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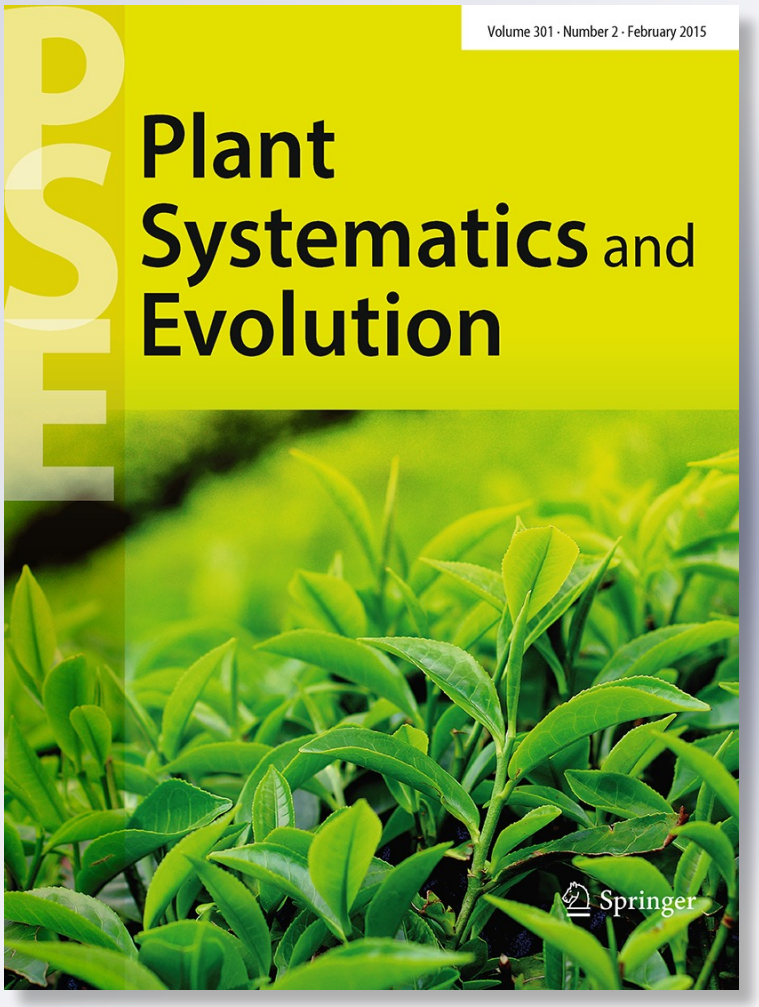

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ORIGINAL ARTICLE

Phylogeography and demographic history of *Zamia paucijuga* Wieland (Zamiaceae), a cycad species from the Mexican Pacific slope

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Abstract We have investigated the phylogeographic structure and demographic history of *Zamia paucijuga*, based on 120 *ITS2* and 117 *psbK/I* sequences from 13 populations distributed along the entire distribution range of the species. We have detected 15 *ITS2* and four *psbK/I* haplotypes, for a total of 19. The genetic diversity estimated for *psbK/I* was relatively lower than the *ITS2* diversity. These results imply that the average genetic diversity in *Z. paucijuga* is lower in comparison with other cycad species, but relatively higher than the diversity found in conifers. Non-hierarchical and hierarchical AMOVAs for *ITS2* and *psbK/I* showed both low and high levels of genetic structure. This discrepancy likely reflects a decrease in gene flow intensity for seeds but high pollen gene flow, which correlates with the divergent inheritance processes in nuclear *vis-à-vis* organellar DNAs. SAMOVA tests for both loci (*ITS2* and *psbK/I*) showed high correspondence with the regional geographic structure defined a priori, indicating that the use of both nuclear and chloroplast gene regions improves inferences concerning the evolutionary processes that affect population dynamics in *Z. paucijuga*. Furthermore, our results are consistent with the conclusions of other studies on the origin of the genus *Zamia*, which support locating the diversification times of *Z. paucijuga* populations in the Pleistocene.

Keywords Cycads · Demographic history · Genetic structure · *ITS2* · Mexico · Phylogeography · Pleistocene · *psbK/I* · Diversification timing · *Zamia paucijuga*

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Introduction

The distribution patterns of species are largely determined by historical processes, for instance, geological, climatic and geographical changes, among others (Zunino and Zullini 2003). However, those patterns can also be influenced by contemporary processes, such as changes in land use, habitat fragmentation and edge effects. Therefore, geographical distribution patterns are significantly correlated with the way in which genetic diversity is spatially apportioned both within and between populations, and provide a window to diverse aspects of their evolution (Avise 2000).

Several examples of phylogeographic studies in Mexican gymnosperms have accumulated already, in which organellar DNA has been used to infer demographic

structure and the processes responsible for genetic differentiation between regions or populations, as a result of geological and climatic events—e.g., *Pinus nelsonii* Shaw (Cuenca et al. 2003); *Pinus radiata* P. Don. (Karhuet et al. 2006); *Picea chihuahuana* Martínez (Jaramillo-Correa et al. 2006) and *Pinus ayacahuite* C. Ehrenb. ex Schltdl. (Ortíz-Medrano et al. 2008). From a phylogeographic perspective, species from Mexico are interesting due to its complex topography, which involves convergence of the Nearctic and Neotropical zones in the middle regions of the country (Ferrusquía-Villafranca 1993; Marshall and Liebherr 2000). These geological and biogeographical conditions have strongly interacted to determine the overall high level of endemism displayed by several Mexican supraspecific taxa, among which cycads in the family Zamiaceae occupy an important place (Nicolalde-Morejón et al. 2014).

The order Cycadales represents a monophyletic lineage originated approximately 300 million years ago, during the Upper Carboniferous-Lower Permian transition (Norstog and Nicholls 1997). However, recent molecular systematic analyses have suggested that the extant cycads (which amount to ca. 331 species; Osborne et al. 2012) are the product of divergence events that took place in the late Miocene (i.e., around 12 million years ago; Nagalingum et al. 2011). This surprising discovery has undermined the traditional 'living fossil' status that the taxonomic order used to have, although it does not invalidate the antiquity of bona fide remains of much older age (e.g., Delevoryas 1982), some of which have been recently described in the paleobotanical literature (e.g., Zhang et al. 2010).

Research in the phylogeography of cycads has been carried out mainly in Asian species such as *Cycas debaoensis* Y. C. Zhong & C. J. Chen (Zhan et al. 2011) and *C. revoluta* Thunb. (Chiang et al. 2009; Kyoda and Setoguchi 2010), using diverse chloroplast and mitochondrial intergenic spacers. These studies have unveiled low levels of genetic diversity, interpreted as indicators of population bottlenecks and of geographical events that took place during the Quaternary interglacial period. Huang et al. (2001) found two populations of *C. taitungensis* C. F. Shen, K. D. Hill, C. H. Tsou & C. J. Chen, from Taiwan with high genetic variation but low population differentiation, suggesting that a relatively short period of isolation precluded the fixation of ancestral polymorphic alleles. On the other hand, working in Mexico, Gutiérrez-Ortega (2010) inferred phylogeographic structure and low genetic diversity in nine locations for *Dioon sonorense* (De Luca, Sabato & Vázq. Torres) Chemnick, T. J. Greg. & S. Salas-Mor., located in Sonora. This author also associated the observed diversity to population bottlenecks that might have taken place during climatic fluctuations in the Quaternary, and to subsequent demographic expansions derived from populations with relatively low effective population sizes.

