ORGANISMAL BIOLOGY

# An ancient push-pull pollination mechanism in cycads

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**Most cycads engage in brood-site pollination mutualisms, yet the mechanism by which the Cycadales entice pollination services from diverse insect mutualists remains unknown. Here, we characterize a push-pull pollination mechanism between a New World cycad and its weevil pollinators that mirrors the mechanism between a distantly related Old World cycad and its thrips pollinators. The behavioral convergence between weevils and thrips, combined with molecular phylogenetic dating and a meta-analysis of thermogenesis and coordinated patterns of volatile attraction and repulsion suggest that a push-pull pollination mutualism strategy is ancestral in this ancient, dioecious plant group. Hence, it may represent one of the earliest insect/plant pollination mechanisms, arising long before the evolution of visual floral signaling commonly used by flowering plants.**

## INTRODUCTION

Highly specialized coevolved systems are uncommon in nature and are sometimes considered evolutionary dead ends (*1*). However, the plant order Cycadales is one of the oldest living lineages of seed plants (*2*), and most species form obligate brood-site pollination mutualisms with insects (*3*, *4*). Here, we investigate whether unrelated pollination mutualists exhibit matching behavioral responses to conserved plant behaviors, representing a shared pollination strategy. The push-pull pollination mechanism of the Australian cycad *Macrozamia lucida* is characterized by a daily peak in plant thermogenesis and volatile production whereby thrips pollination mutualists are attracted to lower levels of a plant volatile and repelled by high levels of the same compound (*5*). Across the Cycadales, pollination-stage cones undergo a daily cycle of thermogenesis (*6*) and respiratory processes that similarly culminate in a peak of volatile production (*7*). By analyzing a distantly related New World cycad species that, like most cycads, is associated with a coleopteran pollination mutualist, we investigate whether this characteristic cycle of thermogenesis and coordinated volatile production is broadly indicative of an ancient and conserved pollination strategy.

Within the dioecious Cycadales, specialized insect pollinators, mostly beetles and weevils, live and feed almost exclusively within the male pollen cone tissue (*3*, *4*, *8*–*10*), although one obligate ovule parasite may play a minor role in pollination of *Encephalartos villosus* (*9*). Successful cycad reproduction requires these brood-site mutualists to leave the host pollen cone and transfer pollen to a female ovulate cone. Over the course of a day, pollination-stage cones produce a predictable thermogenic and volatile pattern (*7*). Both pollen and ovulate cones of an individual species produce similar thermogenic and volatile profiles (*6*, *11*–*14*), yet across all species, pollen cones produce higher temperature peaks (*6*) and more concentrated volatile compounds (*3*, *11*, *12*, *14*). Pollinator exit from pollen cones has been observed to coincide with thermogenic and volatile peaks (*5*, *12*, *15*–*17*). The specific behavioral mechanism by which a brood-site mutualist is repelled from its host pollen cone and enticed into ovulate cones has been described in *M. lucida* (*5*), where *Cycadothrips chadwickii* thrips (order: Thysanoptera) are attracted ("pulled") by lower concentrations of the dominant plant volatile compound, β-myrcene, and repelled ("pushed") by higher concentrations of the same compound. The daily plant thermogenic and volatile cycle induces pollinator movement between pollen and ovulate cones, enabling pollination and seed set (*5*). This push-pull pollination mechanism differs from most pollination syndromes because it has a repulsive component that expels pollinators from pollen cones at a certain point in the cycle in addition to the attractive mechanisms commonly seen in the visual and chemical cues of flowers. Furthermore, the same chemical cue is used for attraction and repulsion.

The Mexican cycad *Zamia furfuracea* (Zamiacea), which is more than 150 million years (Ma) diverged from *M. lucida*, has an obligate pollination mutualism with *Rhopalotria furfuracea* (Coleoptera: Belidae) weevils (formally *R. mollis*) (*17*). *R. furfuracea* complete their entire development within the plants' disposable pollen cone parenchyma tissue (*10*), and the plants do not set seed without pollinators (*17*). Specialized coleopteran pollination is widespread in extant Cycadales (*3*, *4*) and has existed in the lineage since at least the mid-Mesozoic where fossilized Boganiidae beetle pollinators have been found (*18*) well before the rise of flowering plants (*19*) or the earliest known direct evidence of thrips pollination (*20*). While push-pull pollination has also been hypothesized for Coleoptera-pollinated cycads (*21*, *22*), the mechanism by which the plants manipulate their behavior to carry out pollination has not yet been explored.

