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ZAMIA (ZAMIACEAE) PHENOLOGY IN A PHYLOGENETIC CONTEXT: DOES *IN SITU* REPRODUCTIVE TIMING CORRELATE WITH ANCESTRY?

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The Cycadales are a group of significant global conservation concern and have the highest extinction risk of all seed plants. Understanding the synchronisation of reproductive phenology of Cycadales may be useful for conservation by enabling the targeting of pollen and seed collection from wild populations and identifying the window of fertilisation to aid in the cultivation of Cycadales. Phenological data for 11 species of *Zamia* were gathered from herbarium specimens. Four phenological characters were coded with monthly character states. DNA was isolated and sequenced for 26S, *CAB*, *NEEDLY*, *matK* and *rbcL*, and a simultaneous phylogenetic analysis of phenology and DNA sequence data was carried out. Three major clades were recovered: a Caribbean clade, a Central American clade and a South American clade. Eight species showed statistically significant synchronisation in microsporangiate and ovulate phenological phases, indicating the time of fertilisation. Close reproductive synchronisation was consistently observed throughout the Caribbean clade (statistically significant in four of five species) but was less consistent in the Central American clade (statistically significant in one of two species) and South American clade (statistically significant in three of four species). Ultimately, phenology is shown to be a potential driver of speciation in some clades of *Zamia* and in others to be a potential barrier to hybridisation.

Keywords. Circular statistics, Cycadales, dioecy, phenograms. phenology, reproductive timing, *Zamia*.

Introduction

Shifts in the reproductive timing of plants have attracted increasing interest among researchers. For example, climate change is considered to have a significant effect on the phenological cycles and synchrony of plants, which could ultimately affect the diversity and abundance of plant species (Gordo & Sanz, 2010; Ovaskainen *et al.*, 2013).

The Cycadales are a group of significant global conservation concern and have the highest extinction risk of all seed plants (IUCN, 2015). Asynchronous phenological

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patterns can be problematic in small isolated populations, negatively affecting the fitness of these populations and threatening their survival (Schneider *et al.*, 2002; López-Gallego, 2007; Reed *et al.*, 2013). Understanding the reproductive phenology of plants can further our understanding of reproductive isolation mechanisms, which can, in turn, be used directly in conservation by aiding in the cultivation and propagation of *ex situ* collections (Gorelick & Marler, 2012). Cycadales cannot self-pollinate, because they are dioecious and therefore require reproductive synchronisation. Dioecy in species means obligate outcrossing, and synchronisation is essential. This may be problematic in smaller populations, and exacerbated by their push–pull pollination mechanism (an odour-mediated interaction between cycads and their pollinators) that is essential for pollination to occur (Terry *et al.*, 2005; Suinyuy *et al.*, 2009, 2013). Therefore the phenology of Cycadales is particularly biologically significant.

Zamia L. is the largest genus in Zamiaceae, consisting of more than 70 species (Osborne *et al.*, 2012) that are widely distributed throughout the Neotropics. *Zamia* inhabits rain forest, coastal swamps, deserts, beaches and epiphytic habitats. It is perhaps the most morphologically and ecologically diverse genus of the extant Cycadales (Jones, 2002). Wild populations of *Zamia fairchildiana* L.D.Gómez produce a flush of leaves during and after the wet season (López-Gallego & O'Neil, 2010). In *Zamia*, a new flush of leaves often coincides with the production of strobili (Prado *et al.*, 2014). Under certain environmental conditions, such as water stress, zamias have been known to abort strobili to favour the production or retention of leaves (Negrón-Ortiz & Breckon, 1989). However, plants that are in an active state of seed maturation initiate the senescence-leaves in unfavourable conditions and retain the strobili to allow the maturation of seeds (Negrón-Ortiz and Breckon, 1989).

Wild *Zamia* populations often consist of a large number of mature plants, yet only a small number might participate in a given reproductive event (Ornduff, 1987, 1992). Microsporangiate *Zamia* often produce multiple strobili within a single growing season, presumably because microsporangiate strobili require fewer resources than megasporangiate strobili (Tang, 1990). Microsporangiate strobili produced in multiples often mature at different times (Schutzman, 2004). Clark & Clark (1987, 1988) produced one of the most detailed studies of cycad phenology, focused on *Zamia skinneri* Warsz. (now regarded as a synonym of *Z. neurophyllidia* D.W.Stev.), with observations of more than 200 individuals over a 6-year period. Clark & Clark (1987, 1988) found that microsporangiate plants began to release pollen before receptivity of megasporangiate plants, with larger microsporangiate plants being able to shed pollen over a longer period of time; that 26% of adult plants produced megasporangiate strobili within the 6-year period; and that rates of leaf production were negatively correlated with the production of strobili, demonstrating the drain on resources that the reproductive process imposes.

Clugston *et al.* (in press) collected data from both wild-collected herbarium specimens and living collections of 22 species of *Zamia* and found significant variation in phenological patterns among species as well as between wild and cultivated plants

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of the same species. The pollination mechanism in *Zamia* has been well documented (Norstog *et al.*, 1986; Tang, 1987, Norstog & Fawcett, 1989; Vovides *et al.*, 1993; Terry *et al.*, 2012) and is often directly linked to the life cycle of the beetle pollinators (Curculionidae). This suggests that timing is an important factor: the dehiscence of the pollen sacs must coincide with the opening of the megasporophylls and the secretion of pollination droplets for fertilisation to occur (Donaldson, 1997; Terry *et al.*, 2007). Some species of *Zamia* share the same pollinator (Blake & Holzman, 2012) and occur within a small geographical range. In these species, asynchronous reproduction is a potential prezygotic isolation mechanism (Clugston *et al.*, in press).

Our aim in doing this study was to provide insight into the phenology of wild *Zamia* by analysing reproductive phenological phases in the context of a phylogeny inferred from nuclear, plastid and mitochondrial DNA. In doing so, we assume that phenology is a heritable character (i.e. phenological synapomorphies are a result of common ancestry).

We hoped to answer a number of questions. First, are phenological data more or less consistent with phylogeny? Second, is it the microsporangiate or the megasporangiate phase that drives evolutionary changes in the phenology of *Zamia*? Third, is phenology a barrier to hybridisation or a driver of speciation and radiation?

Materials and Methods

Phenology

Phenological observations were obtained from herbarium specimens of known wild collections of 11 species of *Zamia* L., i.e. *Z. acuminata* Oerst. ex Dyer, *Z. angustifolia* Jacq., *Z. erosa* O.F.Cook & G.N.Collins, *Z. integrifolia* L.f., *Z. lecointei* Ducke, *Z. manicata* Linden ex Regel, *Z. muricata* Willd., *Z. neurophyllidia* D.W.Stev., *Z. pumila* L., *Z. pygmaea* Sims and *Z. roezlii* Linden, and two outgroup taxa, *Ceratozamia robusta* Miq. and *Microcycas calocoma* (Miq.) A.DC. These observations were gathered from 25 herbaria (AAH, AAU, AHUC, B, BM, COAH, COL, CR, E, F, FTG, GH, HUA, JBSD, K, LE, MGR, MO, NY, P, PENN, SEL, U, US and VEN; herbarium codes follow Thiers *et al.*, continuously updated). All herbarium specimens were verified against relevant floras and checklists (Vovides, 1992; Stevenson, 2004; Nicolalde-Morejón *et al.*, 2009; Osborne *et al.*, 2012). Specimens examined are listed in Appendix 1. Each specimen was categorised into one of the following four states: 1, closed pollen (condensed central axis, closed microsporangia with included microspores); 2, open pollen (OP; elongated central axis, dehisced microsporangia, free microspores); 3, early ovule (EO; immature megagametophyte, ovulate cone open and receptive); or 4, late ovule (LO; mature megagametophyte and degraded sarcotesta; seeds lacking evidence of sarcotesta were excluded). Phenological phases were recorded as days of the year and then converted to angles before analysis. Statistical analyses and visualisations were carried out using R framework 3.01 (R Core Team, 2013). The Circular Statistics package version 0.4-3 (Agostinelli & Lund, 2011) was used to plot

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rose diagrams ('phenographs' *sensu* Griffith *et al.*, 2012) and to calculate Wallraff test values (Wallraff, 1979).

Estimation of relationships

DNA isolation and sequencing of the large ribosomal subunit (26S; high-copy nuclear), chlorophyll a/b–binding protein (*CAB*; single-copy nuclear, chloroplast expressed), maturase K (*matK*; plastid), *NEEDLY* (single-copy nuclear) and the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*; plastid) follow Gorelick *et al.* (2014). Finished sequences were aligned with MUSCLE 3.8 (Edgar, 2004). Inferred insertions or deletions were coded as binary characters using simple indel coding (Simmons & Ochoterena, 2000), as implemented in 2matrix (Salinas & Little, 2014). Sequences and voucher information were archived in GenBank (Appendix table 1, Appendix 2). For each species, the dates of closed microsporangia, OP, EO and LO were coded as four characters with monthly character states. The circular relationship among phenological character states was represented using a symmetrical matrix (Sankoff, 1975), with a maximum distance of 6 steps between states (e.g. a change from November to January was counted as 2 steps, not 10). A simultaneous analysis of molecular data and phenological characters was conducted using the implicit enumeration option in TNT 1.1 (Goloboff *et al.*, 2008). Clade support was assessed using 1000 jackknife replicates (Farris *et al.*, 1996). Each resampled matrix was searched using implicit enumeration. The strict consensus of each jackknife replicate was used to calculate the jackknife frequency of each clade in the strict consensus of the original data.