Climatic fluctuations occurred during the Pleistocene had a great impact in the current worldwide distribution of species in the order Cycadales (González and Vovides 2002). In line with this, Contreras-Medina and Luna-Vega (2002) concluded –for work carried out in Mexican species of cycads—that Pleistocene refugia have been instrumental in defining phylogeographic dynamics. Furthermore, González-Astorga et al. (2003a, b, 2005) studied the effect of Pleistocene glaciations upon the distribution of genetic diversity in *Dioon edule* Lindl., and based their hypothesis on the origin of *Dioon angustifolium* Miq. along the coastal plateau of the Gulf of Mexico on the notion of refugia. In concert with other geodynamic processes, fluctuations in climate due to glaciations might have been crucial to restrict the distribution of species in the order Cycadales to contrasting habitats within the tropical and subtropical regions of Africa, Australia, Asia, the Greater Antilles and the Americas (Salas-Leiva et al. 2013).

Within the cycads, the genus *Zamia* L. has the widest geographic distribution in the Neotropical region, covering an area that starts in Georgia and Florida (United States) down to Bolivia and Mato Grosso (Brazil) in South America (Stevenson 2001; Nicolalde-Morejón et al. 2009). *Zamia paucijuga* is located in the Mexican Pacific slope, throughout a ca. 1,000 km-long region that encompasses territories between the states of Nayarit and Oaxaca. The distribution of *Z. paucijuga* is one of the widest for the genus, and is associated with several vegetation types—e.g., semi-evergreen seasonal forest, deciduous seasonal forest and *Quercus* forest (Nicolalde-Morejón et al. 2009). More specifically, *Z. paucijuga* populations are found in three well-defined geographical regions: a northern region, comprising the Pacific edge of the Trans-Mexican Volcanic Belt in the neighboring states of Nayarit and Jalisco (labeled 'NR' throughout this paper); a middle region (labeled 'CR'), located along the Sierra Madre del Sur, in Michoacán and Guerrero; and a southern region (labeled 'SR'), corresponding to Oaxaca.

In this study, we have examined genetic variation in *Zamia paucijuga* throughout its distribution. For this purpose, we have determined the relationship between genetic and geographic structure in terms of populations and regions, with the aim of elucidating evolutionary and ecological patterns and processes that might have determined its current geographic distribution. We have employed two genetic loci as molecular markers—namely, *ITS2*, a transcribed spacer from the nuclear compartment (nDNA), and *psbK/I*, an intergenic spacer from the chloroplast genome compartment (cpDNA). In cycads, cpDNA is inherited maternally throughout seeds (Cafasso et al. 2001), although there are no equivalent studies that allow

an unequivocal assessment of the pattern of inheritance of nDNA (Petit et al. 2005). However, since autogamy has not been demonstrated in cycads, here we assume that its inheritance pattern is biparental, as in some conifer species (Petit et al. 2005). As a guide for our study, we have formulated and attempted to answer the following four questions: (1) which are the levels of genetic variation throughout the distributional range in *Zamia paucijuga*?; (2) are the geographical distribution patterns of haplotypes defined by the type of marker employed?; (3) are the populations and groups of populations identified throughout the distributional range genetically differentiated?; and (4) how is the distribution of genetic diversity in the species related to historical events?

Materials and methods

Sample collection and DNA sequencing

We collected foliar tissue from 120 individuals, distributed across 13 *Zamia paucijuga* populations that occur throughout the known geographic circumscription for the species. Samples were collected in silica gel and stored at −70 °C until further processing.

Genomic DNA was extracted from 200 mg of foliar tissue with the DNeasy extraction kit (QIAGEN, Valencia, CA, USA). Two loci were amplified: the second internal transcribed spacer (*ITS2*) from the ribosomal DNA coding complex in the nuclear compartment (nDNA), and an intergenic spacer between genes *psbK* and *psbI* (*psbK/I*) located in the chloroplast genome compartment (cpDNA). Each PCR (20 µl) was carried out employing 4 µl of 5× buffer (Promega), 2 µl of 25 mM $MgCl_2$, 2.5 µl of a 8 mM dNTPs mix, 0.8 µl of each amplification primer at 10 µM, 0.3 µl of Taq polymerase (Promega) and 5 µl of template DNA (20 ng). Amplification conditions were standard: an initial cycle was carried out at 94 °C for 5 min, followed by 30 cycles of denaturation/annealing/extension at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and a final extension period of 7 min at 72 °C. Amplification products were inspected in ethidium bromide-stained 1 % agarose gels and were directly purified with the QIAquick gel purification kit (QIAGEN). Bidirectional automated sequencing was carried out by Macrogen (South Korea; http://dna.macrogen.com).

Sequence files were edited with Sequencher 5 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned with Bio Edit 7.0.9 (Hall 1999), using the multiple alignment option in Clustal X (Thompson et al. 1997). Aligned sequences were imported and edited with Mesquite 2.73 (Madison and Madison 2010), and files were saved in Nexus format for subsequent analyses.

Table 1 Substitution rates used to date the crown radiation of *Zamia paucijuga*

Radiation age (Mya)	*ITS* substitution rate (s/s/My)	*psbK* substitution rate (s/s/My)	Lower 95 % HPD (Mya)	Mean age (Mya)	Upper 95 % HPD (Mya)
(A)					
4.77	0.00219	0.00067	31.41	2.71	4.16
5.77	0.00181	0.00056	1.70	3.29	5.05
11.25	0.00093	0.00029	3.35	6.40	9.94
(B)					
27.6	0.00498	0.00108	0.84	1.61	2.50
36.5	0.00376	0.00081	1.10	2.13	3.32
45.6	0.00301	0.00065	1.37	2.67	4.16

A: Dates published by Nagalingum et al. (2012) for the crown-age of *Zamia* spp.; B: dates published by Salas-Leiva et al. (2013) for the splitting event between *Zamia* spp. and *Microcycas calocoma*; substitution rate is given as substitution per site per million years (s/s/My)

DNA data analyses

Nucleotide and genetic diversities (π and Hd, respectively; Nei 1987) were estimated with DnaSP 5.10 (Librado and Rozas 2009). These analyses were performed for individual loci, population and population group (i.e., NR, CR and SR).

Both non-hierarchical and hierarchical analyses of molecular variance (AMOVA) were performed with 1,000 permutations in Arlequin 3.5 (Excoffier et al. 2005). A spatial analysis of molecular variance (SAMOVA) was additionally carried out (100 permutations in 'annealing step') in SAMOVA 1.0 (Dupanloup et al. 2002), and the fixation index (F_{CT})—which indicates the proportion of the total genetic variance due to differences between population groups (Excoffier et al. 1992)—was also calculated. These analyses incorporate the geographical locations of populations (down to decimal degrees) and the corresponding individual haplotypes; they were carried out both for each gene separately and combined in a concatenated ("total evidence") matrix, with the aim of detecting each gene's differential effect. Likewise, we evaluated the percentage of correspondence between the a priori defined geographic structure—namely, the regionalization into NR, CR and SR—with respect to the structure generated by SAMOVA (with $K = 3$) for all loci, either separately or in conjunction. The comparison between groups of populations defined a priori and those generated with SAMOVA was based on the percentage of coincidences between groups, weighted by the number of comparisons within groups.

