPBP, and haplotypes H3, H8 were much more frequent in eastern YPBP. BARRIER analysis showed, for both regions (*atpF-atpH* and ITS2), that the most probable genetic discontinuities among populations correspond to the east and west regions of the PBYP: the group in the east includes Puerto Morelos, Carrillo Puerto, Bacalar, José María Morelos, Cobá, Panto-Ha, X-Hazil, Pedro A. Santos, Nachi Cocom, Calakmul, Hormiguero and Virgencita; the group in the west includes Valladolid, Peto,



**Figure 2.** (A) Haplotype network obtained with chloroplast sequence *atpF-atpH*. Circle size is proportional to the frequency of each haplotype; lines between the haplotypes represent the mutational steps. (B) Geographic distribution of haplotypes in the 23 populations of *Zamia prasina* sampled in the YPBP. Pie charts represent the haplotypes found for each population; section size is proportional to the number of individuals with that haplotype. Gray dotted lines represent the location of the most probable barriers obtained with BARRIER. Numbers in the pie are population codes (see Figure 1 for locations).



**Figure 3.** (A) Haplotype network obtained with nuclear sequence ITS2. Circle size is proportional to the frequency of each haplotype; lines between the haplotypes represent the mutational steps. (B) Geographic distribution of haplotypes in 22 populations of *Zamia prasina* in YPBP. Pie charts represent the haplotypes found for each population; section size is proportional to the number of individuals with that haplotype. Gray dotted lines represent the location of the most probable barriers obtained with BARRIER. Numbers in the pie are populations codes (see Figure 1 for locations).

**Table 2.** Non-hierarchical Molecular variance analysis (AMOVA) of 23 populations of *Zamia prasina* using the chloroplast region *atpF-atpH* and the nuclear region ITS2.

Source of variation	<i>atpF-atpH</i>				ITS2			
	Sum of squares	Variance components	Percent variation	Fixation index	Sum of squares	Variance components	Percent variation	Fixation index
Among populations	23.989	0.17088	33.85	$F_{ST} = 0.338^*$	64.778	0.53492	37.23	$F_{ST} = 0.372^{**}$
Within populations	26.383	0.33397	66.15		61.333	0.90196	62.77	
Total	50.373	0.50484			126.111	1.43688		

Becancién, Rancho Duarte, Kaxil Kiic, Escarcega, Chamotón, Palenque and Tenosique (Figures 2-B and 3-B). This analysis supported the geographic distribution of haplotypes mentioned before.

Considering the evidence obtained regarding the population groups in the eastern and western YPBP, we then determined their genetic diversity (Table 3). For *atpF-atpH*, the mean haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were low for both groups:  $h = 0.373$  and  $\pi = 0.00080$  for the group in the east;  $h = 0.406$  and  $\pi = 0.00210$  for the group in the west. For ITS2 in both groups, diversity values were moderate than for *atpF-atpH* ( $h = 0.566$  and  $\pi = 0.00701$  for the group in the east;  $h = 0.600$  and  $\pi = 0.00281$  for the group in the west).

The principal component analysis (PCA) shows two groups of populations, where the populations in the east of the YPBP are located and another group where most of the western populations of the YPBP are found (Appendix 5). Most of the variation was obtained in two components: PCA1 contributed 55.03 % of the variation and PCA2 contributed 21.22 % explaining in total 76.25 % of the variation. For PCA1, six variables were associated, with two temperature variables (BIO2 and BIO7) contributing the most; however, four of the variables in this component corresponded to variables related to precipitation (BIO15, BIO17, BIO18 and BIO19). Two variables were associated with PCA2, one related to temperature (BIO3) and another to precipitation (BIO12), which contributed the most to the component.

*Divergence time.* The comparison of all the coalescence analyses revealed high convergence between the inferred parameters, and the effective sample sizes were always greater than 200. The trees obtained for *atpF-atpH* and ITS2 (Figures 4

and 5, respectively) were congruent in the principal nodes and with the phylogeny of *Zamia* (Calonje *et al.* 2019): *Microcycas* A. DC. as sister group of *Zamia*; the clade formed by *Z. furfuracea*, *Z. paucijuga* Wieland, *Z. loddigesii* and *Z. spartea* as a sister group of the clade formed by *Z. variegata* and *Z. prasina*; and *Z. variegata* as sister species of *Z. prasina*. The genealogy for *atpF-atpH* showed that the haplotypes of *Z. prasina* grouped into two clades: (1) haplotypes A1, A2, A7 and A9 and (2) A3, A4, A5, A6 and A8. The greatest divergence age between these two haplotypes groups was 1.37 (95 % HPD, 2.3-0.46 Ma), and the ancestral haplotype is A7 (Figure 4, Appendix 6), which was found exclusively in the Hormiguero population (Campeche) (Table 1).

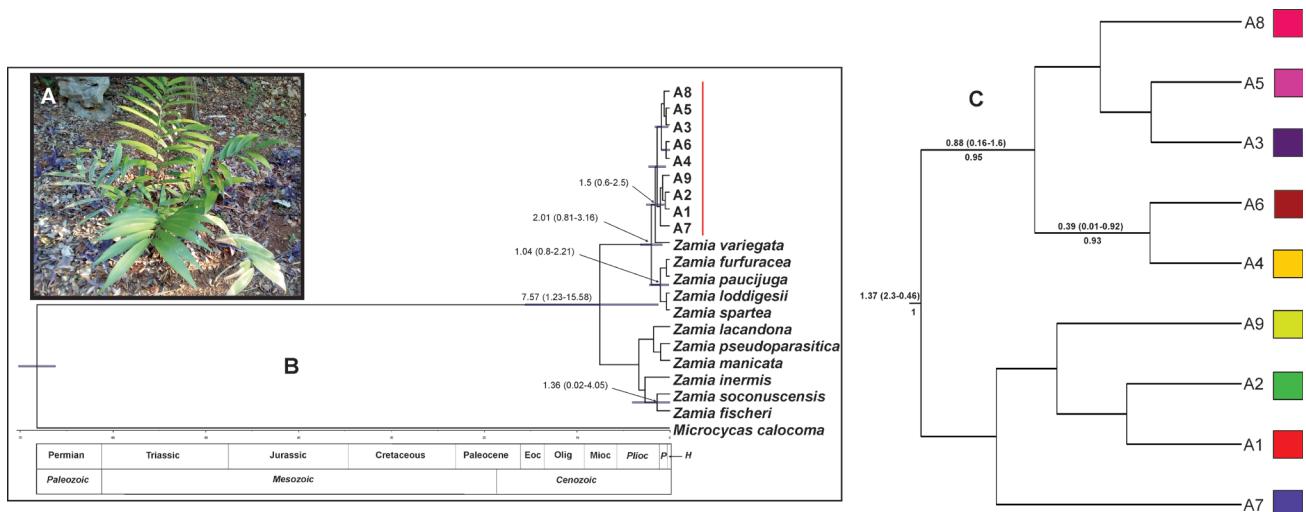
The topology obtained with ITS2 had several clades with short branches, indicating low differentiation between the haplotypes and that, based on molecular clock theory, the times of divergence must be recent. The haplotypes of *Z. prasina* grouped into six clades: (1) haplotypes H13, H14 and H16; (2) H17, 2, 6, 4, 1 and H10; (3) H3, H5, H7; (4) H18; (5) H8 and H9; and (6) H15, H11, H12. The greatest divergence age among group of haplotypes was 1.36 (95 % HPD, 2.23-0.48 Ma). The ancestral haplotype was H12 with a divergence time of 69,000 years (Figure 5, Appendix 7). This haplotype was present in individuals from Panto-Ha, Nachi Cocom, Carrillo Puerto and was the only haplotype present in the population Belize-Cayo (Table 1).