Synchronisation of open pollen and early ovule phases

The Wallraff test was used to assess the statistical significance of differences in OP and EO timing within and among species. The method of Benjamini & Hochberg (1995) was used to correct for multiple comparisons. To visualise the synchronisation of the OP and EO phases, data from four species with the largest numbers of phenological observations (*Zamia integrifolia*, *Z. lecointei*, *Z. pumila* and *Z. pygmaea*) were plotted using the Histogram function of Microsoft Excel 2010 (Microsoft Corp.) with 20 degree categories.

Results

Phenology

Figures 1–4 show phenographs for 11 species of *Zamia* and four phenological phases. Table 1 shows the total number of phenological observations, the median observation month and the interquartile range of the median observation for each species. Here, species with 10 or more phenological observations are described, because species with fewer phenological observations are more likely to result in phenological mismatch and

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TABLE 1. Phenological observations for all 11 species of *Zamia*, showing the total number of observations per species

Species	No. of observations*	Median observation	Interquartile range
Closed pollen			
Z. acuminata	**10**	January	January
Z. angustifolia	5	July	May to December
Z. erosa	2	April	March to May
Z. integrifolia	**16**	November	June to November
Z. lecointei	**11**	July	April to November
Z. manicata	1	February	February
Z. muricata	5	July	April to August
Z. neurophyllidia	4	September	August to October
Z. pumila	**11**	January	January
Z. pygmaea	2	July	April to September
Z. roezlii	2	March	March
Open pollen			
Z. acuminata	3	January	January
Z. angustifolia	6	May	March to May
Z. erosa	8	March	February to April
Z. integrifolia	**30**	March	February to June
Z. lecointei	**13**	September	April to November
Z. manicata	5	December	December
Z. muricata	5	April	April to June
Z. neurophyllidia	2	August	July to September
Z. pumila	**30**	March	March
Z. pygmaea	**13**	March	February to May
Z. roezlii	5	February	February to March
Early ovule			
Z. acuminata	4	January	January
Z. angustifolia	**10**	April	February to May
Z. erosa	8	March	February to April
Z. integrifolia	**55**	April	March to June
Z. lecointei	**10**	July	May to October
Z. manicata	**10**	March	February to April
Z. muricata	6	May	April to August
Z. neurophyllidia	3	August	May to October
Z. pumila	**32**	April	March to October
Z. pygmaea	**27**	March	March to August
Z. roezlii	4	March	March
Late ovule			
Z. acuminata	3	January	January
Z. angustifolia	5	June	June
Z. erosa	**10**	February	February to June
Z. integrifolia	**26**	May	March to July
Z. lecointei	**11**	July	April to September
Z. manicata	**11**	October	July to December
Z. muricata	**20**	April	February to July
Z. neurophyllidia	**11**	January	January to September
Z. pumila	**36**	July	March to October
Z. pygmaea	**14**	June	March to October
Z. roezlii	**12**	March	March to June

*Ten or more observations are highlighted in bold.

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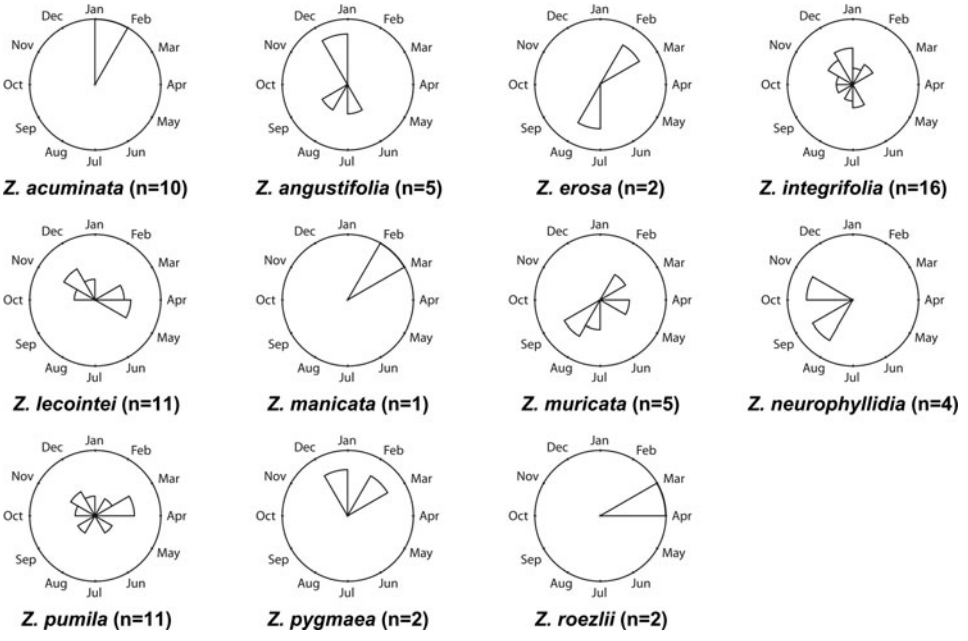

FIG. 1. Phenographs of the closed pollen phenological phase for data from 11 species of *Zamia* gathered from herbarium specimens.

less accurate results, although these species still provide insights into the phenological timing of a species.

Closed pollen. The closed pollen phase, which is defined by the pre-elongation of the central axis and the prerelease of pollen, is shown in phenographs of 11 *Zamia* species in Fig. 1. The corresponding phenological observation data are shown in Table 1. *Zamia acuminata*, *Z. integrifolia*, *Z. lecointei* and *Z. pumila* all have 10 or more observations (Table 1) and therefore greater statistical support. Both *Zamia pumila* and *Z. integrifolia* show a rather extended phenological cycle, with observations almost year round, and with the highest recorded frequencies of observations among all the phenographs. The median observations for *Zamia pumila* and *Z. integrifolia* differ from each other, however.

Open pollen. The OP phase is defined by the release of pollen and the elongation of the central axis (Table 1 and Fig. 2). *Zamia integrifolia*, *Z. lecointei*, *Z. pumila* and *Z. pygmaea* all have 10 or more observations, with *Z. integrifolia* having the greatest number at 30. However, *Z. lecointei* has the greatest range of phenological observations based on its interquartile range.

Early ovule. The EO phase is defined by the point of ovulate receptivity (Table 1 and Fig. 3). *Zamia angustifolia*, *Z. integrifolia*, *Z. lecointei*, *Z. manicata*, *Z. pumila* and *Z. pygmaea* all have more than 10 observations, and *Z. integrifolia* has the greatest number

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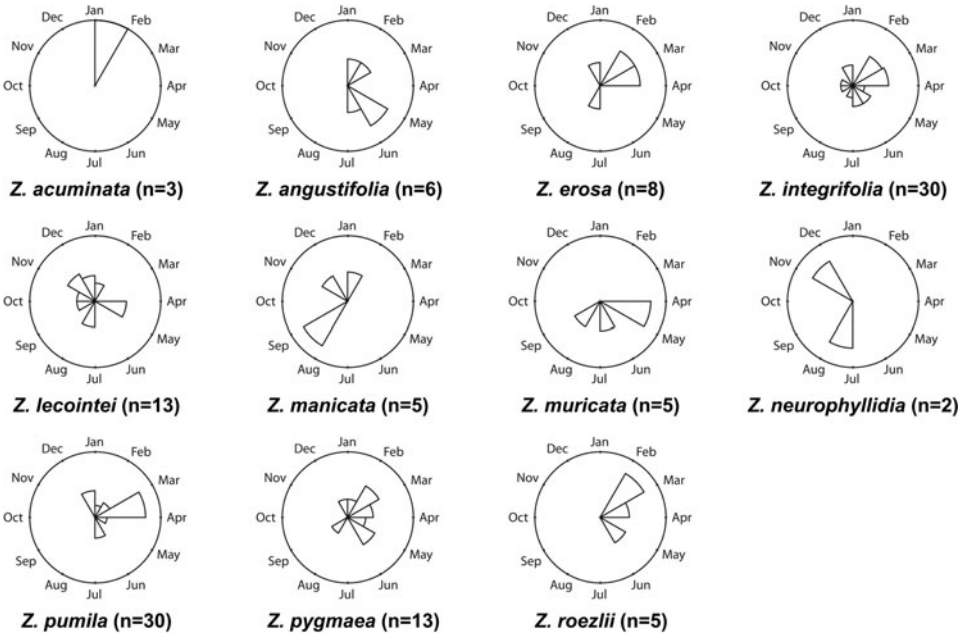

FIG. 2. Phenographs of the open pollen phenological phase for data from 11 species of *Zamia* gathered from herbarium specimens.