Table 2 Number of individuals registered in ca. 1 ha (*N*) and analyzed (*n*) per population and geographical region of *Zamia paucijuga,* with molecular markers *ITS2* and *psbK/I*, nucleotide diversity (π), number of haplotypes (*H*) and diversity of haplotypes (Hd)

Geographical region		No. of population (*N*)	*ITS2*				*psbK/I*			
			n	π	*H*	Hd	*n*	π	*H*	Hd
North region (NR)										
1	Compostela, Nayarit	30	10	0.0047	3	0.622	9	0	1	0
2	San Sebastián, Nayarit	15	8	0.0062	4	0.75	9	0.0008	2	0.5
3	Cabo Corrientes, Jalisco	21	10	0.0053	3	0.511	10	0	1	0
4	El Tuito, Jalisco	35	10	0.0054	5	0.8	9	0	1	0
5	Cuantitlán, Jalisco	13	8	0.0061	4	0.75	10	0	1	0
Subtotal			46	0.0059	7	0.722	47	0.0009	3	0.5245
Central region (CR)										
6	El Guayabo, Michoacán	40	9	0.0041	4	0.833	10	0	1	0
7	Las Mecillas 1, Guerrero	30	10	0.0057	6	0.889	10	0	1	0
8	Las Mecillas 2, Guerrero	6	6	0.0038	4	0.867	5	0	1	0
9	Chilpancingo, Guerrero	30	10	0.0047	7	0.911	10	0	1	0
10	Tlacuachistlahuacan, Guerrero	18	10	0.0049	3	0.6	9	0	1	0
Subtotal			45	0.0061	12	0.863	44	0.0008	2	0.5074
South region (SR)										
11	San Gabriel Mixtepec, Oaxaca	20	9	0.0059	6	0.833	8	0	1	0
12	San Bartolomé Loxicha 1, Oaxaca	32	10	0.0034	2	0.356	8	0	1	0
13	San Bartolomé Loxicha 2, Oaxaca	28	10	0.0044	5	0.822	10	0	1	0
Subtotal			29	0.0047	9	0.756	26	0	1	0
Total		318	120[a]	0.0063 ± 0.0003	15	0.8431 ± 0.0170	117	0.0013 ± 0.0001	4	0.6690 ± 0.0180

[a] Represented 38 % of all registered plants

Haplotype networks were constructed for each locus using the median-joining (MJ) algorithm in Network 4.5 (Bandelt et al. 1999); this method combines the topology of a minimum expansion tree with a maximum parsimony tree. For the inference of demographic processes, extended Bayesian skyline plot analyses were performed with BEAST 1.8.0 (Drummond and Rambaut 2007). The specific search settings and prior will be specified below (i.e., Beast search). Extended Bayesian skyline plot analyses allow the use of independent substitution and clock models, as well as the assignment of specific mutation rates to each locus. This type of analysis is adequate for data sets that involve non-correlated molecular partitions. The model for the prior tree was set as linear, and was kept linked for both loci, allowing the effective population size to change continuously along each interval (Ho and Shapiro 2001). Plots were inferred for each geographic region as well as for the global distribution (all populations together). No outgroup was used for any of these analyses. To corroborate the Bayesian skyline plots results, we performed Fu's Fs test (Fu 1997), which employs information from haplotype distribution to infer population demographic growth. In this test, negative and significantly different from zero Fs values indicate range expansion at the population level.

Prior to the demographic analyses, we performed a Bayesian Inference (BI) for inferring a reliable tree that would be used later for the BEAST analysis as a starting tree (i.e., tree prior). We used the Akaike Information Criterion (Alfaro and Huelsenbeck 2006) in jMODELTEST 2.0.2 (Posada 2008) to select an appropriate model of nucleotide substitution, and the model priors during BI. This analysis was performed on the total-evidence dataset

Table 3 Analysis of molecular variance (AMOVA) of 13 populations of *Zamia paucijuga* with *ITS2* and *psbK/I* (A) and hierarchical AMOVA among geographical groups (B)

Source	*ITS2*					*psbK/I*				
	df	SS	Est. var.	Variation (%)	Fixation index	*df*	SS	Est. var.	Variation (%)	Fixation index
(A)										
Among populations	12	44.061	0.285	21.5	$F_{ST} = 0.215$*	12	49.624	0.458	96	$F_{ST} = 0.960$*
Within populations	107	111.389	1.041	78.5		104	2.000	0.019	4	
Total	119	156.450	1.326			116	51.624	0.477		
(B)										
Among regions	2	17.086	0.148	10.79	$F_{SC} = 0.148$*	2	26.800	0.292	51.8	$F_{SC} = 0.929$*
Among populations within regions	10	26.975	0.181	13.19	$F_{ST} = 0.239$*	10	22.824	0.252	44.8	$F_{ST} = 0.965$*
Within populations	107	111.389	1.041	76.02	$F_{CT} = 0.108$*	104	2.000	0.019	3.4	$F_{CT} = 0.518$*
Total						116	51.624	0.563		

* $P < 0.05$

using MRBAYES 3.2.1 (Ronquist and Huelsenbeck 2003). Nonetheless, we employed partition-specific DNA evolution models of each gene. Two parallel Markov chain Monte Carlo (MCMC) analyses were executed simultaneously, each of them run for a minimum of 20 million generations, with sampling every 1,000 generations. A majority consensus tree was calculated from the sampled trees remaining after 25 % of the burn-in samples were discarded (Ronquist and Huelsenbeck 2003). For inferring the BI tree and subsequent analyses, nine species of *Zamia* (i.e., *Zamia cunaria*, *Z. fischeri*, *Z. integrifolia*, *Z. manicata*, *Z. prasina*, *Z. soconuscensis*, *Z. standleyi*, *Z. tuerckheimii*, and *Z. vazquezii*) were chosen as outgroups. These taxa were chosen, instead of a single outgroup, because currently there is no consensus about phylogenetic relationships among the species of this genus. These nine species are closely related species to *Z. paucijuga*; sequences for both molecular markers were available for them (Nicolalde-Morejón et al. 2011). The final tree was rooted with *Z. integrifolia*, which was the most distant taxon from *Z. paucijuga*.

Dates for potential isolation events within the *Z. paucijuga* phylogeny were calculated using BEAST 1.8.0 (Drummond and Rambaut 2007). The tree inferred from the BI analyses was used as a starting tree. A Yule process was set as prior for the tree model. For date estimation, we used the total-evidence data set; however, the evolutionary model of each partition as chosen by jMODELTEST was used as prior (i.e., GTR, gamma shape = 0.029 for *psbK/I*; GTR, gamma shape 0.047 for *ITS2*). To allow for differences in the molecular characteristics of each sequence, the substitution and clock models were set as unlinked. Recent studies have addressed the radiation (i.e., evolutionary diversification) history between the different lineages of

Table 4 Fixation index (F_{CT}) corresponding to population groups ($K = 3$) from SAMOVA for the populations of *Zamia paucijuga*

Markers	Grouped populations	F_{CT}	*P*
psbK/I + ITS2	(2, 3, 4, 5, 6, 7) (1, 8, 9, 10) (11, 12, 13)	0.419	<0.0001
psbK/I	(1, 2, 8, 9, 10) (3, 4, 5, 6, 7) (11, 12, 13)	0.910	<0.00001
ITS2	(1, 2, 3, 4, 5, 6, 12, 13) (9) (7, 8, 10, 11)	0.265	<0.00001

North Region (NR): 1, 2, 3, 4 and 5; Central Region (CR): 6, 7, 8, 9 and 10; South Region (SR): 11, 12 and 13

cycads (Nagalingum et al. 2011; Salas-Leiva et al. 2013); however, the split times presented differ significantly. For instance, Nagalingum et al. (2011) place the crown radiation of the genus *Zamia* between 4.77 and 11.25 Mya, whereas Salas-Leiva et al. (2013) set the split time between the genera *Zamia* and *Microcycas* from 27.6 to 45.6 Mya. To accommodate for those discrepancies, we did not use specific calibration points; instead, we used the different published dates to infer alternative nucleotide substitution rates for each locus to be set as priors for the ucld.mean parameter in the BEAST analyses (see Table 1). For this, substitution rates were obtained after dividing one half of the mean genetic distance between either the different species of *Zamia*, or between *Zamia* spp. and *Microcycas* by the respective diversification date. Additionally, we used the age confidence intervals published by Nagalingum et al. (2011) and Salas-Leiva et al. (2013) to define bounds for the ucld.mean prior, and chose the relaxed clock (uncorrelated) model with a log-normal distribution of rates. Other priors were set from the best-fit model, and a normal distribution was selected. No topological constraints were enforced in any analyses. The MCMC