*Historical demography.* Tajima's  $D$  and Fu's  $F_s$  were negative values at the species and population levels; however, these values were not significant ( $p > 0.05$ ). This result was similar for *atpF-atpH* and for ITS2 (Table 4). The mismatch distribution analysis showed a distinctive unimodal pattern, indicating recent demography expansion ( $R_2 = 0.0477$  and  $R_2 = 0.0455$  for *atpF-atpH* and ITS2 respectively). The skyline plot for the species level indicated that the effective size of the population decreased, and much more so for ITS2, then increased recently, in the last 10,000 years approximately, which can be interpreted as population growth, which we expect would be followed by an expansion (Figures 6-A and 6-D). At the population group level, the skyline plot showed one pattern of demography expansion only for the population groups in the east for ITS2 (Figure 6-F).

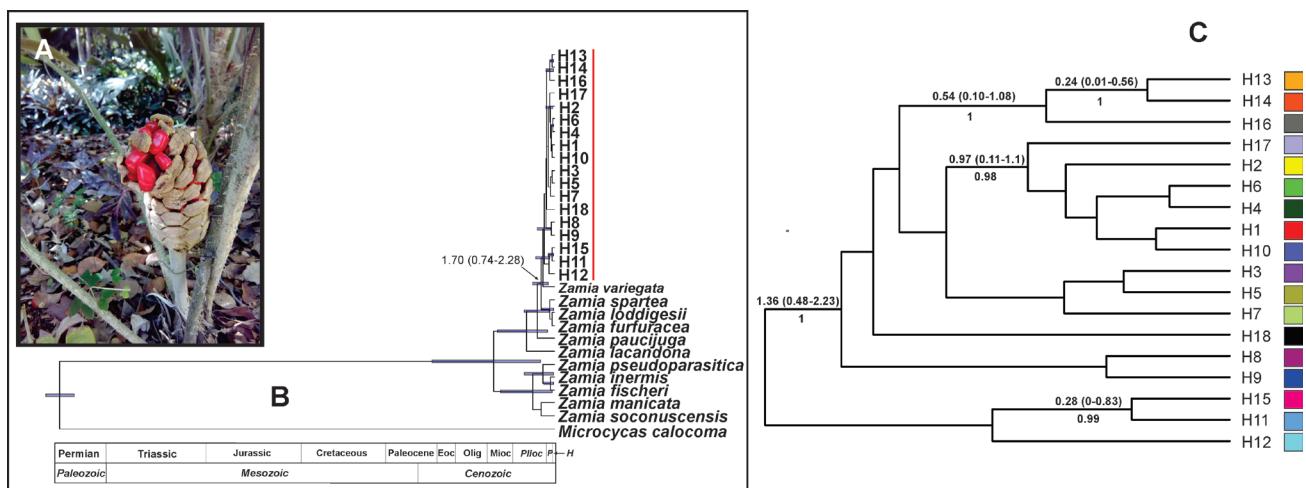
*Potential distribution.* The ENM performed well (AUC =  $0.907 \pm 0.018$ ) and the predicted distribution (Figure 7)

**Table 3.** Average genetic diversity in the eastern and western groups using the chloroplast region *atpF-atpH* and nuclear region ITS2.

Statistic	<i>atpF-atpH</i>	
	Eastern group	Western group
$h$	$0.373 \pm 0.0075$	$0.406 \pm 0.1290$ ( $p = 0.002$ )
$\pi$	$0.0008 \pm 0.0001$	$0.0021 \pm 0.0010$ ( $p < 0.0001$ )
ITS2		
$h$	$0.566 \pm 0.100$	$0.600 \pm 0.106$ ( $p = 0.822$ )
$\pi$	$0.007 \pm 0.0039$	$0.002 \pm 0.0005$ ( $p < 0.0001$ )



**Figure 4.** (A) Plant of *Zamia prasina* in its natural habit. (B) Bayesian chronogram for chloroplast sequence *atpF-atpH*. The divergence time of *Zamia prasina* haplotypes in the YPBP and other cycad species is shown. The 95 % confidence intervals are shown with purple bars; the numbers at the nodes indicate the estimated age, the numbers below the branches indicate the posterior probability (Appendix 6). (C) Extract from the complete chronogram corresponding to the *Zamia prasina* haplotypes (marked with the red line), the colors of the squares correspond to the haplotypes shown in Figure 2-A.



**Figure 5.** (A) Bayesian chronogram for nuclear sequence ITS2. The divergence time of *Zamia prasina* haplotypes in the YPBP and other cycad species is shown. The 95 % confidence intervals are shown with purple bars; the numbers at the nodes indicate the estimated age, the numbers below the branches indicate the posterior probability (Appendix 7). (B) Female cone of *Zamia prasina*. (C) Extract from the complete chronogram corresponding to the *Zamia prasina* haplotypes (marked with the red line), the colors of the squares correspond to the haplotypes shown in Figure 3-A.