## RESULTS

### Plant volatile profile and weevil physiological response

We identify the chemical communication mechanism underlying the mutualism between *R. furfuracea* and *Z. furfuracea*. Using headspace collection methods, plant volatile compounds were collected from *Z. furfuracea* pollen ($n = 8$) and ovulate cones ($n = 7$) to determine the plant volatile profile and the physiological response of *R. furfuracea* to host plant volatiles. *Z. furfuracea* pollen and ovulate cones produce two major compounds: the hydrocarbon 1,3-octadiene and the alcohol linalool (Fig. 1). Electroantenograph detection (EAD) demonstrated that *R. furfuracea* male and female weevils are physiologically capable of perceiving only 1,3-octadiene ($n = 10$;

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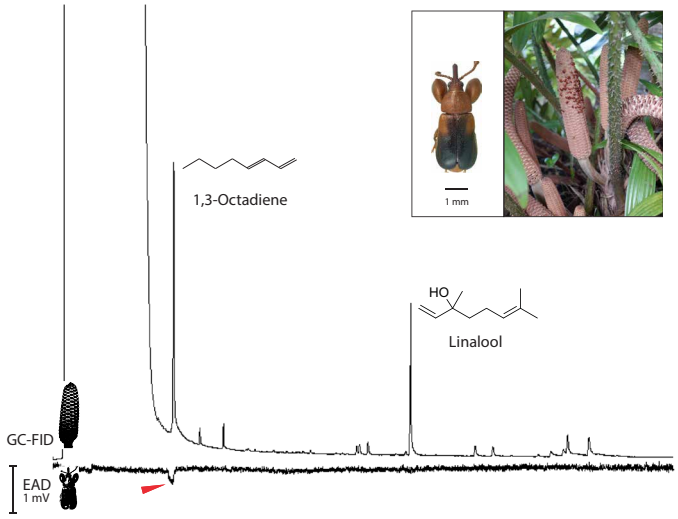


**Fig. 1. *Z. furfuracea* produce two main volatile components, 1,3-octadiene and linalool, yet *R. furfuracea* only physiologically respond to 1,3-octadiene.** Gas chromatography–flame ionization detector (GC-FID) of *Z. furfuracea* plant cone volatiles is shown at the top with an example male *R. furfuracea* weevil electroantenograph detection (EAD) on the bottom. Scale bar, 1 mV for EAD channel. Red arrow denotes a positive physiological response of weevils to 1,3-octadiene that is not seen for linalool ($n = 10$). Weevil response to 1,3-octadiene and lack of response to linalool were confirmed with standards ($n = 6$). Photo credit: Shayla Salzman, Cornell University.

Fig. 1), explaining why previous attempts to elicit behavioral responses in *R. furfuracea* using linalool were unsuccessful (*11*).

## Weevil behavioral response to plant volatiles

Because a physiological response does not equate with attraction and the production of 1,3-octadiene was found to vary over the course of the day (fig. S1), we analyzed the behavioral response of *R. furfuracea* to different amounts of 1,3-octadiene, covering the range of intrinsic plant production. Using pitfall tests where groups of 4 to 10 weevils are introduced for a period of 30 min to a dilution of 1,3-octadiene in dichloromethane or a control (fig. S2), we show that *R. furfuracea* are positively attracted to 1,3-octadiene and that weevil attraction to increasing amounts of 1,3-octadiene is nonlinear (Fig. 2A). Weevils are more attracted to the intermediate amount of 10,000 ng of 1,3-octadiene than to all lower quantities ($P < 0.012$ for all lower amounts; table S2) and to the highest quantity ($P = 0.0102$ for 650,000 ng). The decrease in attraction suggests, but does not conclusively demonstrate, that weevils are repelled (i.e., pushed) by high concentrations.

We therefore tested whether intermediate (10,000 ng) and high (650,000 ng) quantities of 1,3-octadiene elicited different movement responses in weevils. The push-pull hypothesis predicts that weevils should move toward 10,000 ng of 1,3-octadiene and move away from 650,000 ng of 1,3-octadiene. We set up enclosed arenas (fig. S3) that included ~80 weevils, exposing five groups to either 10,000 ng of 1,3-octadiene plus a control or that of 650,000 ng plus a control, and videotaped their movements over a period of 31 min. We measured the change in distance toward the 1,3-octadiene sample from the beginning of the video to the end. As predicted, *R. furfuracea* move toward 10,000 ng of 1,3-octadiene but move away from 650,000 ng of 1,3-octadiene ($P = 0.0033$; fig. S4 and Fig. 2B).