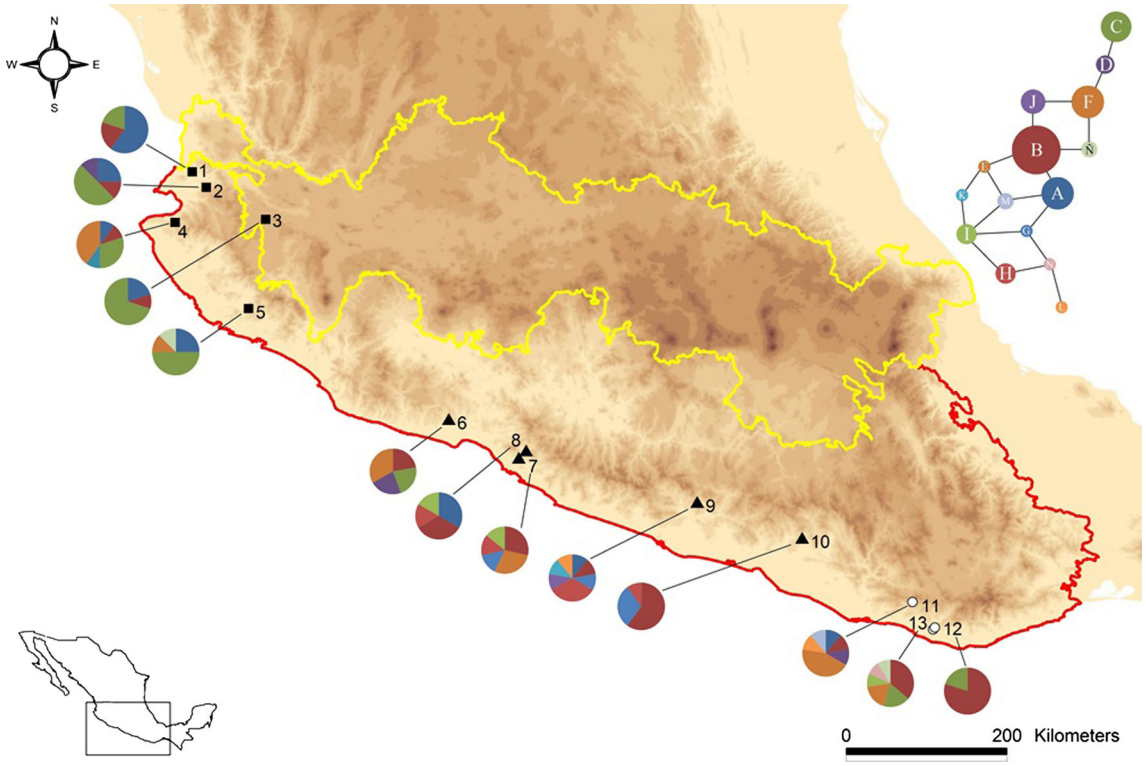


Fig. 1 Geographic distribution of *ITS2* (nDNA) haplotypes in *Zamia paucijuga*. Haplotype *names/numbers* are identified in the legend. *Circle size* is proportional to haplotype frequency in populations, and *lines* between haplotypes indicate one mutational step. The *yellow line* corresponds to Trans-Mexican Volcanic Belt and the *red one* to the Sierra Madre del Sur. The network of nDNA haplotypes is shown in the *upper right corner*

analyses were run at least ten times for 10 million generations each, and parameters were sampled every 1,000th generation. Finally, the program TRACER 1.5 (Drummond and Rambaut 2007) was used for assessing stationarity of the MCMC, effective sample sizes (ESSs; values >200), and posterior intervals spanning the 95 % highest posterior density. The single runs from TRACER were combined with LogCombiner, as implemented in BEAST. Resulting trees obtained after applying a 15 % burn-in were compiled with Tree Annotator 1.8.0, and results were visualized with FigTree 1.3.1.

Biogeographic scenario

Spatial patterns of geographic diversification within *Zamia paucijuga* were inferred using statistical dispersal–vicariance analysis as implemented in RASP v. 2.1 beta (Yu et al. 2010, 2013). The software uses the collection of trees from a Bayesian MCMC analysis and can handle phylogenetic uncertainty in reconstructing biogeographical histories (Nylander et al. 2008). The three main geographic areas were defined as indicated above (i.e., NR, CR and SR). The ancestral areas of terminal species representing higher taxa were scored for all portions of the genus or clade range. Multiple representatives of *Zamia paucijuga* were each scored based on their own distribution. We defined five alternative biogeographic areas for this analysis: (1) Central America [*Z. cunaria*, *Z. soconuscensis*, *Z. standleyi*, and *Z. tuerckheimii*], (2) Gulf of Mexico [*Z. fischeri* and *Z. vazquezii*], (3) Caribbean [*Z. integrifolia*], (4) South America [*Z. manicata*] and (5) Yucatán [*Z. prasina*]. *Z. integrifolia* was declared as the outgroup. Post burn-in trees from the BEAST output were fed into RASP to estimate probabilities of ancestral areas at each node; these amounted to 59,973 trees (i.e., the complete posterior distribution after burn-in removal) to account for uncertainties in phylogeny. The maximum number of areas in ancestral ranges was constrained to four, and the ancestral areas for all nodes were visualized on the condensed tree.

Results

Genetic diversity

All populations ($n = 13$) considered here were polymorphic for both *ITS2* and *psbK/I*. The total number of haplotypes detected was 19 (15 for *ITS2* and four for *psbK/I*; Table 2). The maximum length of the matrix of *ITS2*

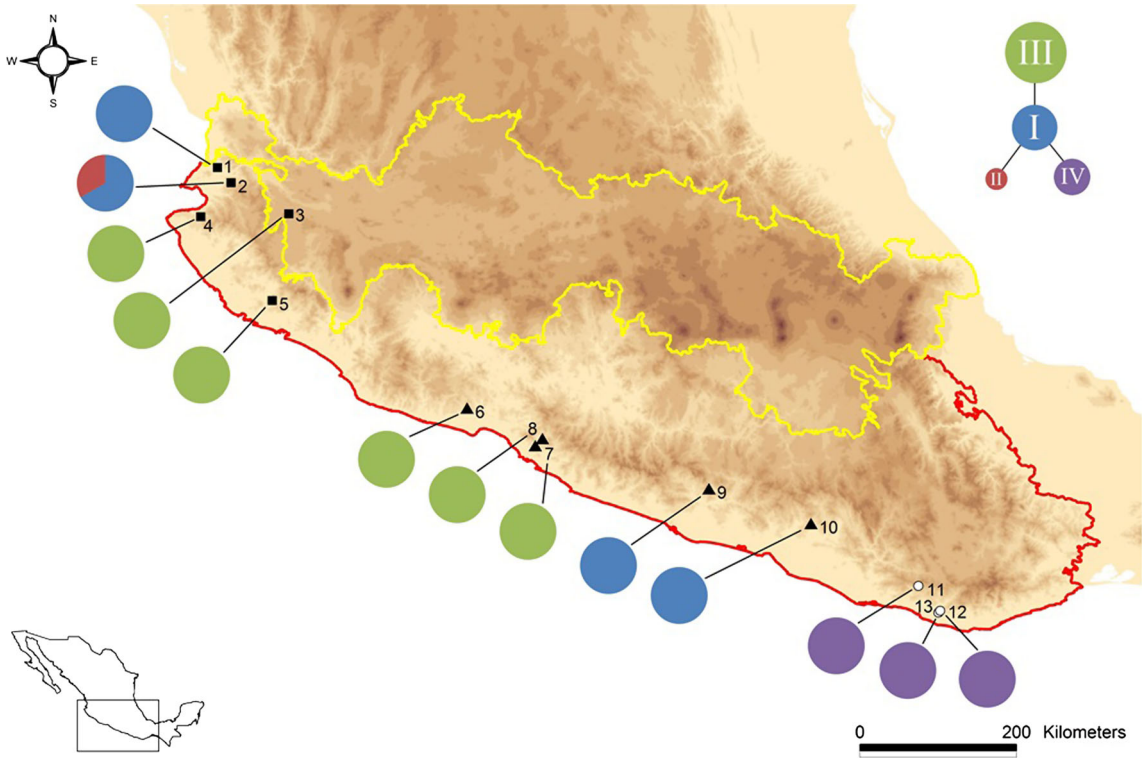


Fig. 2 Geographic distribution of *psbK/I* (cpDNA) haplotypes in *Zamia paucijuga*. Haplotype names/numbers are identified in the legend. *Circle size* is proportional to haplotype frequency in populations, and *lines* between haplotypes indicate one mutational step. The *yellow line* corresponds to Trans-Mexican Volcanic Belt and the red one to the Sierra Madre del Sur. The network of nDNA haplotypes is shown in the *upper right corner*