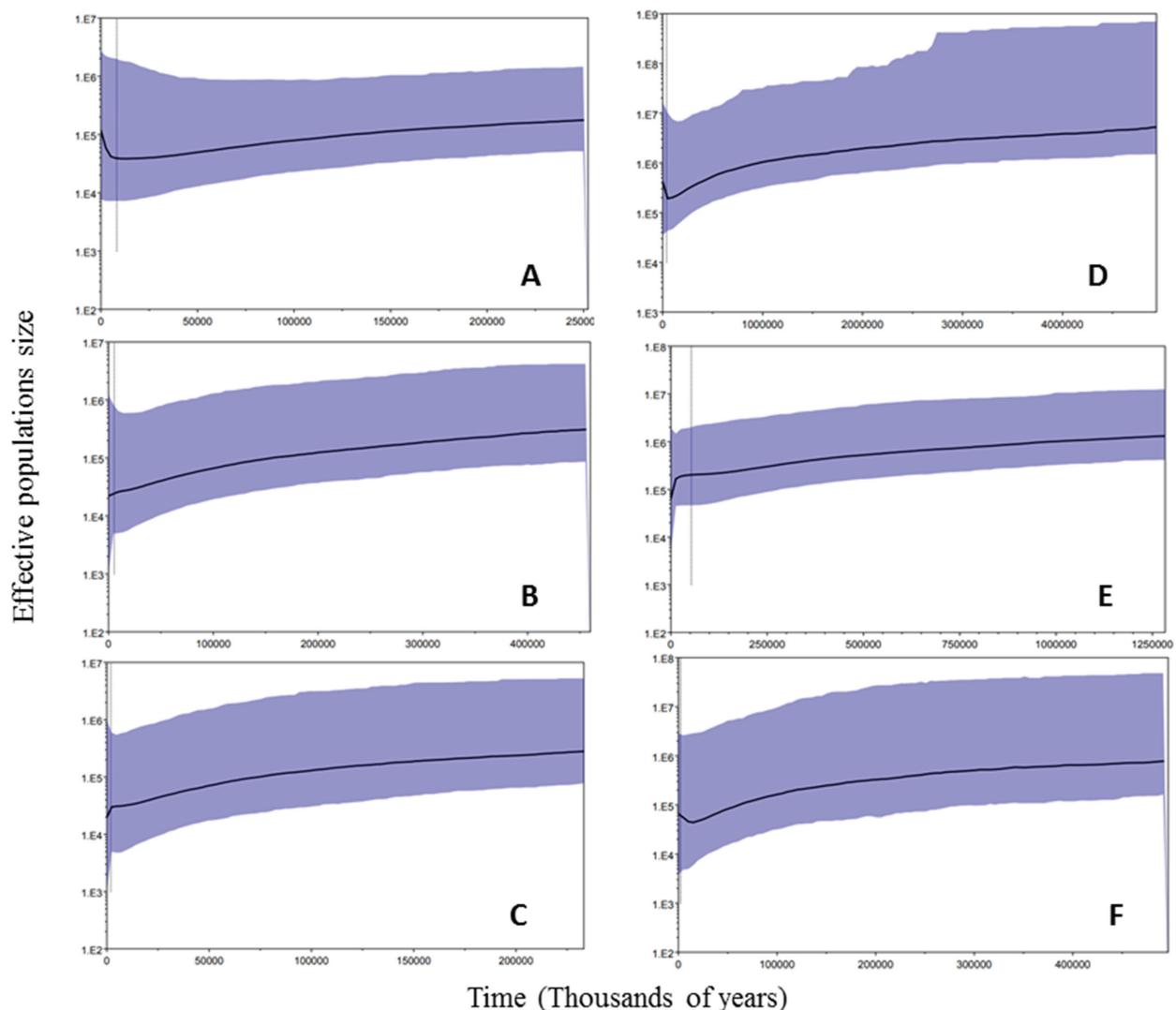
matched the actual known range of *Z. prasina* (Vovides & Nicolalde-Morejón 2010) associated with tropical evergreen and tropical deciduous forests in the YPBP and north of Nicaragua where other *Zamia* species are distributed. The ENM transferred into a past climatic scenarios shows drastic range shift since the LIG from present. During the last interglacial period (LIG  $\approx$  140-120 ka), only the northern area of Guatemala and Chiapas were suitable for *Z. prasina*. The suitable

area during the Last Glacial Maximum (LGM  $\approx$  21-18 ka) for the two GCM were highly contracted. According to the CCSM model, during the LGM the species was possibly distributed on the eastern coast of the YPBP and in some areas of Central America. The MIROC model, on the other hand, indicated that conditions were suitable in a small area in the eastern part of the YPBP and western part of the Yucatan Peninsula. During the Middle Holocene (MH) for both mod-

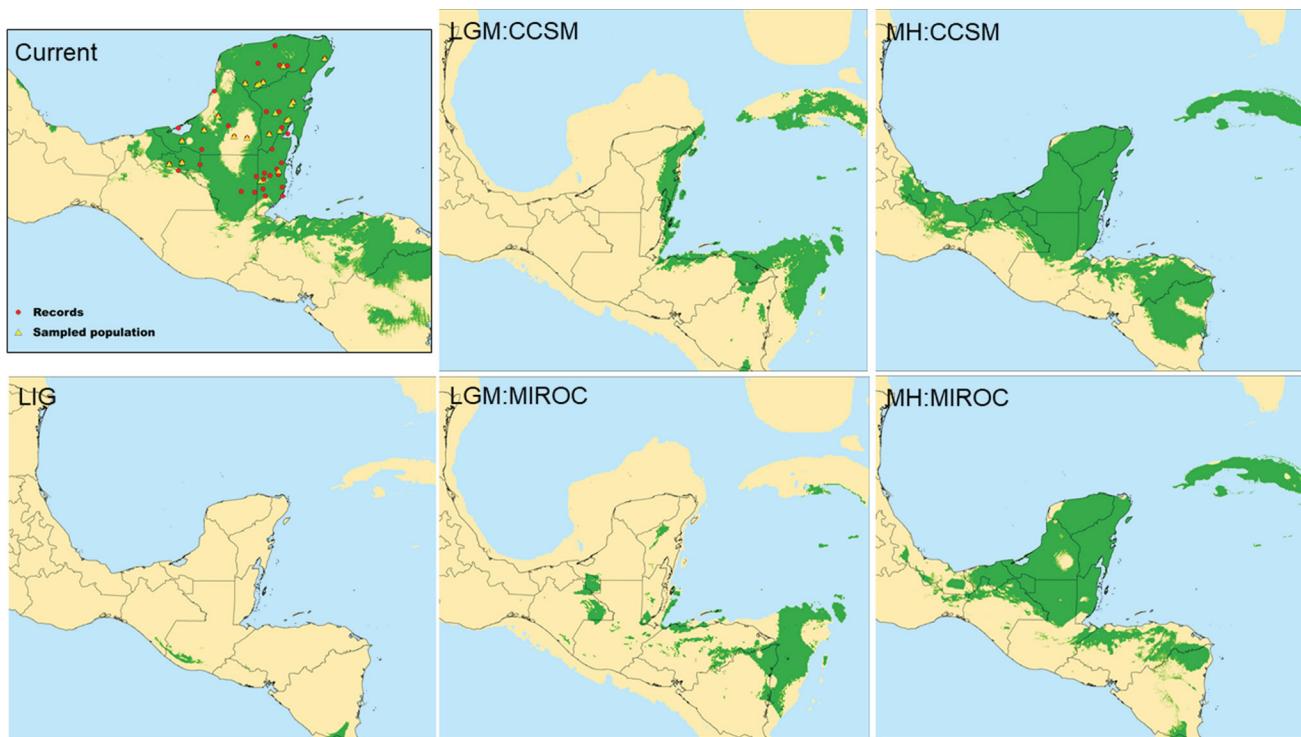
**Table 4.** Average values of the neutrality indexes Tajima's  $D$  and Fu's  $F_S$  for the population groups in the eastern and western parts of the peninsula and for all populations.