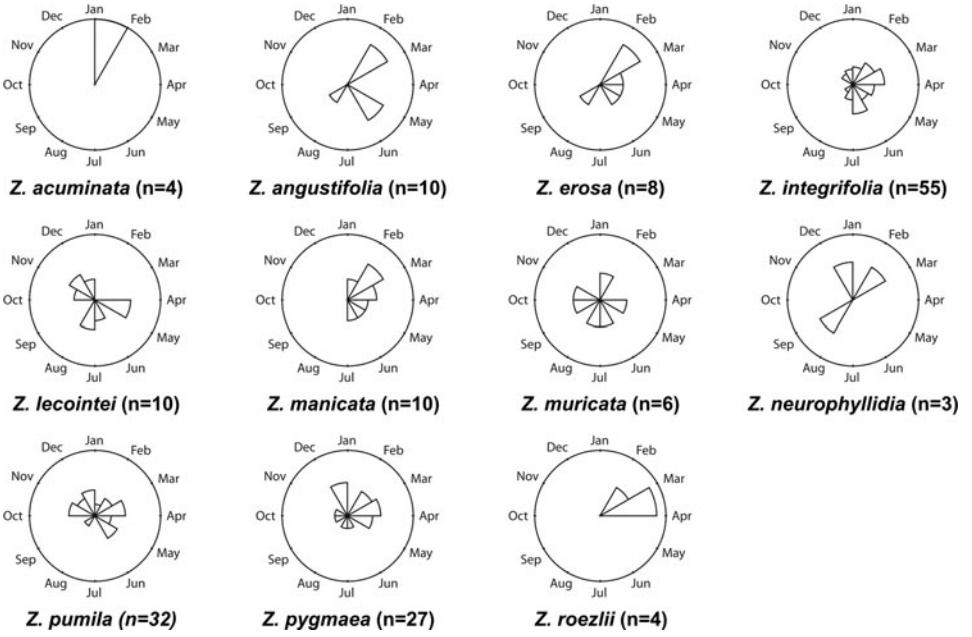

FIG. 3. Phenographs of the early ovule phenological phase for data from 11 species of *Zamia* gathered from herbarium specimens.

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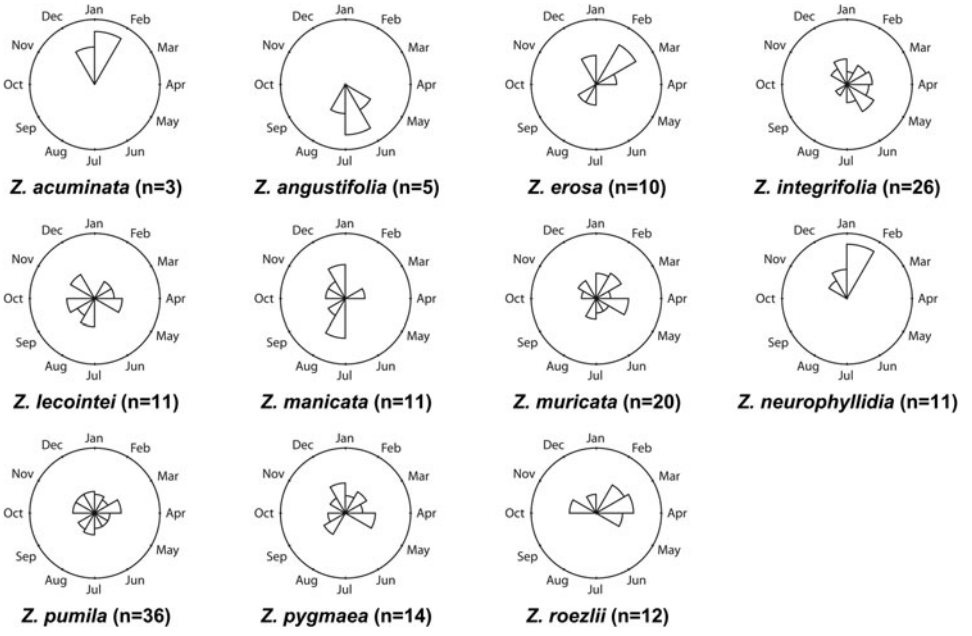

FIG. 4. Phenographs of the late ovule phenological phase for data from 11 species of *Zamia* gathered from herbarium specimens.

at 55. *Zamia pumila* has the greatest range of phenological observations from March to October.

Late ovule. The LO phase (Table 1 and Fig. 4) is defined by the hardening of the sclerotesta and the release of seeds. *Zamia erosa*, *Z. integrifolia*, *Z. lecointei*, *Z. manicata*, *Z. muricata*, *Z. neurophyllidia*, *Z. pumila*, *Z. pygmaea* and *Z. roezlii* all have more than 10 observations, with *Z. pumila* having the greatest number.

Phylogeny

The 180 parsimony-informative characters are unevenly distributed among the five markers: 26S, 69; *matK,* 45; *NEEDLY*, 33; *rbcL*, 21; and *CAB*, 8. The strict consensus of the 12 most parsimonious trees from the simultaneous analysis (Fig. 5) has three well-supported clades, with the most parsimonious trees having a consistency index of 0.850 and a retention index of 0.873. The mean jackknife support value is 60.9%. The 'Caribbean clade' has a 99% jackknife support value and contains five species: *Zamia integrifolia*, *Z. pumila*, *Z. pygmaea*, *Z. erosa* and *Z. angustifolia*. The 'Central American clade' contains two species: *Zamia neurophyllidia* and *Z. acuminata*, united with a 66% jackknife support value. The 'South American clade' has an 88% support value and contains four species: *Zamia roezlii*, *Z. manicata*, *Z. muricata* and *Z. lecointei*.

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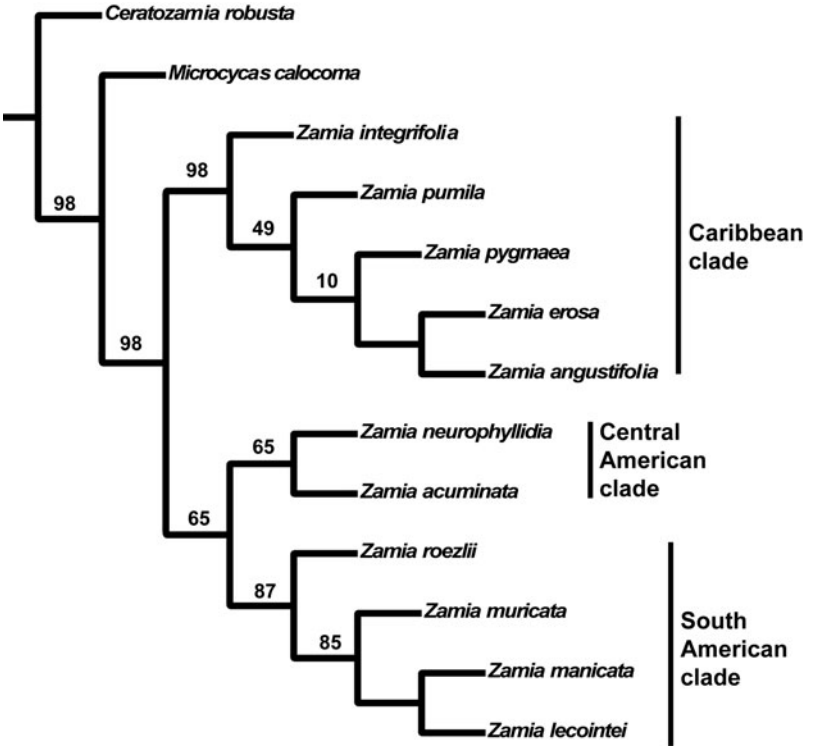

FIG. 5. Parsimony tree of *Zamia* generated from DNA sequence data from the large ribosomal subunit (26S; high-copy nuclear), chlorophyll a/b–binding protein (CAB; single-copy nuclear, chloroplast expressed), maturase K (*mat*K; plastid), *NEEDLY* (single-copy nuclear) and the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L; plastid). The tree is a strict consensus of 12 trees, with a consistency index of 0.850 and a retention index of 0.873. Jackknife support values are shown at nodes with a mean group support of 60.9%.

Phenological character optimisation. For discussion, a single tree from the simultaneous analysis was randomly selected from the 12 most parsimonious trees, and phenological data for closed pollen, OP, EO and LO were optimised (Sankoff, 1975) onto the nodes (Fig. 6). Fig. 6 indicates that the EO phase is 2 (February) for all species of *Zamia*, and there are resulting parallel shifts in OP phase to 2 on both of the diverging branches. Later, there is a change in EO phase to 4 (April) and 6 (June), and again a change in OP phase to include 4.

Caribbean clade. The Caribbean clade has a number of synapomorphies in the four phenological characters. The reconstruction indicates that there is a change in OP timing in the clade, which results in synchronisation of the OP and EO phases. *Zamia erosa* and *Z. angustifolia* show a further change in the observations for the LO phase during July and December.