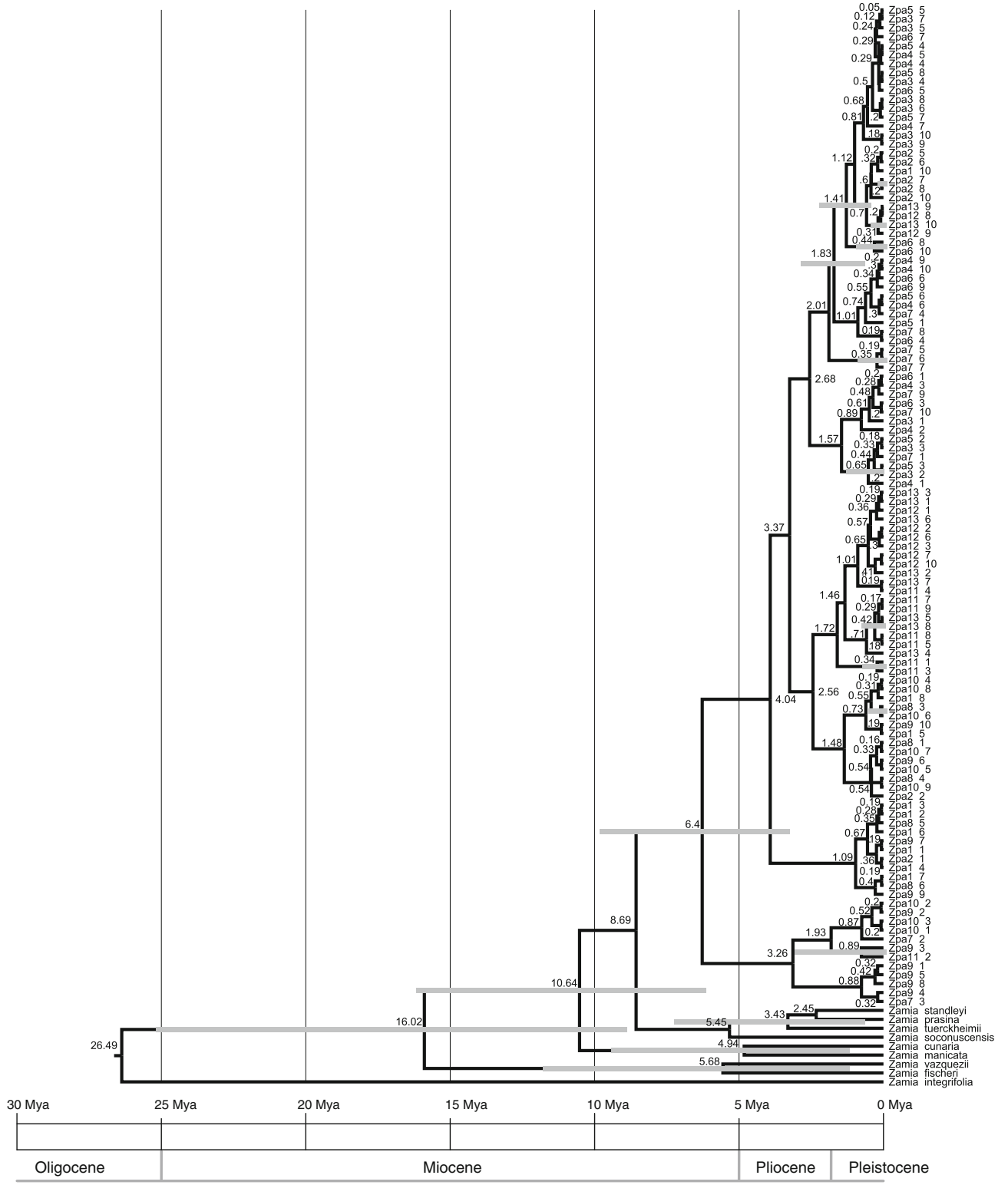
aligned sequences was 418 bp, and the *psbK/I* matrix was 670 bp long. The overall estimated genetic diversity was larger for *ITS2* (Hd = 0.843, $\pi = 0.0063$) than for *psbK/I* (Hd = 0.669, $\pi = 0.0013$). When analyzed individually, the CR and NR populations held the highest genetic diversity for *ITS2* and *psbK/I*, respectively. Whereas for *ITS2* all values were similar (NR = 0.722, CR = 0.863 and SR = 0.756), the SR population had zero diversity for *psbK/I* (i.e., it contained only one haplotype; Table 2).

Genetic structure

The non-hierarchical AMOVA showed that 78.5 % of the total genetic variation for *ITS2* is due to differences within populations, whereas the remaining 21.4 % of the variation can be attributed to interpopulational differences (Table 3A). For *psbK/I*, 4 % of the total variation is due to differences within populations and 96 % to differences among them. The hierarchical AMOVA (which assumes the geographic partition NR, CR and SR) indicated that for *ITS2* the total genetic variation is partly due to differences between regions (10.79 %) and to interpopulational differences within regions (13.19 %), but that the largest difference is found within populations (76.02 %; Table 3B). For *psbK/I*, the AMOVA tests indicated that total genetic variation is mainly due to interregional differences (51.8 %) and secondarily to internal differences within regions (44.8 %). Only the remaining 3.4 % of the variation was due to intrapopulational differences (Table 3B).

Considering both *ITS2* and *psbK/I* together, the SAMOVA test with $K = 3$ showed a fixation index F_{CT} of 0.419 ($P < 0.0001$), whereas the individual *psbK/I* and *ITS2* values were $F_{CT} = 0.910$ ($P < 0.00001$) and 0.265 ($P < 0.00001$), respectively (Table 4). Correspondence between SAMOVA for $K = 3$ and the a priori geographic structure was 53.9 % for the joint analyses, a value that contrasts sharply with the percentages obtained for each individual locus (i.e., 38.5 % for *psbK/I* and 23.5 % for *ITS2*).

The *ITS2* network indicated that all interconnected haplotypes differ by one mutational step (Fig. 1). CR was the region with the highest number of haplotypes (12, out of 15 total), followed by SR (9 haplotypes) and NR (7; Table 2). Five haplotypes (A, B, C, D and F) were found in all three regions; among these, haplotype B has the widest distribution and is, therefore, a likely ancestral haplotype candidate. On the other hand, several private haplotypes



◀**Fig. 3** Chronogram inferred from the TE dataset encompassing nuclear *ITS2* and chloroplast *psbK/I* sequences. *Zamia paucijuga* terminals are recovered as a monophyletic lineage with a Miocene-Pliocene origin (ca. 6 Mya). The *numbers* by the nodes indicate splitting dates. *Gray bars* show 95 % age confidence limits. *Tip labels* indicate population origin and sample number, respectively

were found for each geographical region: (1) haplotype E for NR, (2) G, K and L for CR, and (3) N for SR.

For *psbK/I*, the haplotype network included four haplotypes that differ by one mutational step (Fig. 2). Although haplotype I was not the most frequent in the network, its internal position might suggest an ancestral haplotype status. The geographic distribution of *psbK/I* haplotypes allows distinguishing a geographic structure formed by two groups: (1) one that includes NR and CR populations, with haplotypes I, II and III, and (2) a separate, SR-related group that bears haplotype IV. Haplotypes I and III were predominant in NR and CR; however, population 2 included two haplotypes (I and II). Haplotype II was exclusive for this population (Fig. 2).