Statistic	atpF-atpH		
	Eastern	Western	Total
Tajima's $D$	0.01856 ( $p = 0.68700$ )	-0.17476 ( $p = 0.83408$ )	-0.05336 ( $p = 0.80242$ )
Fu's $F_S$	0.31829 (NA)	0.09316 (NA)	0.24214 (NA)
		ITS2	
Tajima's $D$	0.0397 ( $p = 0.71136$ )	-0.006129 ( $p = 0.68373$ )	-0.03263 ( $p = 0.69850$ )
Fu's $F_S$	-0.05500 (NA)	0.05405 (NA)	0.11408 (NA)

Positive values for  $D$  and  $F_S$  are indicative of mutation-drift equilibrium, typical of stable populations; negative and significant values ( $p < 0.05$ ) resulting from an excess of rare haplotypes indicate that populations have experienced recent expansions, often preceded by a bottleneck.



**Figure 6.** Demography history of *Zamia prasina* in YPBP based on Bayesian skyline plot. Substitution rates reported by Nolasco-Soto *et al.* (2015) were used. The vertical axis corresponds to the effective size of the population (Ne), the black line indicates the trend for the median Ne over time; purple lines are the 95% confidence intervals. The horizontal axis represents time in thousands of years. Graphs on left atpF-atpH (A: All populations, B: populations of the east group of YPBP, C: populations of the west group). Graphs on right ITS2 (D: All populations, E: populations of the east group of the YPBP, F: populations of the west group).



**Figure 7.** Ecological niche modelling for *Zamia prasina*. Current potential distribution. (LIG) Predicted distribution during the interglacial period ( $\approx 140$ - $120$  ka). (LGM-CCSM) Predicted distribution during the Last Glacial Maximum, CCSM model ( $\approx 21$ - $18$  ka). (LGM-MIROC) Predicted distribution during the Last Glacial Maximum, MIROC model. (HM-CCSM) Predicted distribution during the Middle Holocene, CCSM model ( $\approx 6$  ka). (HM-MIROC) Predicted distribution during the Middle Holocene, MIROC model. Green: high suitability, light brown: low suitability.

els, the suitable area for *Z. prasina* increased, and expanded more to the northwest, so that it inhabited a large part of the YPBP and the southern coast of the Gulf of Mexico.

## Discussion

**Phylogeography.** The moderate and high haplotype diversity for *atpF-atpH* and *ITS2*, respectively, and their low nucleotide diversity indicate the presence of many haplotypes at high frequency but with few nucleotide differences among them; thus, the diversification of these haplotypes is recent. Similar results have been reported by Nolasco-Soto *et al.* (2015) for *Z. paucijuga*, with  $h = 0.669$  and  $\pi = 0.0013$  for the chloroplast region *psbK-psbI* and  $h = 0.843$  and  $\pi = 0.0063$  for *ITS2*. For *Dioon sonorense*, Gutiérrez-Ortega *et al.* (2014) reported  $h = 0.629$  and  $\pi = 0.0004$  for the chloroplast region *trnL-trnF*. For the cycads in Mexico, there are reports of high genetic diversity in some species, with few populations and restricted habitats, as in the case of *Zamia loddigesii* Miq. (González-Astorga *et al.* 2006), *Dioon caputoi* De Luca, Sabato & Vázq. Torres, *D. merolae* De Luca, Sabato & Vázq. Torres (Cabrera-Toledo *et al.* 2010) and *D. sonorense* (De Luca, Sabato & Vázq. Torres) Chemnick, T. J. Greg. & Salas-Mor (González-Astorga *et al.* 2008).

Disagreements in the estimated genetic diversity values for *atpF-atpH* and *ITS2* in *Zamia prasina* may be due to

the differences between these two DNA types. cpDNA is inherited maternally in cycads (Cafasso *et al.* 2001) so there is no genetic recombination, whereas it is reasonable to assume that nDNA is inherited bipaternally. In plants, cpDNA markers display average evolutionary rates in the range of  $10^{-9}$  substitutions/site/year, relatively slower than nDNA rates (Wolf *et al.* 1987); our results are consistent with this difference.

The moderately-high genetic diversity found in *Z. prasina* may be due to three factors. (1) Although its geographical distribution is restricted and its habitat perturbed by anthropogenic activities, this species is abundant, and there are still large extension of vegetation where the species is common (Vovides & Nicolalde-Morejón 2010). The Global Biodiversity Information Facility (GBIF, <http://data.gbif.org/species/browse/taxon/>) reports more than 100 records of *Z. prasina*. The majority of them are in Mexico and Belize, but there are still areas that have been little explored to the south of Petén in Guatemala (Districts of Izabal and Alta Verapaz), where the species can be found (M. Veliz, curator of the BIGU herbarium pers. comm.). (2) The species is dioecious with a cross-mating system that is mediated by pollinators, which implies that genetic material is exchanged between individuals, thus generating greater genetic diversity (Vovides & Nicolalde-Morejón 2010). (3) Most likely, a recent population expansion increased the haplotype diversity with

low values of nucleotide diversity; this pattern was reported for other zamias (*i.e.*, *Z. paucijuga*) (Nolasco-Soto *et al.* 2015). As a population expands, it may come in contact with populations that had been isolated during the glaciation and subjected to different selection pressures. This contact between divergent populations can increase the diversity due to genetic flow between them.

The number of samples analysed also can influences the amount of genetic diversity detected. In cycads, a range of sample sizes have been used for phylogeographic studies, for example, one individual was used per population for *Dioon sonorense* (Zamiaceae), and moderate values of nucleotide diversity ( $h = 0.629$ ) were obtained using the chloroplast region *trnL-trnF* (Gutiérrez-Ortega *et al.* 2014). For *Zamia paucijuga* (Zamiaceae) with 8-10 individuals per population, high haploid diversity was found using ITS2 ( $h = 0.843$ ) and moderate diversity for the chloroplast region *psbK-psbI* ( $h = 0.669$ ) (Nolasco-Soto *et al.* 2015). Here we used a sample size between these two examples among these reported (five individuals per population) and still managed to detect a moderate to-high genetic diversity (*atpF-atpH*,  $h = 0.564$ ; ITS2,  $h = 0.827$ ) in agreement with the previous studies. Since we are working with non-coding regions that have low mutation rates, small sample sizes might not detected, the actual diversity.