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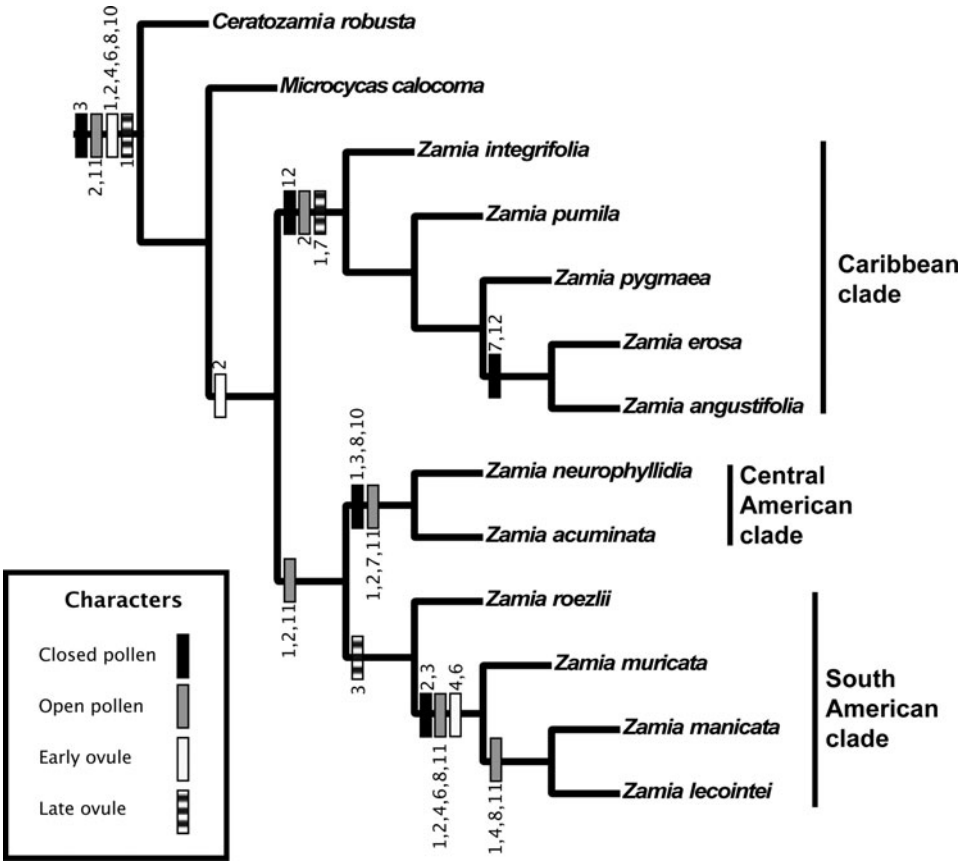

FIG. 6. Phylogenetic tree showing unambiguous synapomorphic character state changes for four phenological characters (autapomorphies are not shown). Numbers at the site of each character state change indicate month(s) of the year. All phenological characters have a consistency index of 0.850 and a retention index of 0.873.

Central American clade. Zamia neurophyllidia and *Z. acuminata* are synapomorphic in OP and closed pollen. OP and EO phases are synchronised during February, but the OP phase is also active during January, July and November (Fig. 6).

South American clade. The South American clade has a synapomorphy in the LO phase. The *Zamia muricata* subclade has an increase in the OP during April, June and August, whereas *Z. lecointei* and *Z. manicata* show a slight reduction in observations during February (Fig. 6).

Synchronisation

Zamia integrifolia, *Z. lecointei*, *Z. pumila* and *Z. pygmaea* had the highest number of observations for the EO and OP phases of all 11 species of *Zamia*, with the

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four species examined in detail (Figs 7–10) being synchronised between EO and OP phases.

Table 2 shows the significance of OP and EO, whether they show asynchronisation in their phenological pattern for each species and whether the species are significantly differentiated based on phenological data. Significant ($P < 0.001$) differences were found in the timing of OP and EO in *Zamia pumila* ($P = 0.0002$), indicating asynchronisation in phenology.

Significant ($P < 0.01$) differences were noted between species in the timing of the OP and EO phases; *Zamia acuminata* differs significantly from all species, with the exception of *Z. neurophyllidia* and *Z. roezlii*. *Zamia pumila* is significantly different from *Z. acuminata* ($P = 0.001$), *Z. integrifolia* ($P = 0.003$) and *Z. lecointei* ($P = 0.005$); however, no significant infraspecific differences were found.

Significant infraspecific differences ($P < 0.05$) were found in OP and EO timing for both *Zamia acuminata* ($P = 0.02$) and *Z. roezlii* ($P = 0.02$). Significant interspecific differences were found between a number of species: *Zamia acuminata* differs significantly from *Z. erosa* ($P = 0.01$), *Z. lecointei* ($P = 0.01$), *Z. manicata* ($P = 0.01$), *Z. muricata* ($P = 0.02$), *Z. neurophyllidia* ($P = 0.046$) and *Z. roezlii* ($P = 0.03$). *Zamia angustifolia* has borderline difference from *Z. roezlii* ($P = 0.051$), which is also the case for *Z. integrifolia* ($P = 0.03$), *Z. manicata* ($P = 0.01$), *Z. muricata* ($P = 0.01$) and *Z. pygmaea* ($P = 0.02$), which in turn differ significantly from *Z. roezlii*. *Zamia erosa* differs significantly in its OP and EO timing from *Z. angustifolia* ($P = 0.048$), *Z. pumila* ($P = 0.03$) and *Z. roezlii* ($P = 0.02$). *Zamia muricata* differs significantly from *Z. angustifolia* ($P = 0.04$) and *Z. roezlii* ($P = 0.01$). The OP and EO of *Z. neurophyllidia* differ significantly from those of *Z. acuminata* ($P = 0.03$) and *Z. roezlii* ($P = 0.046$). *Zamia pumila* differs significantly from *Z. angustifolia* ($P = 0.01$), *Z. muricata* ($P = 0.02$) and *Z. pygmaea* ($P = 0.02$). *Zamia roezlii* differs significantly in its OP and EO from those of *Z. acuminata* ($P = 0.01$), *Z. angustifolia* ($P = 0.04$) and *Z. pumila* ($P = 0.01$).

Discussion

Strengths and weaknesses of the data

The data were gathered from 1265 herbarium specimens providing phenological data and represent one of the largest and most complete phenological datasets available for *Zamia* even when other in-depth studies (e.g. Clark & Clark, 1987; Ornduff, 1987; Clark & Clark, 1988; López-Gallego & O'Neil, 2010; Ornduff, 1992; and Griffith *et al.*, 2012) are included. The OP and closed pollen can both be clearly seen on herbarium specimens because of the elongation of the central axis of the microsporangiate strobili and the release of pollen (dehisced microsporangia). In contrast, EO was particularly difficult to see in herbarium specimens, because pollen drops are not preserved in dried specimens, and for this reason the event was recorded as an immature megagametophyte phase. The LO phase was easy to confirm because of the presence of a mature megagametophyte.

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TABLE 2. Statistical differences (*P* values) between the timing of open pollen and early ovule phases in *Zamia* species, assessed via the Wallraff test*

	Caribbean clade					Central American clade		South American clade			
Species	*Z. angustifolia*	*Z. erosa*	*Z. integrifolia*	*Z. pumila*	*Z. pygmaea*	*Z. acuminata*	*Z. neurophyllidia*	*Z. lecointei*	*Z. manicata*	*Z. muricata*	*Z. roezlii*
Z. angustifolia	0.74	0.80	0.73	0.15	0.82	**0.008**	1	0.28	0.83	0.42	0.051
Z. erosa	**0.048**	0.29	0.35	**0.03**	0.31	**0.005**	0.41	**0.050**	0.79	0.12	**0.016**
Z. integrifolia	0.45	0.91	0.73	0.09	0.74	**0.001**	0.66	0.08	0.57	0.25	**0.03**
Z. pumila	**0.015**	**0.015**	**0.003**	**0.0002**	**0.02**	**0.001**	0.23	**0.0002**	0.15	**0.02**	0.56
Z. pygmaea	0.31	0.94	0.31	0.06	0.32	**0.003**	0.74	0.11	0.62	0.22	**0.017**
Z. acuminata	**0.009**	**0.014**	**0.009**	**0.008**	**0.02**	**0.018**	**0.046**	**0.011**	**0.011**	**0.019**	**0.03**
Z. neurophyllidia	0.07	0.29	0.83	1	0.67	**0.03**	0.55	1	0.19	1	**0.046**
Z. lecointei	1	0.51	0.23	0.92	0.55	**0.003**	0.64	0.29	0.21	0.73	**0.017**
Z. manicata	0.38	0.77	0.88	0.50	0.78	**0.009**	0.45	0.33	0.17	0.46	**0.011**
Z. muricata	**0.04**	0.77	0.58	0.23	0.94	**0.009**	0.88	0.18	0.46	0.20	**0.011**
Z. roezlii	**0.04**	0.14	0.19	**0.011**	0.17	**0.010**	0.65	0.07	0.27	0.14	**0.02**

*Significant differences (shown in bold) indicate statistically significant asynchronous event timing.