Diversification timing

The starting trees obtained with the *ITS2*, *psbK/I* and total-evidence (TE) datasets from BI were mutually congruent, with main nodes showing good support. Although coalescent analyses were performed for the individual loci as well as for the TE dataset, to avoid confusion here we present only the results inferred from the TE dataset (that was the most informative partition). The results from each partitioning strategy [i.e., individual genes, concatenated matrix (not partitioned by genes), and TE (concatenated matrix partitioned by genes)] were compared using Bayes factor (Newton and Raftery 1994). Bayes factor indicated that the BI tree inferred from the TE dataset was more informative (2ln: TE & *ITS2* = 4.6; TE & *psbK/I* = 3.4), although this difference was not necessarily significant (Kass and Raftery 1995).

Besides small differences in the position of several terminal branches, the main relationships observed in the chronogram inferred from the TE dataset (Fig. 3) are consistent with those observed in the trees resulting from the analysis of each individual marker (see supplementary materials). The comparison of all coalescence analyses (i.e., BEAST runs) revealed high convergence among the inferred parameters, and ESSs were large (ranging from 653 to 1,152). Divergence time estimation analyses resulted in similar values, both for the TE and the individual genes (not presented here, but available as supplementary material). The genealogy shows that, although some geographical structure is recovered, the a priori defined geographic regions (i.e., NR, CR and SR) have not been isolated from each other through time. The mean age for the diversification of *Z. paucijuga* (Table 1) was dated between 6.4 and 1.6 million years ago (Mya), although most of our estimations set such event during the Pliocene. On the other hand, the ecological, evolutionary and phylogeographic events relevant to such diversification (radiation sensu Nagalingum et al. 2011) most likely occurred during the Pleistocene.

Biogeographic scenario

Based on the Bayes-DIVA analyses, the CR is supported as the source region of the diversification (Fig. 4, node I; ancestral area probability: CR = 94.9 %, NR + CR = 3.2 %, CR + SR = 1.5 %, NR + CR + SR = 0.3 %, or NR + SR = 0.1 %). Apparently, the SR was the first region to detach as a unit from the CR. Based on the more conservative chronogram (inferred with the slowest mutation rate), such separation occurred close to the Pliocene–Pleistocene boundary between 2.6 and 1.7 Mya (Fig. 4, node II). The NR region was in turn separated from the CR region more recently (1.4 and 1.1 Mya; Fig. 4, node III). On the other hand, our results indicate that there were historical migration event between the three geographic regions, although dispersal was not symmetrical. This analysis recovered 17 dispersal events. Apparently, a bi-directional interchange has taken place between the CR and the NR (8 from CR to NR, 7 from NR to CR), whereas migration seems to have been unidirectional between these two regions and the SR. Historical migration has only occurred once from CR to SR (ca. 0.9 Mya; Fig. 4, node IV), and one long-distance dispersal event took place from NR to SR (ca. 0.7 Mya; Fig. 4, node V).

Historical demography

The extended Bayesian skyline plot analyses suggest a complex demographic history in *Zamia paucijuga* (Fig. 5). None of the corresponding plots for the NR and SR were significant. However, skyline plots show demographic growth and values of Fu's Fs were significant for the CR (Fs = −6.729) as well as for the global distribution (Fs = −15.975). Overall, these results indicate that the effective population size (Ne) has been stable throughout time; however, demographic growth has apparently taken place during the last half million years (Fig. 5).

Discussion

Genetic diversity

Xiao et al. (2010) showed the existence of non-functional translocated copies (i.e., pseudogenes) of ribosomal DNA

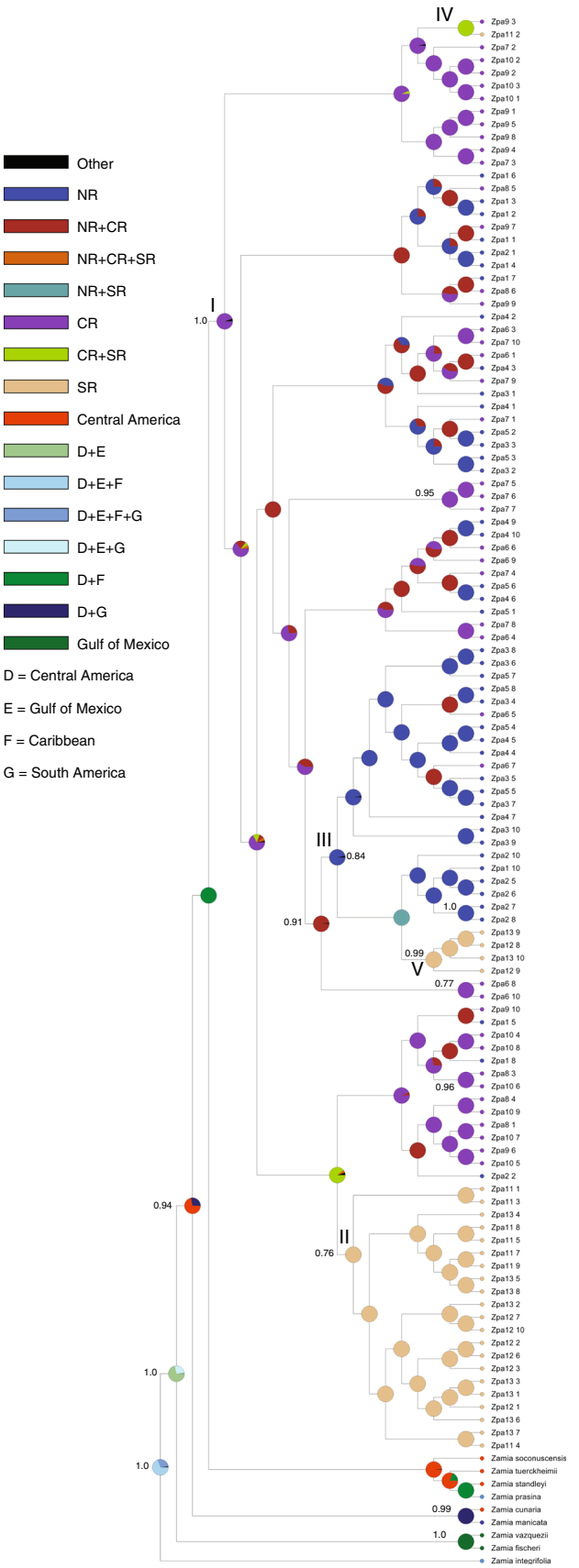


Fig. 4 Dispersal–vicariance scenario based on 59,973 trees obtained from the Yule BEAST analysis. This set of trees comprises the complete posterior distribution that remained after burn-in removal. *Pie charts* show the marginal probabilities for each putative ancestral area. *Numbers* are node posterior probabilities >0.75. Key events during the population history of *Zamia paucijuga* are indicated by *roman numerals* (see text). The South region (SR) was inferred as the ancestral area for the species

in the genome of the six species from the genus *Cycas*; apparently, multiple *ITS* pseudogene copies are especially common in those taxa. Such pseudogenes are characterized by a significant reduction in GC content (<50 % in pseudogenes vs. >60 % in functional *ITS*), and a high rate of nucleotide substitutions (six times higher than functional *ITS*). Besides the potential formation of chimeric sequences (Wei et al. 2003), pseudogenes might recover spurious relationships by joining taxa randomly due to nucleotide saturation. In contrast, Ochieng et al. (2007) concluded that this fast accumulation of changes in pseudogenes might be advantageous for phylogenetic studies involving very close related species (or population analyses) in which the divergence from the common ancestor is too recent.