According to our chronogram (Figures 4 and 5), the diversification within *Z. prasina* happened during the Pleistocene. During this period, neotropical montane forests experienced extremely complex glacial-interglacial dynamics and the effect of climatic fluctuations on the genetic structure and population history of species distributed in these habitats led to different outcomes, such as rapid radiation or local extinction (Ramírez-Barahona & Eguiarte 2013). (4) The moderate genetic diversity detected for *Z. prasina* might also be the result of a still unfinished process of habitat fragmentation, in which subsequent isolation has not yet affected the distribution of its molecular variants.

The greatest variation is within the populations and not between them may be because not enough time has elapsed for a significant divergence between them. The comparison of genetic diversity between the eastern and western population groups on peninsula indicates that there are no differences between these two. We found populations that stand out due to their high genetic diversity, for *atpF-atpH* in the Virgen-cita and Hormiguero populations and for ITS2 in the Jose Maria Morelos, Carrillo Puerto and Bekanche populations.

The east-west phylogeographic structure in the populations of *Z. prasina* (suggested by the geographic distribution of the haplotypes and BARRIER analysis) can be explained by climatic factors. The PCA supports the east - west divergence and indicates that the variables related to precipitation contributed greatly to PCA1 and PCA2; thus, the genetic differentiation between the group of populations from the eastern and western parts of the peninsula may be due to the precipitation gradient in the YPBP. There is a humid region in the southeast and a dry region in the north-northeast (Carnevali *et al.* 2010). The eastern part of the YPBP is humid with 1,200-1,500 mm of annual rainfall, and the basin of

Laguna de Terminos in southeastern YPBP receives up to 1,400 mm, compared with 500-1,000 mm in the northwest part of the YPBP (Orellana & Espada 2009). This gradient of precipitation determines the type of vegetation; in the northeast, low-elevation deciduous forest predominates, in the middle is low-elevation deciduous forest, in the east is predominantly medium-elevation semideciduous forest, and in the southeast, high-elevation evergreen forest predominates (Carnevali *et al.* 2012). Thus, the geographic distribution of *Zamia prasina* in the YPBP could be determined by precipitation. Although no published studies have focused on the influence of precipitation, the drier northeastern strip constitutes a barrier to the distribution of this species, as indicated by the absence of historical records in this area (GBIF, <http://data.gbif.org/species/browse/taxon/>). The populations group in the west is found in the medium subdeciduous forest, bordering the dry northeast portion of the YPBP, while, the eastern population group is in a medium evergreen forest in a more humid area and thus more suitable for *Z. prasina*. Differences in geography, forest and climate may thus also contribute to the differentiation (Vovides & Nicolalde-Morejón 2010). To test this hypothesis, a more comprehensive study with a landscape genomics approach should be carried out.

Regarding the influence of the precipitation gradient of the YPBP on *Zamia prasina*, Vovides & Olivares (1996) analyzed the caryotypical variation in 11 individuals of *Z. prasina* in the Mexican part of the YPBP. They found that the plants collected in the northeastern part of this region, where habitats are drier, have higher chromosome numbers ( $2n = 24-27$ ) than in plants collected in the southeastern region ( $2n = 17$ ), where the habitats are more humid. Vovides & Olivares (1996) concluded that the stressful drought conditions present in the northeast generate chromosomal changes. The chromosomal changes in *Zamia* are attributed to chromosomal fusion or fission. The fusion occurs between two acrocentric chromosomes (chromosomes whose centromere is closer to one of the ends), giving rise to a metacentric chromosome (with the centromere in the center of the chromosome) and thus reducing the number of chromosomes. During fission, a metacentric chromosome arises by fission or rupture of a metacentric chromosome into two acrocentric chromosomes, in this case increasing the number of chromosomes. Chromosomal fissions might confer adaptive advantages to survive in dry environments. Similar results were reported for other *Zamia* species of the Caribbean, which live in more stable conditions than do the continental *Zamia* species, which are exposed to a wide range of ecological conditions, from humid to dry and semi-xeric (Balduzzi *et al.* 1982).

In a study of the influence of environmental conditions on phenotypic diversity of *Z. prasina*, Limón *et al.* (2016) found that in populations with low precipitation and high temperature, the plants have fewer but wider leafs; in contrast, in areas with greater precipitation, the plants have more but thinner leafs. These authors proposed that *Z. prasina* inhabits areas under the dense canopy of tropical rainforests and semi-deciduous forests; therefore, the leaflets may be an adaptation to facilitate evapotranspiration and light capture in

the shade. According to Stevenson *et al.* (1996), individuals of *Z. prasina* in intermediate environments have intermediate morphological forms. Thus, evidence of phylogeographic structure, in karyotype and morphology indicate that the precipitation gradient in the YPBP greatly influences the populations of *Z. prasina*. A study on the influence of precipitation on the genetic diversity of this species is necessary.

The present study shows that the estimated time of divergence among the most ancestral haplotypes of *Zamia prasina* was approximately 1.37 Ma based on *atpF-atpH* and 1.36 Ma based on ITS2; although most of the haplotypes have a more recent origin, indicating a recent population expansion that coincides with the Pleistocene (1.8-0.10 Ma), a period of events relevant to the evolution of the cycads. During the climatic changes of the Pleistocene, the tropical forests were subjected to a complex dynamic, an aspect that favored the presentation of rapid radiations and local extinctions (Ramírez-Barahona & Eguiarte 2013), and in the case of *Z. prasina*, there was an increase in genetic diversification.

Based on molecular phylogeny, Nagalingum *et al.* (2011) pointed out that, diversification increased in the genus *Zamia* during the Pleistocene and that it was one of the most speciose rich genera of cycads at that time. Pleistocene speciation prevailed in the neotropics, mainly for *Zamia* and *Ceratozamia* with the glacial-interglacial cycles being the predominant environmental force. The climate during that period also became more seasonal. Nolasco-Soto *et al.* (2015), employing nuclear and chloroplast markers determined the origin of *Z. paucijuga* between 6.4-1.6 Ma. Nolasco-Soto *et al.* (2015) suggested that the historical factors that affected *Z. paucijuga* at the population level during the Late Pleistocene similar affected other *Zamia* species, as our results showed for *Z. prasina*.

The values of the Tajima's *D* and the Fu's *F<sub>s</sub>* were negative but not significant, indicating that there was no population expansion. Because *Z. prasina* is a perennial with a long generational cycle the mutation rate is very low (Loveless & Hamrick 1984), thus, the significance of these two statistics is difficult to assess. However, the present work generated several lines of evidence that suggest that populations of *Z. prasina* underwent a recent demography expansion. (1) In the populations sampled, haplotype diversity is high, and nucleotide diversity is low. The low genetic divergence between the haplotypes can be explained because not enough time has elapsed for a significant divergence between the haplotypes (Lavery *et al.* 1996). (2) The star-shaped network of haplotypes found for *Z. prasina* in *atpF-atpH* and ITS2 regions also indicate a population expansion and one or a few haplotypes were more frequent, from which many differing haplotypes were derived in one or a few mutational steps; moreover, these haplotypes are found in low frequency. (3) The skyline plot showed a recent population expansion at species level, the effective size of the populations underwent a recent increase in the late Pleistocene-Holocene. (4) The diversification of haplotypes increased in the Late Pleistocene-Holocene.