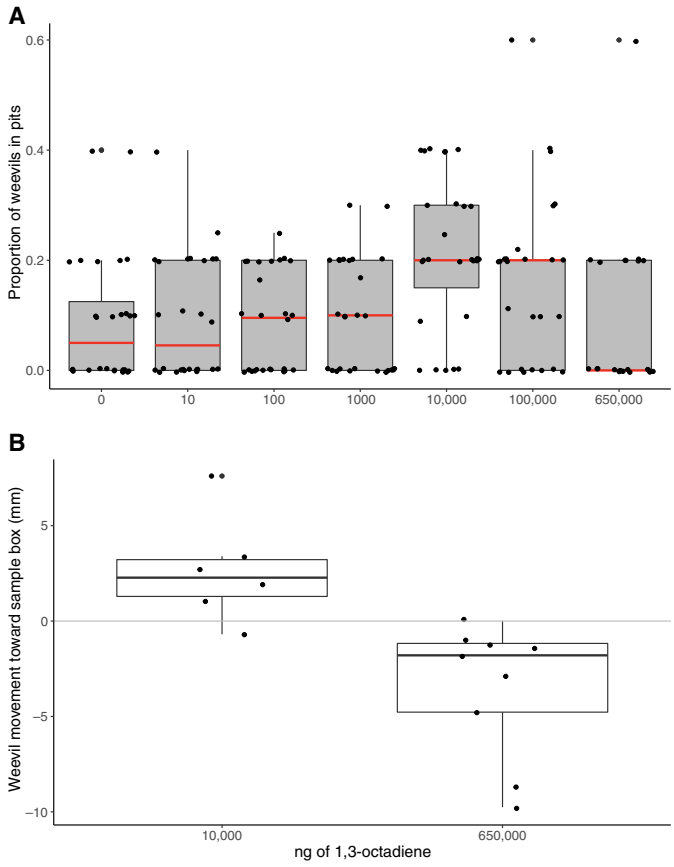


**Fig. 2. Weevil behavior follows push-pull pollination where they are attracted to lower concentrations of 1,3-octadiene and repelled by higher amounts.** (**A**) Weevils are preferentially attracted to mid-level amounts of 1,3-octadiene in pitfall tests (shown in fig. S2) and less attracted to more concentrated amounts. The proportion of weevils in the bait pit is shown on the *y* axis. Sequential Bonferonni-adjusted *P* values using an ordinary least squares model are found in table S2. (**B**) Weevils are attracted to and move toward 10,000 ng of 1,3-octadiene and are repelled and move away from that of 650,000 ng in behavioral arenas (shown in fig. S3). Significance is determined using an ordinary least squares model. Measurements above the gray line show a change in average weevil position toward the 1,3-octadiene sample and below the line a shift away from the sample. Individual points represent one trial of 4 to 10 weevils in (A) and one trial of ~80 weevils in (B). Absolute amounts of 1,3-octadiene in 10 μl of dichloromethane are shown on the *x* axis in both graphs.

Together, these experiments demonstrate that weevil attraction preference is for an intermediate amount of 1,3-octadiene and that their movement can be induced by varying the quantities of plant compounds, consistent with the push-pull pollination observed in *M. lucida* (*5*).

## Weevil and plant behavior

We assessed the attraction of weevils to pollen microsporophylls and ovulate megasporophylls (hereafter referred to as pollen and ovulate cone scales) in the laboratory over an 8-hour period to look for differences between the pollen cone, which serves as a feeding site, and the ovulate cone, which is not fed upon. Using small arenas containing four weevils with either a pollen cone scale or an ovulate cone scale, we defined "attraction" as a weevil being on or within one body length of the cone scale. We find that overall, weevils are

more attracted to pollen cone scales than ovulate cone scales ($P = 0.0177$). However, attraction to both pollen and ovulate cone scales changes over the course of the day ($P = 0$ for pollen cone scales and $P = 0.0249$ for ovulate cone scales). The decrease in attraction to pollen cone scales in the evening is much greater than the decrease in attraction to ovulate cone scales (test of variance between pollen and ovulate cone scale attraction, $P = 0.0003199$) (Fig. 3A). Because excised cones are known to continue both thermogenic and volatile patterning (*5*, *7*), we hypothesized that this pattern may be related to plant volatile production and not just the artificial nature of laboratory experimentation.

We therefore investigated the pattern of *Z. furfuracea* volatile release and its correlation to *R. furfuracea* movement in the field. We hypothesized that there would be an increase in volatile production in pollen cones and *R. furfuracea* activity around 19:00 to 20:00 after the thermogenic peak at about 18:30 to 19:00 (fig. S4) (*6*). We collected volatiles from pollination-stage pollen and ovulate cones ($n = 5$; table S1) at hourly intervals and identify a typical diurnal pattern in volatile production in both sexes of *Z. furfuracea*, whereby cones change the ratio of the two major volatile compounds over the course of the day, approaching 100% production of 1,3-octadiene in the evening (Fig. 3B). Pollen cones consistently produce a greater quantity of volatile compounds ($P = 0.0007$) than ovulate cones and show a clear pattern of increase in production of 1,3-octadiene in the evening ($P = 0.0184$; Fig. 3C) that coincides with *R. furfuracea* expulsion from pollen cones around 20:00 (Fig. 3D). Ovulate cone production of 1,3-octadiene (fig. S1 and Fig. 3C) may act as a "cheating" mechanism in that they mimic pollen cones but do not provide food or a brood site.