After performing BLAST searches with our data, we confirmed that both the *ITS2* and *psbK/I* sequence datasets used in the present analyses belong to functional regions and not to pseudogenes. More specifically, (1) we detected that sequence lengths do not differ significantly from those deposited in Genbank; (2) homologous motives (variable and conserve regions) within and among the sequences are recognizable; (3) GC content fell within the range of functional sequences; (4) genetic distances (i.e., the number of nucleotide substitutions) among sequences do not show significant differences; and (5) nucleotide bias observed in the different gene partitions is nearly identical.

The length of sequences used in our analyses approximates the values reported in other studies—e.g., 577 bp for *psbA-trnH* (cpDNA) in *Cycas debaoensis* (Zhan et al. 2011) or 960 bp for *trnL-F* in *Dioon sonorense* (Gutiérrez-Ortega 2010). Genetic and haplotype diversity (π and Hd, respectively) in *Zamia paucijuga* is lower than the average registered for Asiatic cycad species (see Table 5), but relatively higher when compared to *Dioon sonorense* (Gutiérrez-Ortega 2010) and *Pseudotsuga menziesii* (Hd = 0.690, $\pi = 0.00072$; Gugger et al. 2011), both species with Mexican distribution. In turn, for both *ITS2* and *psbK/I*, the genetic diversity estimated for *Z. paucijuga* (Hd = 0.756 ± 0.12) is similar than the average genetic diversity estimated in seven gymnosperm species from North America in the genera *Pseudotsuga* and *Pinus* (0.723 ± 0.16; Cuenca et al. 2003; Jaramillo-Correa et al. 2006; Karhu et al. 2006; Ortíz-Medrano et al. 2008; Moreno-Letelier and Piñero 2009; Rodríguez-Banderas et al. 2009; Gugger et al. 2011).

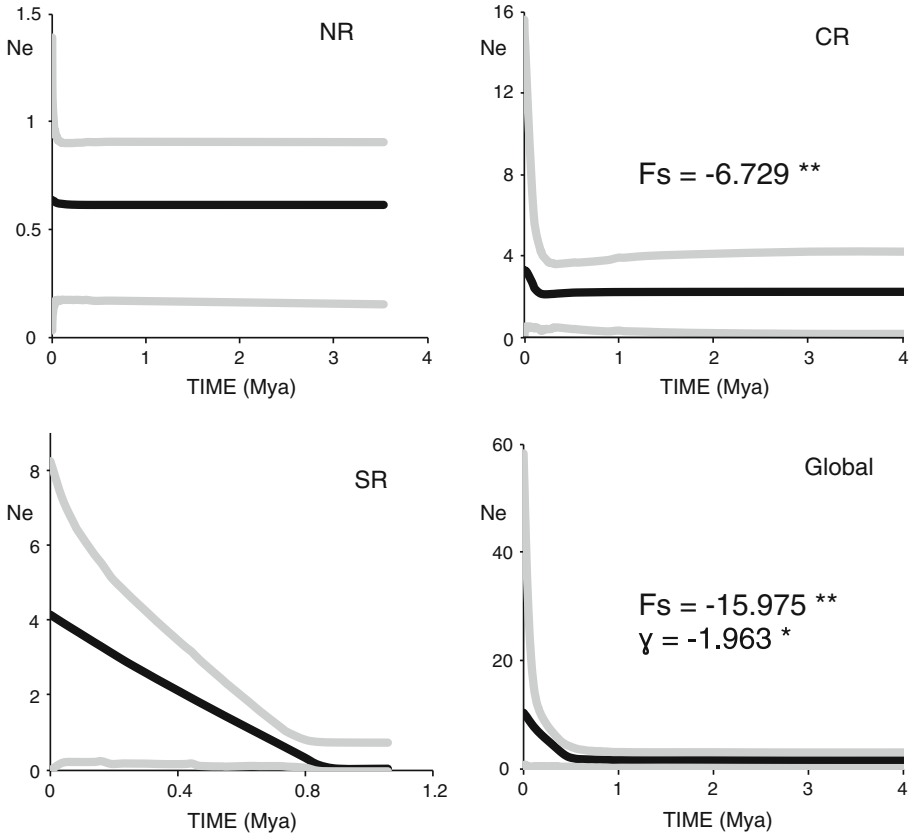


Fig. 5 Demographic history of *Zamia paucijuga* based on extended Bayesian skyline plots analyses. The *plots* presented here are based on the next substitution rates ITS: 0.00181 s/s/My, and psbK: 0.00056 s/s/My. Although the analyses were run using all the estimated substitution rates (see Table 1), the general patterns recovered did not differ. The *vertical axis* corresponds to the effective population size (Ne). The *black line* indicates the trend of the median Ne throughout time, and *gray lines* establish 95 % confidence limits. Fs is Fu's statistical and γ stands for the rate of lineage splitting

In plants, chloroplast DNA (cpDNA) markers display average evolutionary rates in the range of 10^{-9} substitutions/site/year, which are relatively slower than nuclear DNA (nDNA) rates (Wolf et al. 1987). Disagreements in the estimated genetic diversity values for *ITS2* and *psbK/I* in *Zamia paucijuga* are consistent with this difference. For instance, haplotype diversity (Hd) as well as nucleotide diversity (π) is relatively larger in *ITS2* (nDNA) than in *psbK/I* (cpDNA). It is known that cpDNA inheritance in cycads occurs along the maternal line (Cafasso et al. 2001), whereas it is reasonable to assume that nDNA is inherited bipaternally, as in conifers (Petit et al. 2005). In this context, our results suggest that information, provided by different DNA compartments, is useful for evaluating patterns of differentiation among and within populations.

It has been documented that the evolutionary history of taxa and genes might differ significantly (Rosenberg and Nordborg 2002; Lynch et al. 2006; Degman and Rosenberg 2009). Although the nuclear and the chloroplast gene compartments have different nucleotide substitution rates, as reflected in the loci studied here (i.e., *ITS2* accumulates between 3.2 and 4.6 more mutations than *psbK/I*), the results obtained from analyses performed separately for each were congruent to those based on the TE dataset. To infer a reliable evolutionary history for the populations of *Zamia paucijuga*, here we suggest that the TE dataset-based results should be preferentially discussed.

Overall, Mexican cycads display a definite pattern in their genetic diversity levels. In some species, genetic diversity is relatively high, despite their small number of populations and restricted distributions—e.g., *Zamia loddigesii* Miq. (González-Astorga et al. 2006), *Dioon caputoi* De Luca, Sabato & Vázq. Torres and *D. merolae* De Luca, Sabato & Vázq. Torres (Cabrera-Toledo et al. 2010) and *D. sonorense* (González-Astorga et al. 2009). However, other species –such as *D. holmgrenii* De Luca, Sabato & Vázq. Torres– have exactly the opposite pattern (González-Astorga et al. 2008). The *Z. paucijuga* populations studied here fall within the first category, despite the fact that its known populations are geographically isolated and have low population numbers (see Table 2). The relatively high genetic diversity displayed by *Z. paucijuga* with respect to its Mexican congeners might 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.