**Current and palaeodistribution modelling.** As mentioned earlier, the palaeoecological studies of fossil pollen in the

YPBP that reconstructed past of vegetation distribution and climate (Carrillo-Bastos *et al.* 2010, Sánchez-Sánchez & Islebe 2002) showed that the distribution in the YPBP was governed mainly by precipitation levels. A clear example is given by the certain ones semi-evergreen forest species, which grow in areas with a precipitation between 1,000 and 1,500 mm/year (Sánchez-Sánchez & Islebe 2002). The mosaic of current vegetation formed during the early Holocene, which implies that the modern pattern of isohyets was also established then (Leyden 2002). In the present, the climate is more seasonal, with rainier summers and less-intense winters, which encourages the regeneration and survival of seedlings. *Z. prasina* is actually more widely distributed in the YPBP, in ecosystems with seasonal climates (evergreen, deciduous and subdeciduous tropical forest).

In a geospatial analysis study of pollen records from the YPBP, Carrillo-Bastos *et al.* (2012) found that changes in vegetation during the Holocene in the peninsula were caused by changes in climate. In addition to climate influences during the Classic period (2,500-1,200 BC), the activities carried out by the Mayan people placed pressure on the forest coverage. In spite of this pressure, the vegetation coverage was not severely diminished; thus, the use of forest resources did not result in total deforestation. Eventually, vegetation recovered in relation to an increase of precipitation that coincided with the Medieval warm period during the Holocene. The highest pollen percentages taxa of semi-evergreen forests (85-92 %) were predicted to be in the southern and central parts of the peninsula and the lowest in the northern part. This distribution is similar to the modern distribution according to the isohyets, with precipitation increasing toward the eastern coast (Carrillo-Bastos *et al.* (2012) and consistent with our results that, during the Middle Holocene (MH), the environmentally suitable area for *Z. prasina* increased and expanded from the southeast to the center, covering a large part of the YPBP.

Although the LGM was a very dry period, some places have been proposed to be relatively wet, *e.g.*, center-west of Mexico (Bradbury 2000) and south of the YPBP (Hodell *et al.* 2008, Bush *et al.* 2009). Drill cores obtained from Lake Petén Itzá, Petén, Guatemala, contain a ~85-kyr record of terrestrial climate from lowland Central America that was used to reconstruct hydrologic changes in the northern Neotropics during the last glaciation.

Sediments are composed of clay, reflecting a relatively wet climate, and pollen from the same period indicates vegetation consisted of a temperate pine-oak forest. This finding contradicts previous inferences that the climate was arid during the Last Glacial Maximum (LGM) at least in the south of the YPBP (Hodell *et al.* 2008). This result coincides with ours that, during the LGM, the environment was suitable for the establishment of *Z. prasina* was concentrated in the eastern YPBP where the species may have found refuge during the cold, dry periods of the glaciation.

We propose that the absence of environmental suitability in the YPBP for the establishment of *Z. prasina* in the Last Interglacial ( $\approx$  120 ka) could be due to some *Zamia* species needing a seasonal climate, while others have a tree habit and are adapted to very humid, woodland habitats (Nicolalde-

Morejón *et al.* 2011). It is possible that the last interglacial was warmer, humid and without seasonality, in comparison with the Holocene (Walker *et al.* 2012, Cornejo-Romero *et al.* 2017), and thus was not propitious for the growth of *Z. prasina*.

The predictions by the MIROC and the CCSM models for the paleodistribution of *Z. prasina* in the LGM are different. The CCSM model predicts environmental suitability areas on the eastern coast of the YPBP, by the MIROC model does not predict any favorable areas on the peninsula (Figure 7). This difference may be due to these models basing their simulations on different initial experimental conditions using in different algorithms (Harrison *et al.* 2016). CCSM model estimates a lower temperature and greater precipitation than the MIROC model does (Otto-Bliesner *et al.* 2007) although the differences vary depending on the area of study (Taylor *et al.* 2012, Harrison *et al.* 2016).

The genetic data (phylogeographic structure and historical demography) suggest that the populations of *Z. prasina* expanded during the warmest periods of the Mid-Holocene, a pattern consistent with the glacial refugium hypothesis and validated by its paleodistribution. Post-Pleistocene expansion has been proposed for other species of the genus *Zamia*, such as *Z. paucijuga* (Nolasco-Soto *et al.* 2015); after the glaciations, its populations that originated in central Mexico became established to the south in the Pacific watershed.

In our study, the presence of ancestral haplotypes on the southeastern peninsula (based on ITS2 evidence) indicates possible center of origin, although based on *atpF-atpH* the center could be in Campeche. This difference may be due to the differing type of inheritance between the two markers. However, these results are only preliminary because they are based on information from two short genome regions. Future studies should address this incongruity by using sampling more populations and genomic regions.

In general, our results indicate that climatic fluctuations during the Pleistocene-Holocene influenced the evolutionary history of *Z. prasina*. The ecological niche modelling suggests that this species prevail during glacial events in restricted populations on the eastern coast of the peninsula, and expanded post-glacially into the northwest to occupy almost the entire territory of the YPBP. This result are congruent with the phylogeographic pattern of other *Zamia* species (Nolasco-Soto *et al.* 2015) and helps us understand the historical phylogeographic patterns of the Zamiaceae in the Neotropics. An analysis of the comparative phylogeography at the genus level for *Zamia* with greater genomic sampling will allow us to make inferences about the temporal congruence of species and about the influence of climate on the composition of the genus.

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**Author Contributions:** GMF (<https://orcid.org/0000-0002-0627-3347>). Collected most of the botanical material, conceived, designed, and performed the experiments, analyzed the data, and drafted the first version of the paper as well as reviewed drafts of the manuscript. LFST Drafted the first version of the paper. Funded the sequences analyses. GCFC Conceived and designed parts of the experiments and analyses. Drafted the first version of the paper as well as reviewed all drafts of the manuscript. APV Drafted the first version of the paper as well as reviewed all drafts of the manuscript. RGL Carried out the analysis of potential distribution with the tool of Ecological Niche Modelling. MMOG Participated in laboratory work, and reviewed drafts of the first version. JAML Participated in the botanical collections, and reviewed drafts of the first version. JMC (<https://orcid.org/0000-0002-6484-6406>). Conceived and designed parts of the experiments and reviewed all drafts of the manuscript. Funded the sequences analysis and the collecting trips.