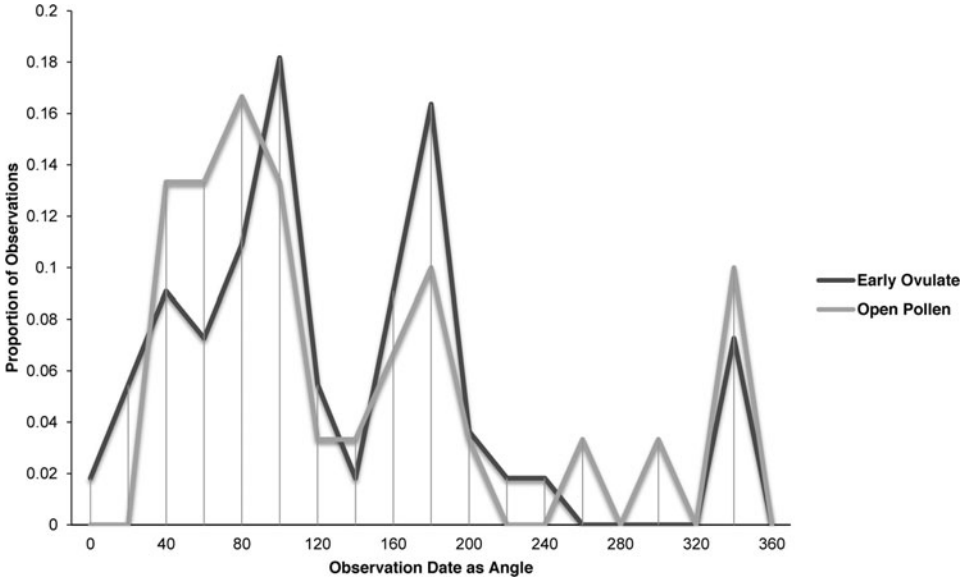

FIG. 7. *Zamia integrifolia*: proportion of phenological observations of cones at the open pollen and early ovule phases during the year.

Importance of synchronisation of open pollen and early ovule phases

Synchronisation between OP and EO phases is clearly manifested in *Zamia integrifolia* (Fig. 7), *Z. lecointei* (Fig. 8), *Z. pumila* (Fig. 9) and *Z. pygmaea* (Fig. 10). This indicates that data gathered from herbarium specimens can be used to understand synchronisation of the EO and OP phases in closely related species with little morphological differentiation, and can therefore also be used as an aid for species classification. Ultimately, these data provide insights into whether phenology is a barrier to hybridisation or a driver in the process of speciation. In fact, the phenological data show distinctive peaks in both OP and EO synchronisation, which indicate the window of fertilisation, because an asynchronous pattern would result in phenological mismatches that would prevent fertilisation in a species. There are similarities in the phenology of *Zamia* species that reside in the same clade, meaning that they would be more likely to hybridise if they occur in the same area. Species in the Caribbean clade share similarities in their geographical ranges, occurring in Florida, the Bahamas and several islands in the Greater Antilles (Osborne *et al.*, 2012), and in some cases also share pollinators (Eckenwalder, 1980). This could allow for hybridisation within the Caribbean clade, providing there is no constraint in geography with the pollinator. This indicates that, in the Caribbean clade, phenology could be acting as a driver for speciation through the hybridisation of closely related species. In contrast, species in the Central and South American clades show lower levels of synchronisation within the clades. However, there is little to no reproductive intraspecific synchronisation

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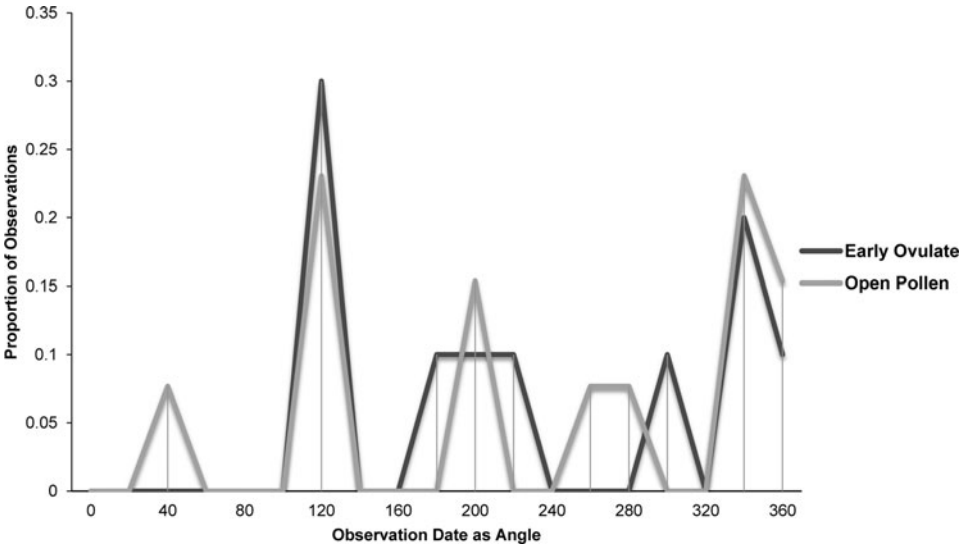

FIG. 8. *Zamia lecointei*: proportion of phenological observations of cones at the open pollen and early ovule phases during the year.

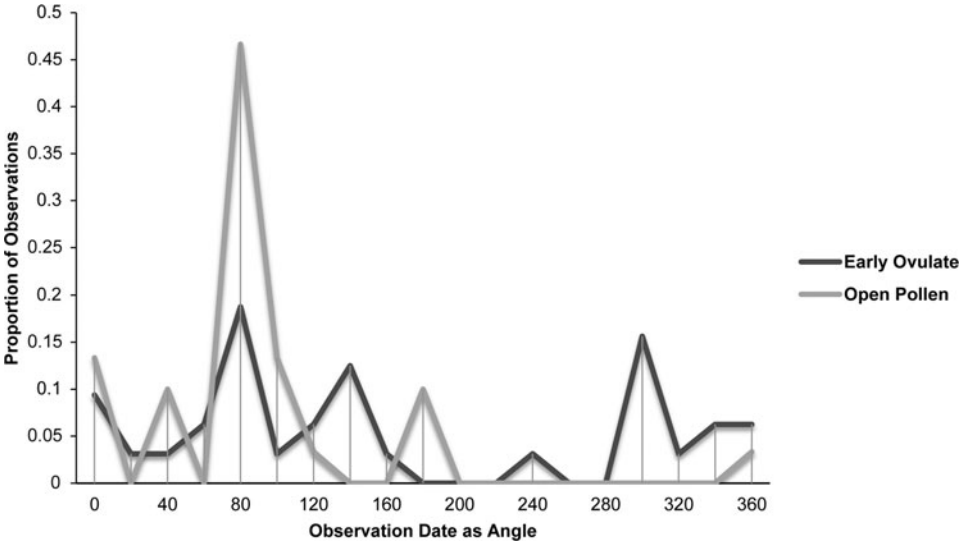

FIG. 9. *Zamia pumila*: proportion of phenological observations of cones at the open pollen and early ovule phases during the year.

in these two clades, and species have a greater level of geographical differentiation (Osborne *et al.*, 2012; Clugston *et al.*, in press). This indicates that within the Central and South American clades there are lower chances of interspecific hybridisation

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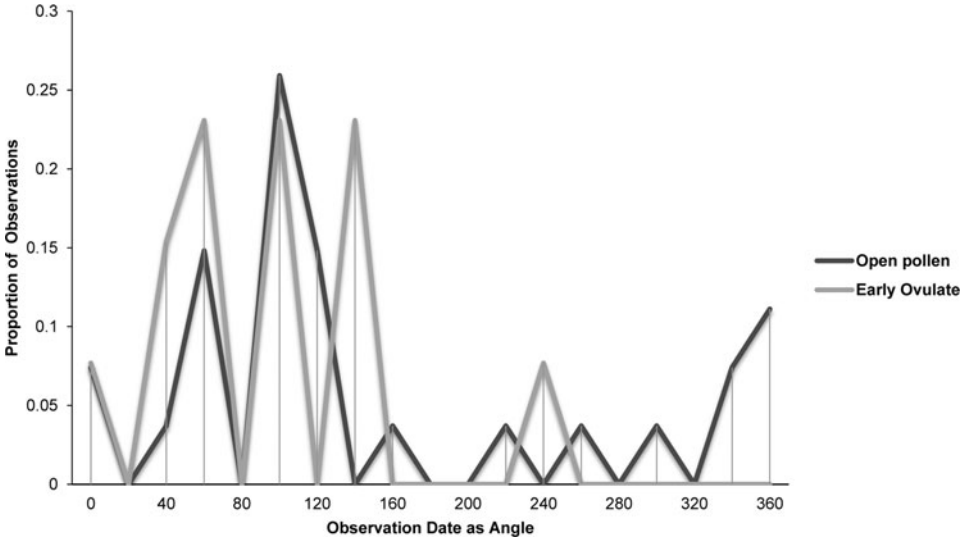

FIG. 10. *Zamia pygmaea*: proportion of phenological observations of cones at the open pollen and early ovule phases during the year.

events occurring, indicating that both phenology and geography act as barriers to hybridisation.

Zamia acuminata, *Z. pumila* and *Z. roezlii* show significant interspecific differentiation between the OP and EO phases. This can be explained in both *Zamia acuminata* and *Z. roezlii*, because both species have five or fewer phenological observations of OP and EO. Fewer phenological observations of both species provide less statistical support and a higher chance of phenological mismatches. With a greater number of phenological observations, *Zamia acuminata* and *Z. roezlii* should show less statistical significance between the OP and EO and a lower chance of phenological mismatches. However, *Zamia pumila* has more than 30 observations of both OP and EO, meaning greater statistical support. The question remains as to why there is significant differentiation between the OP and EO phases in this species. *Zamia pumila*, as currently circumscribed (Osborne *et al.*, 2012), has a wide distribution, including Cuba, Hispaniola (Dominican Republic only) and Puerto Rico, each with similar climatic conditions. The disjunct distribution of the species on three separate islands could also indicate potential differences in the pollinators and seasonality of strobilus production times, indicating a high chance of phenological mismatch in disjunct populations.