Genetic structure

The global, *ITS2* hierarchical AMOVAs data indicate that the largest percentage of genetic variation is due to differences within populations (78.5 %), whereas most of the variation (96.6 %) in *psbK/I* can be attributed to both among regions (51.8 %) and among populations within

Table 5 Summary of studies of phylogeography in cycads

Species	Distribution	GR	*R/P*	Loci	Hd	π	F_{ST}	DE	References
Cycas debaoensis	SE China	ca. 300	2/11	*atpB-rbcL* and *psbA-trnH* (cpDNA)	0.492	0.00130	0.801	No	Zhan et al. (2011)
Cycas revoluta	Islas Ryukyu, Japan	ca. 700	2/22	*trnS-trnfM* and *matK* (cpDNA)	0.641	0.00071	0.827	No	Kyoda and Setoguchi (2010)
Cycas revoluta	Islas Ryukyu, Japan		0/7	*atpB-rbcL* (cpDNA)	0.959	0.05810	0.036-0.159	Yes	Chiang et al. (2009)
				nadI exon B and exon C (mtDNA)	0.932	0.05000	0.006-0.117	Yes	
Dioon sonorense	Sonora, Mexico	ca. 300	0/9	*trnL-F* (cpDNA)	0.363	0.00077	–	–	Gutiérrez-Ortega (2010)
Cycas taitungensis	Taiwan	ca. 100	0/2	*atpB-rbcL* (cpDNA)	0.998	0.01268	0.006	–	Huang et al. (2001)
				ITS (mtDNA)	0.970	0.02637	0.021	–	
Cycas taitungensis	Taiwan		0/2	*atpB-rbcL* (cpDNA)	0.998	0.01810	–	Yes	Chiang et al. (2009)
				nadI exon B and exon C (mtDNA)	0.978	0.03830	–	No	
Zamia paucijuga	Mexico	ca. 1,000	3/13	*psbK-I* (cpDNA)	0.669	0.00130	0.960	No	This study
				ITS2 (nDNA)	0.843	0.00630	0.214	Yes	

GR, geographic range in km; R/P, no. of regions/no. populations; Hd, diversity of haplotypes; π, nucleotide diversity; DE, demographic expansion

regions (44.8) (see Table 3). These differences can be explained by the differential rates of evolution between the genes employed here, as well as by their distinct inheritance types. The low genetic differentiation obtained with nuclear markers (*ITS2*, $F_{ST} = 0.215$), as compared to the results from chloroplast markers (*psbK/I*, $F_{ST} = 0.960$), might indicate that gene flow through seeds is less efficient with respect to pollen movement. Similar patterns have been found in some *Pinus* L. species (Ortíz-Medrano et al. 2008; Rodríguez-Banderas et al. 2009) and in *Cycas debaoensis* from the southeast of China (Zhan et al. 2011). This is coherent with basic knowledge of cycad reproductive biology: whereas it is common that seed vectors are scarce and deficient in the wild, Norstog and Nicholls (1997) indicate that seed dispersers for this group are mainly rodents and birds. In the specific case of the Mexican cycad *Dioon edule*, Vovides (1990) indicated that *Peromyscus mexicanus* Saussure is the seed vector; however, the dispersion process in this species has been inefficient, consequently generating aggregated patterns of local distribution (cf. Octavio-Aguilar et al. 2008, 2009). In contrast to seed dispersion, pollen transport is wider and more efficient, producing relatively high rates of seed fecundity and viability (e.g., in the ecological interaction between *Rhopalotria mollis* Sharp. and *Zamia furfuracea* L. fil: Norstog 1986; Norstog and Fawcett 1989). These contrasting properties of the gametophytes might explain the low values of differentiation detected with nuclear markers.

Using both *psbK/I* and *ITS2*, SAMOVA tests with $K = 3$ showed the highest correspondence with the regional structure defined a priori (53.9 %), whereas such correspondence decreased to 38.5 % for *psbK/I* alone and down to only 23.5 % for *ITS2*. These results indicate that joint consideration of chloroplast and nuclear regions provides more information about genetic and geographic structure than either compartment by itself. This pattern can also be observed in the species' chronogram or genealogy (Fig. 3), which reflects the behavior of a taxon undergoing a complex dynamic process involving colonization and expansion.

Diversification timing

The latest molecular phylogeny reconstructed for the order Cycadales indicates that contemporary species have a recent origin—i.e., Miocene-Pliocene, ca. 12 Mya—whereas species in the genus *Zamia* have diversified approximately 5 million years ago, when climate conditions were colder and seasonal rainfall was higher (Nagalingum et al. 2011). Our analyses place the origin of *Z. paucijuga* within this timeframe (i.e., from 1.61 to 6.4 Mya, see Table 1).

More recent events have also shaped radiation patterns in other seed plant species and genera. For instance, Ortíz-Medrano et al. (2008) detected two demographic expansions in *Pinus ayacahuite* var. *ayacahuite*. The first of these expansions took place before the divergence of two population groups distributed in the center and south of Mexico, and subsequently in populations from southern Chiapas. This pattern was attributed to the impact that the Pleistocene had in southern Mexico and Central America.

In a separate study on the genus *Agave*, an increment in the speciation rate occurred between 3 and 2 million years in the past (Good-Avila et al. 2006). Although *Z. paucijuga* split from its sister clade ca. 8 Mya, the main radiation according to the chronogram (Fig. 3) within this species happened during the Pleistocene. During this period, Neotropical Montane Forest experienced extremely complex glacial–interglacial dynamics (Hewitt 2000), and the effect of climatic fluctuations on the genetic structure and population history of species distributed in these habitats has led to different outcomes, such as rapid radiations or local extinctions (Ramírez-Barahona and Eguiarte 2013). Calculations based on both the nuclear and chloroplast sequences support a recent deceleration in the rate of haplotype formation ($\gamma_{ITS2+psbK/I} = -1.963$). Interestingly, the latter value is not different from what has been found in the genus *Zamia* (Nagalingum et al. 2011); this suggests that the historical factors affecting *Z. paucijuga* at the population level during the late Pleistocene have similar outcomes in other species within the genus. Phylogeographic breaks corresponding to the Tehuantepec Isthmus and the Chiapas Central Depression have been identified for diverse species, including plants, birds and mammals (Ornelas et al. 2013). Such barriers, however, are apparently lineage-specific, revealing a complexity that seems to be the result of differences among taxa in ecological niche requirements and dispersal capabilities. It is likely that within these habitats, there were multiple successive opportunities for populations to diverge in isolation during the Pleistocene.

Biogeographic scenario and historical demography

Demographic analyses with *ITS2* indicate a range expansion for the species, which is most pronounced in populations from CR, a geographic area that corresponds to the states of Michoacán and Guerrero. The coalescence time estimations suggest that the species experienced a vicariance event during the first stage of the Pleistocene followed by a subsequent early Quaternary expansion, when the climate turned similar to its present condition. Similar reports exist for other plants. According to Rodríguez-Banderas et al. (2009), the majority of *Pinus leiophylla* var. *chihuahuana* (Engelm.) Shaw, and *Pinus leiophylla* var. *leiophylla* Schiede ex Schltdl. & Cham. populations distributed in Mexico are currently undergoing a demographic expansion process.

Apparently, the SR behaved like a panmictic unit, restricting the gene flow among those populations in relation to the central and northern populations. This suggests that these populations have more recently diverged from the rest, a scenario already postulated for the evolution of *Dioon edule* Lindl. (González-Astorga et al. 2003a, b) and *D. angustifolium* Miq. (González-Astorga et al. 2005). These two *Dioon* species are distributed along the Sierra Madre Oriental and Gulf of Mexico coastal slope.

In the present work, we established that genetic diversity levels are higher in nDNA (*ITS2*) than in cpDNA (*psbK/I*) sequences from *Z. paucijuga*. However, the populations have a higher phylogeographic structure in terms of cpDNA data, because this genomic compartment is inherited through seeds. We also found a significant relationship between genetic and geographic structure considering both loci. Albeit historical demographic estimates based on Fu's Fs values show significant expansion tendencies in several populations, the fluctuation pattern in Ne is not that clear. Depending on the geographic region, molecular markers may show strong variation in this parameter throughout time. We also observe that demographic analyses like those performed in the present study are sensitive to sample size, sequence length, and the number of markers used (Heled and Drummond 2008). The evolutionary history of the populations of *Zamia paucijuga* is, in general, clearly consistent with previous studies in other seed plant taxa that suggest a post-Pleistocene population expansion, but the results inferred here are a first estimate and need further corroboration. Equivalent studies in other Mexican Zamiaceae species could also help to establish the extent to which this historical demographic pattern is found in other seed plant families or genera occurring in the region.

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