## Appendix

### Appendix 1. Climatic variables per population

Poblaciones	Localidades	bio02	bio03	bio04	bio05	bio07	bio11	bio12	bio15	bio17	bio18	bio19
1	Valladolid	130	68	1899	347	190	227	1220	65	103	452	103
2	Peto	135	68	1914	356	196	231	1109	62	95	296	111
3	Becanchén	134	67	1988	356	198	228	1071	62	92	283	103
4	Rancho Duarte	133	67	2011	353	197	226	1070	61	91	286	101
5	Kaxil Kiuc	135	66	2196	360	204	227	1029	65	81	277	89
6	Champotón	132	65	2222	357	203	226	1223	75	66	280	99
7	Calakmul	117	61	2198	344	189	221	1171	63	89	294	127
8	Escárcega	127	65	2111	357	194	231	1410	73	81	320	114
9	Hormiguero	112	61	2179	334	182	215	1171	57	98	322	142
10	Viergencita	107	62	1986	350	170	237	1624	68	104	352	169
11	Tenosique	110	63	2009	353	174	236	2169	54	206	473	328
12	Palenque	109	60	1981	357	181	238	2443	54	246	649	468
13	Puerto Morelos	100	67	1734	327	149	231	1152	50	129	354	185
14	Cobá	117	69	1755	336	169	229	1151	53	132	404	139
15	Carrillo Puerto	114	69	1787	336	165	232	1290	53	137	481	167
16	Xhazil	111	68	1733	334	161	233	1255	52	135	434	167
17	José María Morelos	110	66	1786	337	166	232	1087	51	118	351	146
18	Pedro A Santos	93	68	1552	321	136	235	1467	52	144	457	226
19	Bacalar	92	68	1528	320	135	236	1445	53	138	530	215
20	Panto-Ha	95	66	1593	324	142	234	1243	54	112	458	167
21	Nachicocom	104	65	1763	329	160	227	1338	59	113	512	166
22	Belize-Belize	85	59	1842	306	142	214	1510	47	155	318	241
23	Belize-Cayo	80	63	1553	310	126	230	2207	51	197	773	364

**Appendix 2.** Genetic diversity of the populations of *Zamia prasina* in the Yucatan Peninsula Biotic Province (YPBP). Biotic Province (YPBP) using the chloroplast DNA region *atpF-atpH*. Number of individuals (N), number of haplotypes (k), haplotype diversity (h) and nucleotide diversity ( $\pi$ ).

Population/state	N	k	h	$\pi$
Valladolid/Yucatán	5	1	0.000	0.00000
Peto/Yucatán	4	2	0.500	0.00110
Becanchén/Yucatán	5	2	0.400	0.00088
Rancho duarte/Yucatán	4	2	0.500	0.00110
Kaxil Kiuc/Yucatán	3	1	0.000	0.00000
Champotón/Campeche	5	1	0.000	0.00000
Escárcega/Campeche	5	1	0.000	0.00000
Virgencita/Campeche	5	4	0.900	0.00574
Calakmul/Campeche	5	1	0.000	0.00000
Hormiguero/Campeche	3	3	1.000	0.01177
Palenque/Chiapa	5	1	0.000	0.00000
Tenosique/Tabasco	5	1	0.000	0.00000

**Appendix 3.** Genetic diversity of populations of *Zamia prasina* in the Yucatan Peninsula Biotic Province (YPBP). Biotic Province (YPBP) using the nuclear DNA region ITS2. Number of individuals (N), number of haplotypes (k), haplotype diversity (h) and nucleotide diversity ( $\pi$ ).

Population/state	N	k	h	$\pi$
Valladolid/Yucatán	5	2	0.400	0.00135
Peto/Yucatán	5	1	0.000	0.00000
Becanchén/Yucatán	4	4	1.000	0.00617
Rancho duarte/Yucatán	5	2	0.600	0.00202
Kaxil Kiuc/Yucatán	3	2	0.666	0.00224
Champotón/Campeche	5	2	0.400	0.00135
Escárcega/Campeche	5	3	0.700	0.00337
Virgencita/Campeche	3	1	0.000	0.00000
Calakmul/Campeche	4	2	0.500	0.00168
Hormiguero/Campeche	3	1	0.000	0.00000
Palenque/Chiapa	5	4	0.900	0.00404
Tenosique/Tabasco	4	3	0.833	0.00505

**Appendix 2.** Continues.

Population/state	N	k	h	π
Xhazil/Quintana Roo	5	2	0.600	0.00132
Pedro A. Santos/Quintana Roo	5	2	0.400	0.00088
Panto Ha/Quintana Roo	5	2	0.600	0.00132
Nachi cocom/Quintana Roo	4	2	0.500	0.00110
Puerto Morelos/Quintana Roo	5	1	0.000	0.00000
Cobá/Quintana Roo	5	2	0.600	0.00132
Carrillo Puerto/Quintana Roo	4	2	0.500	0.00110
Bacalar/Quintana Roo	5	1	0.000	0.00000
José María Morelos/Quintana Roo	5	2	0.400	0.00088
Belize/Belize	2	2	1.000	0.00221
Belize/Cayo	3	2	0.666	0.00147
Total	102	9	0.564	0.00186

**Appendix 3.** Continues.

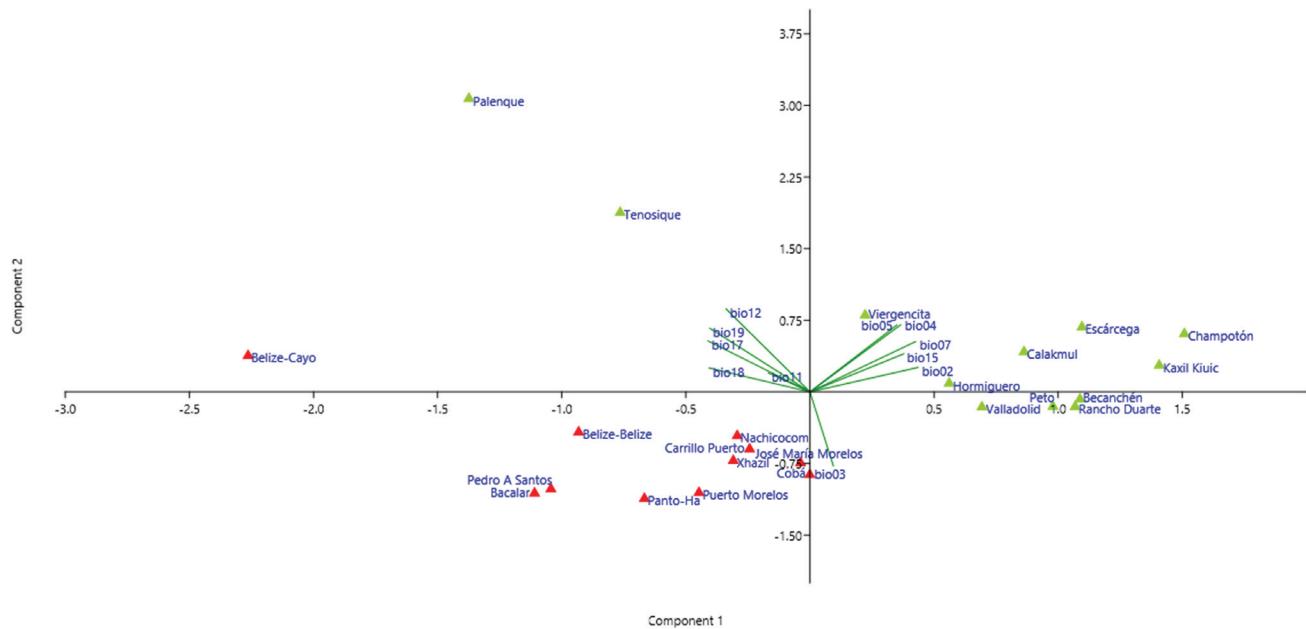
Population/state	N	k	h	π
Xhazil/Quintana Roo	5	2	0.600	0.00202
Pedro A. Santos/Quintana Roo	5	3	0.700	0.00269
Panto Ha/Quintana Roo	5	3	0.700	0.00337
Nachi co com/Quintana Roo	4	2	0.500	0.00168
Puerto Morelos/Quintana Roo	3	2	0.666	0.00224
Cobá/Quintana Roo	3	2	0.666	0.00224
Carrillo Puerto/Quintana Roo	4	4	1.000	0.04433
José M Morelos/Quintana Roo	5	5	1.000	0.01751
Belice/Belize	2	2	1.000	0.00337
Belice/Cayo	3	1	0.000	0.00000
Total	90	18	0.827	0.00852