Phenology and climate

Zamia lecointei and *Z. pumila* share the same overlap between the OP and EO at about 1 month, leaving only a small window for fertilisation to occur. The phenological

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patterns of many plants species are strongly correlated with environmental factors such as precipitation, temperature and carbon dioxide levels (Forkner *et al.*, 2008). Therefore changes in climate may influence phenological patterns of *Zamia*, resulting in a phenological cycle that has become differentiated from that of the pollinators. This would directly affect the period of fertilisation and thereby population fitness and resilience. Reed *et al.* (2013) found that phenology patterns of plants can be shifted in response to climate change. This shift in phenology often results in asynchronous patterns that may ultimately affect plant–pollinator interactions (Willmer, 2012). The often short period for pollination to occur in some species of *Zamia* increases the likelihood that they will develop asynchronous phenological cycles as a result of environmental changes. Cycads often have species-specific pollinators that are directly involved in their push–pull pollination system (Suinyuy *et al.*, 2009, 2013). The pollination system of cycads requires perfect synchronisation between the EO and OP, and depends heavily on the push–pull system and environmental factors, which could be affected by the loss of a pollinator or changes in environmental conditions such as precipitation or drought. *Zamia pumila* has a more extended phenological cycle and has highly significant differences in its OP and EO phases that are more prone to phenological mismatch.

Seasonal environmental changes and mast strobilus production

Seasonal and environmental changes influence the cyclical production of strobili in cycads (Marler & Niklas, 2011). Mast strobilus production, in which many individuals in a population simultaneously produce strobili, is reported in *Macrozamia* Miq. (Ballardie & Whelan, 1986), *Encephalartos* Lehm. (Donaldson, 1993, Suinyuy *et al.*, 2009) and *Zamia* (Negrón-Ortiz & Breckon, 1989). Tang (1990) found that, after fire, 87% of mature *Zamia pumila* plants participated in a mast seeding event. The effects of fire and mast seed production have also been recorded in *Encephalartos* (Grobbelaar *et al.*, 1989), *Dioon* Lindl. (Vovides, 1990) and *Macrozamia* (Grove *et al.*, 1980).

Phenology and phylogeny

Eckenwalder (1980) observed many morphological similarities among the species in the Caribbean clade, which greatly influenced his classification of the genus, implying that the Integrifolia group is a single morphologically plastic species. However, more recent studies have indicated that the Integrifolia group can be split into the nine or more distinct species that are currently recognised in the clade (Stevenson, 1987; Meerow *et al.*, 2012). *Zamia integrifolia* and *Z. pumila* have the greatest number of phenological observations in our dataset, and both species have a large geographical range. Therefore variability in their phenological patterns is unsurprising given the many different environments across their ranges. The EO of both species is scattered throughout the year, indicating a long cycle of receptivity, but the OP has a much more defined cycle, which indicates a shorter window for fertilisation to occur.

Species of *Zamia* in the Caribbean clade share a close molecular relationship, which is further supported by phenological similarities and synchronisation of OP and EO phases, with the exception of *Z. angustifolia*. When compared with other members of the Caribbean clade, *Zamia angustifolia* has a limited geographical range, with populations in the Bahamas and Cuba (Calonje *et al.*, 2013). *Zamia angustifolia* populations are disjunct from the other species (*Z. integrifolia* and *Z. pumila*) that occur in the same regions. *Zamia angustifolia* is also phenologically isolated from closely related species; this further suggests that there is little genetic exchange between *Z. angustifolia* and the other species of *Zamia.* However, perfect phenological synchronisation was recently noted between *Zamia angustifolia* and *Z. integrifolia* in the Bahamas, where microsporangiate strobili were found releasing pollen at the same time as mature seeds were found on megasporangiate strobili (M. Calonje, Montgomery Botanical Center, pers. comm., 30 June 2015).

The phenological patterns of the South American clade show higher levels of differentiation among characters, perhaps because of the much lower availability of material. However, species within the South American clade are in close geographical proximity and resolve in the same clade, indicating that phenology is consistent with the molecular data. The members of the South American clade share few similarities in their phenology. This close geographical proximity indicates that a number of environmental conditions and triggers (e.g. day length) may be shared among species, whereas others (e.g. precipitation) may be variable (Clugston *et al.*, in press); this indicates that strobilus production may be triggered by different environmental conditions, which differentiate the species phenologically. There are few similarities in the OP timing of *Zamia manicata*, *Z. muricata* and *Z. roezlii*. This segregates the month when pollen release occurs for each species, thereby limiting cross-fertilisation.

Species in the Caribbean clade show little variation in their phenological patterns and have a closely synchronised relationship between OP and EO phases. The synchronisation of phenological characters in closely related species is consistent with our assumption that phenology is inherited in this group. There is a distinct relationship between phylogeny and phenology in both the Caribbean clade and the South American clade, which further supports a close relationship between species in the two clades. The consistency index (0.850) of the most parsimonious trees confirms that there is a strong relationship between phenology and phylogeny in *Zamia.* This indicates that species that have a close genetic relationship also share similarities in their phenology.

Directions for further research

Using statistical approaches to build on the earlier cycad phenology work of Clark & Clark (1987, 1988) and Ornduff (1987, 1992) is an effective means to understand phenological patterns in *Zamia* and other members of the Cycadales (Griffith *et al.*, 2012; Clugston *et al.*, in press). One question that still remains to be answered is how factors such as human-mediated habitat fragmentation and habitat loss affect cycad

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phenology. Climatic factors have been shown to affect phenological patterns in other plant groups (Walther *et al.*, 2002; Thuiller *et al.*, 2005; Forkner *et al.*, 2008; Reed *et al.*, 2013), but the effects that climate change may have on the phenological cycles of cycads are still largely unknown.

Our aim in doing this study was to provide insight into the phenology of wild *Zamia* by analysing reproductive phenological phases in the context of a phylogeny inferred from molecular data. We have shown that phenological data are consistent with phylogeny, and that the megasporangiate phase acts as a potential driver of evolutionary changes in the phenology of *Zamia.* Phenology is a potential driver of speciation in some clades of *Zamia*, whereas in others it is a potential barrier to hybridisation. To fully understand the phenological cycles of the Cycadales growing in the wild, further studies need to be completed into other genera of cycads using wild collected herbarium specimens and wild populations. Gaining insights into the phenology of cycads could provide a greater understanding of the relationships among closely related taxa. Phenological data can also be used to provide important insights into the cultivation of *ex situ* conservation collections of *Zamia* and other genera of Cycadales, by enabling better understanding of the timing of strobilus production and period of receptivity for the EO phase. This would guide targeted pollen collection and assisted pollination during that period. Understanding reproductive biology in this way can help with *ex situ* cycad conservation collections, which play a fundamental role in conservation by holding genetic reserves of wild populations (Calonje *et al.*, 2011). Furthermore, this study describes a model that could be applied to other plant groups and further shows the value of wild-collected herbarium specimens in understanding plant phenology.

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Appendix 1

Details of the herbarium specimens of the 11 Zamia *species examined in this study*

***Zamia acuminata* Oerst. ex Dyer**

Stevenson & *Valdespinos* 1147 (FTG), *Stevenson* 1147 (K & NY, 1989) and *Stevenson* DOL31757 (NY), DOL31758 (NY), DOL31759 (NY), DOL31761 (NY), DOL31762 (NY), DOL31767 (NY), DOL31767 (NY), DOL31783 (NY).

Zamia angustifolia Jacq.

Britton & *Millspaugh* 5418 (NY, 1907), *Britton* 408 (NY, 1905), *Bro. Leon* 18095 (GH, 1938), *Correll et al.* 42577 (FTG, 1974), *Correll* & *Kral* 42979 (FTG, 1974), *Correll* & *Evans* 43998 (FTG, 1974), *Correll* & *Hill* 45240 (NY, 1975), *Carabia* 14003 (B & NY, 1939), *Garcia* 1403 (NY, 1939), *Gleuient* 209 (NY, 1920), *Lopéz* 2970 (US, 1959), *Pollard* & *Palmer* 382 (US, NY, MO & GH, 1902), *Norstog* 730 (FTG, 1972), *Proctor* 36937 (FTG, 1977), *Wright* 597 (MO & P, GH, LE, PENN, 1856).

***Zamia erosa* O.F.Cook & G.N.Collins**

Axelrod 3393 (NY, 1991), *Britton* & *Hollick* 2042 (NY, 1908), *Britton* et al. 8903 (NY, 1925), *Carabia* 4102 (F,1940), *Proctor* 31556 (GH, 1970), *Read* and *Drummond* 76–640 (FTG, 1976), *Shafer* 1402 (NY, 1909), *Simson* 3816 (NY, 1966), *Turnbull* 14, 15, 16, 18, 19, 20, 21, 22, 23, 26, 27, 28 & 29 (NY, 2003), *Tryon* & *Tryon* 5491 (MO, 1956), *Tryon* & *Tryon* 5492 (MO, 1956), *Tryon* & *Tryon* 5495 (MO, 1956), *Tryon* & *Tryon* 5496 (MO, 1956), *Wright* 1463 (MO, 1859).