**Appendix 4.** List of GenBank accession for haplotypes.

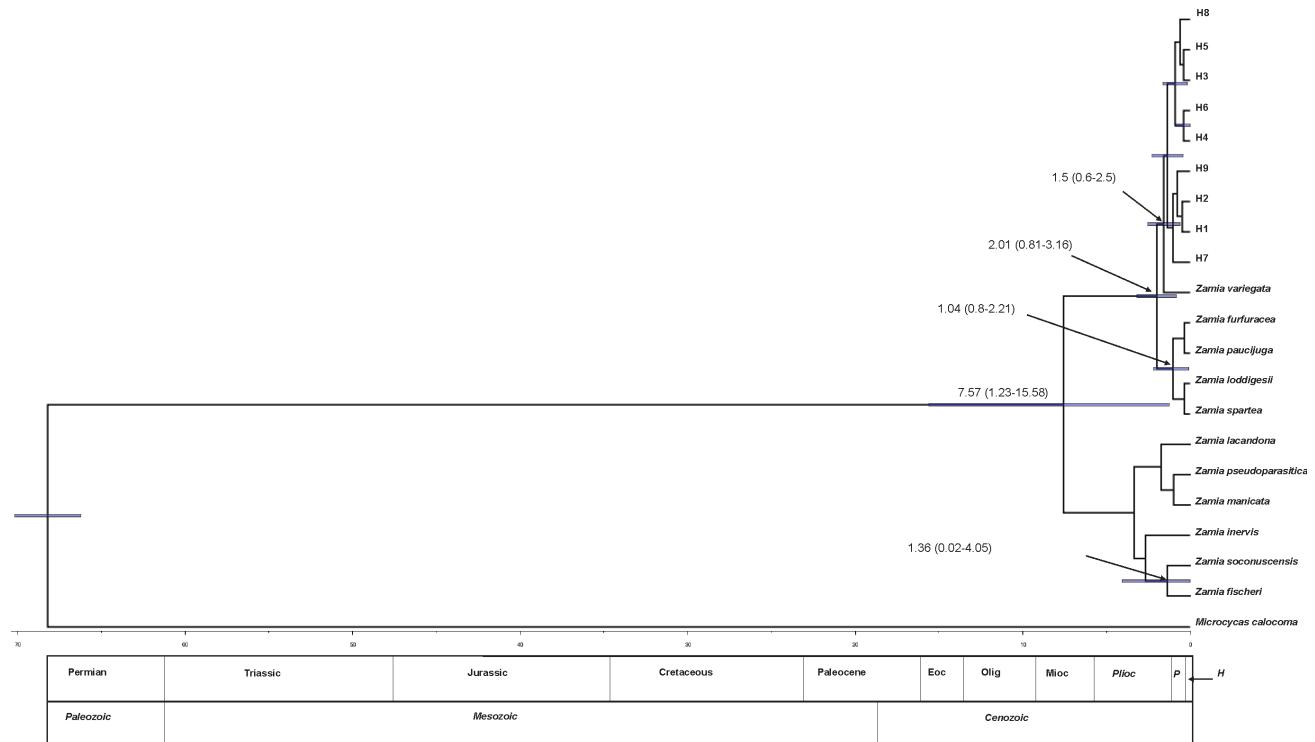
Genome	Code	GenBank accession
Chloroplast ( <i>atpF-atpH</i> )	ZP-A1	MK513628
	ZP-A2	MK513629
	ZP-A3	MK513630
	ZP-A4	MK513631
	ZP-A5	MK513632
	ZP-A6	MK513633
	ZP-A7	MK513634
	ZP-A8	MK513635
	ZP-A9	MK513636
Nuclear (ITS2)	ZP-H1	MK513637
	ZP-H2	MK513638
	ZP-H3	MK513639
	ZP-H4	MK513640
	ZP-H5	MK513641
	ZP-H6	MK513642
	ZP-H7	MK513643
	ZP-H8	MK513644
	ZP-H9	MK513645
	ZP-H10	MK513646
	ZP-H11	MK513647
	ZP-H12	MK513648
	ZP-H13	MK513649
	ZP-H14	MK513650
	ZP-H15	MK513651
	ZP-H16	MK513652
	ZP-H17	MK513653
	ZP-H18	MK513654

## Phylogeography of *Zamia prasina*

**Appendix 5.** Principal component analysis of 11 climatic variables for 23 populations of *Zamia prasina* in the Biotic Province of the Yucatán Peninsula (BPYP) showing the presence of two groups of populations. Red triangles: eastern populations, green triangles: western group.



**Appendix 6.** Bayesian chronogram for chloroplast sequence *atpF-atpH*. The divergence time of *Zamia prasina* haplotypes in the YPBP and other cycad species is shown. The 95 % confidence intervals are shown with purple bars; the numbers at the nodes indicate the estimated age, the numbers below the branches indicate the posterior probability.



**Appendix 7.** Bayesian chronogram for nuclear sequence ITS2. The divergence time of *Zamia prasina* haplotypes in the YPBP and other cycad species is shown. The 95 % confidence intervals are shown with purple bars; the numbers at the nodes indicate the estimated age, the numbers below the branches indicate the posterior probability.