***Zamia integrifolia* L.f.**

Alexander (FTG, 1981), *Blanton* & *O'Neill* 6921 (MGR & PENN, 1931), *Brace* 3693 (NY, 1905), *Brace* 6990 (NY, 1907), *Brace* 6991 (NY, 1907), *Britton* & *Millspaugh* 2576 (NY, 1905), *Britton* & *Wilson* 5443 (NY, 1910), *Britton et al.* 5925 (NY, 1910), *Britton* et al. 1911 (US, 1911), *Britton* et al. 10233 (NY, 1911), *Bros. Leon* & *Alain* 18934 (GH, 1939), *Carabia* 1939 (F, 1939), *Carabia* 4102 (F, 1940), *Curtiss* 2676 (GH, 1911), *Collins*, 237 (NY, 1898), *Collins* 44017 (GH, 1917), *Cooley* et al. 9322 (GH, 1962), *Correll* & *Popenoe* 42713 (FTG, 1974), *Correll et al.* 47030 (FTG, 1976), *Fantz* 413 (FTG, 1979), *Fantz* 3904 (FTG, 1979), *Fantz* 4137 (FTG, 1979), *Godfrey* & *Redfearn* 52817 (GH, 1955), *Godfrey* & *Redfearn* 52834 (GH, NY, 1955), *Garber* (US, 1877), *Hamilton* 179 (NY, 1902), *Hanson et al.* 10748 (FTG, 1985), *Harbison* 57 (AAH, 1918), *Hawkes* 8098 (US, 1898), *Hill* 2334 (FTG, 1974), *Hill* 2513 (FTG, 1975), *Hill* 11090 (NY, 1982), *Howard et al.* 290 (AAH, 1950), *Haynes* JLH05-040 (FTG, 2005), JLH05-046, JLH05-054, JLH05-075, JLH05-106, JLH05-115, JLH05-130, JLH05-145, JLH05-197, JLH05-206, JLH05-217, JLH05-223, JLH05-235, JLH05-261, JLH05-270, JLH05-288 & JLH05-301, *Jack* 5962 (NY, 1927), *Jack* (AAH, 1932), *Jones* (US, 1920), *Landry* & *LaFrankie* 40 (GH, 1979), *Landry* & *LaFrankie* 42 (GH & MGR, 1979), *Landry* & *LaFrankie* 43 (GH & MGR, 1979), *Landry* & *LaFrankie*

44 (GH & MGR, 1979), *Landry* & *LaFrankie* 45 (AAH & MGR, 1979), *Landry* & *LaFrankie* 46 (GH & MGR, 1979), *Landry* & *LaFrankie* 47 (GH & MGR, 1979), *Landry* & *LaFrankie* 49 (GH, 1979), *Leon* 16294 (GH, 1935), *McFarlin* 6349 (MGR, 1931), *Mills* (US, 1926), *Norstog* 728 (FTG, 1972), *Norstog* 932 (FTG, 1972), *Northrop* & *Northrop* 550 (NY, 1890), *O'Neill* (US, 1929), *Ostenfeld* 155 (LE, 1922), *Pilsbey* (PENN, 1937), *Popenoe* 1704 (FTG, 1979), *Proctor* 31556 (NY, 2003), *Rolfs* (NY, 1904), *Safford* (NY, 1917), *Sargent* (AAH, 1886), *Seibert* 1416 (PENN, 1940), *Shafer* 911 (NY, 1903), *Shafer* 1406 (NY, 1909), *Simpson* 394 (US, 1891), *Small* & *Carter* 8909 (NY, 1910), *Smith* 522 (US & PENN, 1882), *Smith* (PENN, 1892), *Sturtevant* 14 (MGR, 1959), *Thorne* & *Benny* 44398 (NY, 1974), *Tracey* 9265 (BM, NY & US,1904), *Tryon* & *Tryon* 5498, 6497 (MO, 1956), *Turnbull* 30, 31, 33 & 35 (NY, 2003), U.O.S. 999 (PENN, 1892), *Van Hermann* 913 (NY, 1905), *Van Hermann* 1940 (F, 1940), *Walker* 1588 (PENN, 1940), *Walker* 1865 (PENN, 1940), *Woods, Coral* & *Rock* 5733 (NY, 1919), *Wright* 1463 (GH, NY & P, 1859).

***Zamia lecointei* Ducke**

Arbelá et al. 644 (HUA, 1996), *Bernal et al.* 1237 (COL, 1986), *Bernal et al.*1239 (COL, 1986), *Bernal et al.* 1240 (COL, 1986), *Cortes* & *Rodriguez* 611 (TARA 133) (COL, 1993), *Ducke* 914 (MO, 1941), *Duke* 915 (NY, AHUC & US, 1941), *Duke* 17889 (U & K 1922), *Duke* 17889 (K, 1929), *Duke* 17890 (U & US, 1925), *Galeano et al.* 661976 (NY, 1976), *Huber* & *Guanchez* 134690 (VEN, 1980), *Madison et al.* 6379 (SEL, 1978), *Plowman* & *Guánchez* 13767 (NY, 1984), *Plowman* & *Guánchez* 13768 (NY, 1984), *Schultes* 12101 (GH, 1951), *Schultes* 12465 (COL, 1951), *Schultes* 13511 (GH, 1951), *Schultes* 14640 (GH, 1951), *Schultes* & *Cabrera* 14956 (NY, 1952), *Schultes* 17663 (GH, 1952), *Schultes* & *Cabrera* 17663 (GH, 1952), *Steyermark* & *Bunting* 85607 (VEN, 1970), *Steyermark* & *Bunting* 102563 (K, US & VEN, 1970), *Wurdack* & *Adderley* 43526 (NY & VEN, 1959).

***Zamia manicata* Linden ex Regel**

Alzate & *Cardona* 1265-A (HUA, 2001), *Betancur et al.* 5999 (HUA, 1995), *Burch* 1112 (MO & US, 1966), *Callejas et al.* 5772 (NY, 1987), *Duke* & *Kirkbride Jr* 14000 (NY, 1967), *Fonnegra et al.* 6128 (HUA, 1996), *Fonnegra G. et al.* 6841 (HUA, 1999), *Forero* 1.577 (COL, 1976), *Forero* 1.614 (COL, 1976), *Gentry* & *Aguirre* 15190 (MO, 1975), *Hahn* 155 (MO, 1980), *Leon* 572 (MO, 1976), *Rentería et al.* 3943 (HUA, 1985), *Rentería et al.* 10981 (HUA, 1995), *Rentería et al.* 10929 (HUA, 1995), *Santa & Brand* 835 (HUA & COL 1983), *Schultes* 18640 (GH, 1953), *Schultes* 18694 (GH, 1953), *Schultes* 18676 (GH, 1953), *Stevenson et al.* 604 (NY, 1984), *Trujillo et al.* 18244 (NY, 1983), *Zuluaga R.* 1033 (COL, 1983).

***Zamia muricata* Willd.**

Allen 3355 (MO, 1945), *Auitegmita* & *Gammei* 1175 (VEN, 1952), *Bernardi* 6934 (VEN, 1959), *Callejas* & *Marulanda* 5911 (MO, 1988), *Curran* 1984 (NY, 1946), *Curran* 1813 (NY, 1952), *Diaz* & *Calderon* 357 (NY, 1991), *Folotats* 3047 (VEN, 1952), *Galitán et al.* 83 (COAH, 2002), *Gallejas* & *Marulanda* 5911 (HUA, 1988), *González* 1092 (VEN, 1977), *Haught* 2601 (COL & US, 1939), *Liesner et al.* 7823 (MO, 1979), *Pittier* 9947 (US, 1921), *Pittier* 13965 (F, NY & US, 1937), *Prance et al.* 14539 (NY, 1971), *Ramírez Arango et al.* 7701 (COAH, 2004), *Ramírez Arango et al*. 8154 (COAH, 2004), *Ramírez Arango et al.* 8356 (COAH, 2004), *Saravia* & *de Saravia* 3584 (COL & US, 1964), *Smith* V879 (VEN, 1967), *Steyermark* 55557 (F, 1944), *Steyermark* & *Steyermark* 69911 (VEN, 1966), *Steyermark* & *Manara* 110702 (NY, 1974), *Steyermark* & *Manara* 110435 (VEN, 1974), *Steyermark* & *Gonzales* 113724 (F & VEN, 1977), *Steyermark et al.* 131608 (NY, 1985), *Stevenson* 2112 (CR, 1998), *Steward et al.* P12944 (NY, 1971), *Sugden* 211 (COL, 1977), *Sugden* 238 (COL, 1977), *Tamayo* 2198 (US, 1942), *Williams* 13802 (F & VEN 1942).

***Zamia neurophyllidia* D.W.Stev.**

Burger 3877 (BM & NY, 1966), *Calonje et al.* MAC06-04 (FTG, 2006), *Calonje et al.* MAC06-23 (FTG, 2006), *Calonje et al.* MAC06-31 (FTG, 2006), *Calonje et al.* MAC06-34 (FTG, 2006), *Cascante et al.* 339 (CR, 1994), *Haber & Bello* 1817 (CR, 1987), *Hammer* 102 (FTG, 1986), *Hammer* 57 (FTG, 1985), *Herrera* 1180 (CR, 1987), *Maas* 1061 (NY & U, 1974), *Molina et al.* 17395 (NY, 1966), *Lent* 621 (CR, 1965), *Robles* 1548 (CR, 1988), *Stevens* 23771 (CR, 1986), *Taylor* 4380 (NY, 1967), *Taylor* 18125 (NY, 1975).

***Zamia pumila* L.**

Abbott 1158 (US, 1921), *Allard* 13572 (US, 1905), *Armour* 819 (COL, 1898), *Britton & Cowell* 1336 (US, 1906), *Britton & Marble* 1278 (NY, 1913), *Britton et al.* 1768 (NY, 1913), *Britton & Cowell* 2038 (NY, 1914), *Britton & Cowell 2*092 (NY & US, 1914), *Britton & Britton* 9282 (NY, 1920), *Britton et al.* 6912 (NY, 1922), *Britton et al.* 8274 (NY, 1925), *Britton et al.* 8299 (NY, 1925), *Calonje et al.* DR09-004 (FTG, 2009), *Calonje et al.* DR09-005 (FT, 2009), *Calonje et al.* DR09-008 (FTG, 2009), *Calonje et al.* DR09-026 (FTG, 2009), *Calonje et al.* DR09-033 (FTG, 2009), *Calonje et al.* DR09-035 (FTG, 2009), *Chrysler* 9261 (NY, 1924), *Dod & Zanoni* 10045 (FTG, 1980), *Ekman* 5800 (GH, 1926), *Farris* 582, (US, 1921), *Garcia & Pimentel* 1050 (NY, 1986), *Howard & Howard* 3774 (NY, 1946), *Howard & Howard* 9774 (US, 1946), *Howard & Howard* 9494 (JBSD, 1946), *Howard & Howard* 9495 (GH, 1946), *Husaw* 4170 (NY, 1920), *Jack* 4544 (US, 1927), *Kena* (NY, 1926), *Liogier*, 12341 (NY, 1968), *Liogier* 14383 (US, 1969), *Mejia & Zanoni* 5081 (NY, 1980), *Mejia & Zanoni* 6204 (NY, 1980), *Mejia et al.* 8930 (JBSD & FTG, 1980), *Mejia & Zanoni* 9145 (NY & FTG, 1980), *Mejia & Zanoni* 9404 (JBSD & FTG 1980), *Mejia et al.* 13075 (FTG & NY, 1981), *Miller* 1001 (US, 1908), *Pelaez* 1651 (NY, 1983), *Rose* 3707 (NY, 1913), *Rose* 3783 (US & NY 1913), *Shafer* 2789, 793, 896, 679 & 2659 (NY, 1909), *Stimson* 3816 (NY, 1966), *Taylor* 112 (NY, 1909), *Taylor* 352 (NY, 1909), *Van Hermann* 2652 (NY, 1906), *Watson* 901 (FTG, 1981), *Wright* 1463 (LE, 1859), *Wright et al.* (US, 1871), *Wright et al.* 873 (US, 1871), *Zanoni et al.* 10624 (FTG & NY 1981), *Zanoni & Pelaez* 16221 (NY, 1981), *Zanoni & Mejia* 17082 (NY, 1981), *Zanoni & Pimentel* 18512 (FTG, 1981 *Zanoni & Pimentel* 18513 (NY, 1981), *Zanoni & Pimentel* 18514 (NY, 1981), *Zanoni et al.* 21138 (NY, 1982), *Zanoni & Pimentel* 32659 (JBSD & NY, 1984), *Zanoni et al.* 29263 (NY, 1984), *Zanoni et al.* 34348 (NY, 1985), *Zanoni & Mejia et al.* 34295 (NY, 1985).

***Zamia pygmaea* Sims**

Acana 4122 (F, 1940), *Ageay* 2928 (NY, 1929), *Baker* 1907 (NY, 1912), *Britton et al.* 638 (NY, 1903), *Britton et al.* 803 (NY, 1903), *Britton et al.* 6105 (NY, 1910), *Britton et al.* 6105a (NY, 1910), *Britton et al.* 7392 (NY, 1910), *Britton et al.* 6267 (NY, 1910), *Britton et al.* 9664 (NY, 1911), *Britton et al.* 14166 (NY, 1916), *Bro. Léon.* 14764 (F, 1940), *Caldwell & Backer* 7157 (F, 1907), *Carabia* 738 (F, 1939), *Carabia* 1902 (F, 1939), *Carabia* 3924 (F, 1940), *Carabia* 4009 (F, 1940), *Combs* 649 (GH, 1895), *Dahlgren* 1266174 (F, 1948), *Gallardo* 1708 (F, 1939), *Gallardo & Leon* 17611 (F, 1940), *Jack* 4017 (US, 1926), *Jean* 4583 (P, 1914), *Killip & Swetland* 41612 (US, 1951), *Killip* 42674 (GH, 1953), *Killip* 43515 (GH, 1954), *Killip* 44071 (US, 1954), *Leon* 1317 (NY, 1909), *Leon & Carabia* 19014 (F, 1939), *Leon & Liogier* 4583 (NY, 1914), *Luca* 585 (NY, 1920), *Leon & Carabia* 1884 (F, 1939), *Leon* 18890 (F, 1939), *Nalsingham* (NY, 1933), *Nateson* 19400 (F, 1937), *Palmer & Riley* 454 (NY, 1900), *Palmer* 16254 (F, 1935), *Shafer* 10652 (NY, 1911), *Shafer* 10715 (NY, 1911), *Tryon & Tryon* 5494 (MO, 1956), *Van Hermann* 744 (F, 1905), *Van Hermann* 7130 (F, 1907), *Van Hermann* 7156 (F, 1907), *Van Hermann* 7160 (F, 1940), *Wilson* 9407 (NY, 1910), *Wright* 3193 (US, 1928).

***Zamia roezlii* Linden**

Bernal & *Galeano* 892 (COL, 1985), *Bernal* & *Correndor* 1459 (COL, 1988), *Cuatrecasas* 14318 (COL, 1943), *Cuatrecasas* 16868 (COL & MO, 1944), *Cuatrecasas* 21902 (NY & MO, 1967), *Cuatrecasas* & *Patino* 27459 (NY, 1969) *Fuchs et al.* 21902 (COL, 1967), *Fuchs et al.* 21903 (COL, 1967), *Fuchs et al.* 22107 (COL & K, 1967), *Gentry et al.* 53395 (MO & NY, 1985), *Kiem* & *Norstog* 38, (FTG, 1976), *Killip* & *Cuatrecasas* 39129 (COL, 1944), *Killip* & *Cuatrecasas* 38967 (COL, US & F, 1944), *Killip* & *Garcia* 33337 (COL & F, 1939).

Appendix 2

GenBank accession numbers for the species examined in this study

Appendix table 1. GenBank accession numbers

Species (identification no.) and markers	GenBank no.
Ceratozamia robusta Miq. (d1105)	
26S	KX503762
CAB	KX503773
matK	KX503752
NEEDLY	KX503784
rbcL	KX503803
Microcycas calocoma (Miq.) A.DC. (d1063)	
26S	KF221117
CAB	KF221128
matK	GQ203854
NEEDLY	GQ203954
rbcL	KF221191
Zamia acuminata Oerst. ex Dyer (d1064)	
26S	KX503772
CAB	KX503774
matK	GQ203856
NEEDLY	GQ203963
rbcL	KX503804
Zamia angustifolia Jacq. (d1066)	
26S	KF221119
CAB	KF221130
matK	GQ203857
NEEDLY	GQ203957
rbcL	KF221193
Zamia erosa O.F.Cook & G.N.Collins (d1065)	
26S	KX503765
CAB	KX503777
matK	KX503756
NEEDLY	KX503786
rbcL	KX503800
Zamia integrifolia L.f. (CC245)	
26S	KX503764
CAB	KX503775

APPENDIX TABLE 1. (*Continued*)

Species (identification no.) and markers	GenBank no.
matK	KX503755
NEEDLY	KX503788
rbcL	KX503799
Zamia lecointei Ducke (d1152)	
26S	KX503767
CAB	KX503783
matK	KX503760
NEEDLY	KX503791
rbcL	KX503795
Zamia manicata Linden ex Regel (d1153)	
26S	KX503768
CAB	KX503782
matK	KX503758
NEEDLY	KX503790
rbcL	KX503797
Zamia muricata Willd. (d1075)	
26S	KX503769
CAB	KX503781
matK	KX503759
NEEDLY	KX503793
rbcL	KX503794
Zamia neurophyllidia D.W.Stev. (d1076)	
26S	KX503771
CAB	KX503778
matK	KX503753
NEEDLY	KX503789
rbcL	KX503798
Zamia pumila L. (d1100)	
26S	KX503766
CAB	KX503776
matK	KX503757
NEEDLY	KX503787
rbcL	KX503801
Zamia pygmaea Sims (CC253)	
26S	KX503763
CAB	KX503779
matK	KX503754
NEEDLY	KX503785
rbcL	KX503802
Zamia roezlii Linden (d1082)	
26S	KX503770
CAB	KX503780
matK	KX503761
NEEDLY	KX503792
rbcL	KX503796