## DISCUSSION

*R. furfuracea* weevils and *C. chadwickii* thrips have converged on the same behavioral response to an ancient plant pollination mechanism. Both pollinators use the pollen cone as a larval development site, as do other known cycad pollinators (*3*, *4*, *11*–*13*). Both insects show differential behavioral responses according to the quantities of one host plant volatile compound: attraction to intermediate amounts and repulsion to higher amounts. In both cases, an increase in volatile production correlates with an expulsion of pollinators from pollen cones, a pattern that has been observed in other cycad genera as well (*5*, *12*, *15*–*17*). In both plants described here, as well as all cycad genera so far tested, the production of key volatile compounds changes throughout the day (Fig. 4). In the *Macrozamia*/thrips system, this is known to be caused by the increased respiration necessary for thermogenesis (*7*), an ancestral trait among the Cycadales (*6*), that by itself (*22*) or together with associated changes in humidity likely acts as an additional cue.

The Cycadales are the basal gymnosperm lineage (*23*) and have an extensive fossil record stretching back 265 Ma (*24*), placing them among the most ancient extant seed plants. Specialized pollination has existed in this group since well before the rise of flowering

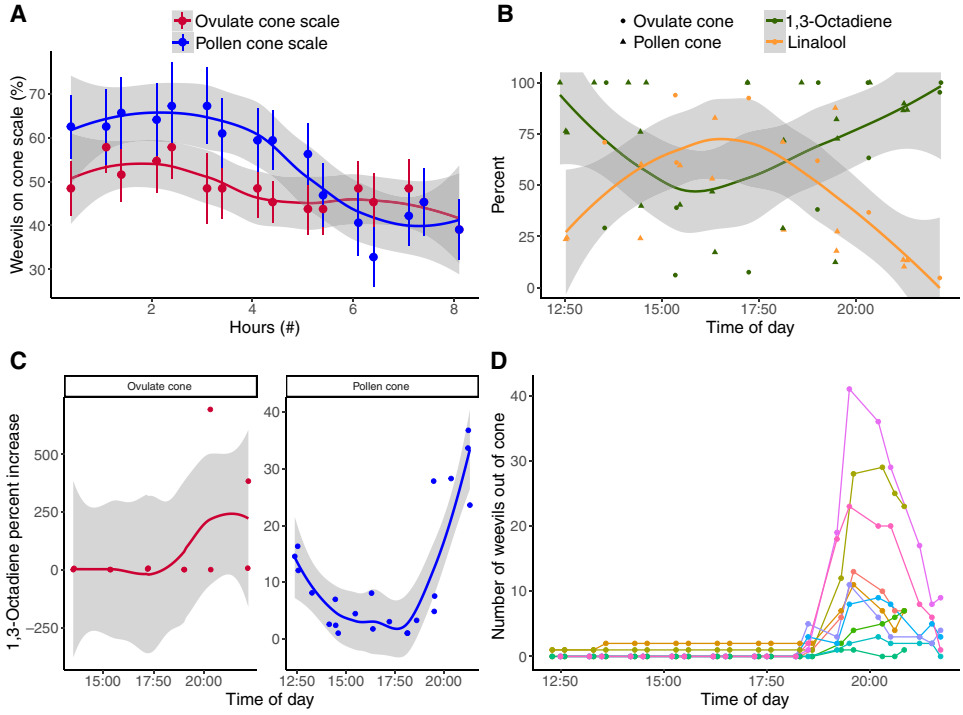


**Fig. 3. An increase in production of 1,3-octadiene by *Z. furfuracea* is positively correlated with an exodus of *R. furfuracea* weevils from the pollen cone.** (**A**) Weevils are more attracted to pollen cone scales, but the percent of weevils on pollen cone scales drops sharply over time in laboratory behavioral arenas. The smoothed conditional mean is shown (method = loess) with one SE at 0.95 confidence in (A) to (C), and significance is determined using a weighted least squares model. Mean values with one SE at 0.95 confidence are overlaid in (A). Raw values are overlaid in (B) and (C). (**B**) The percent composition of the daily volatile profile shifts toward 100% 1,3-octadiene after 20:00 hours in both pollen and ovulate cones. (**C**) The percent increase in 1,3-octadiene production in pollen cones rapidly increases after 20:00 hours. (**D**) Weevils housed in pollen cones are repelled from the cones at 20:00 hours. Each colored line represents an individual pollen cone, and numbers are raw counts of weevils seen in video recordings.

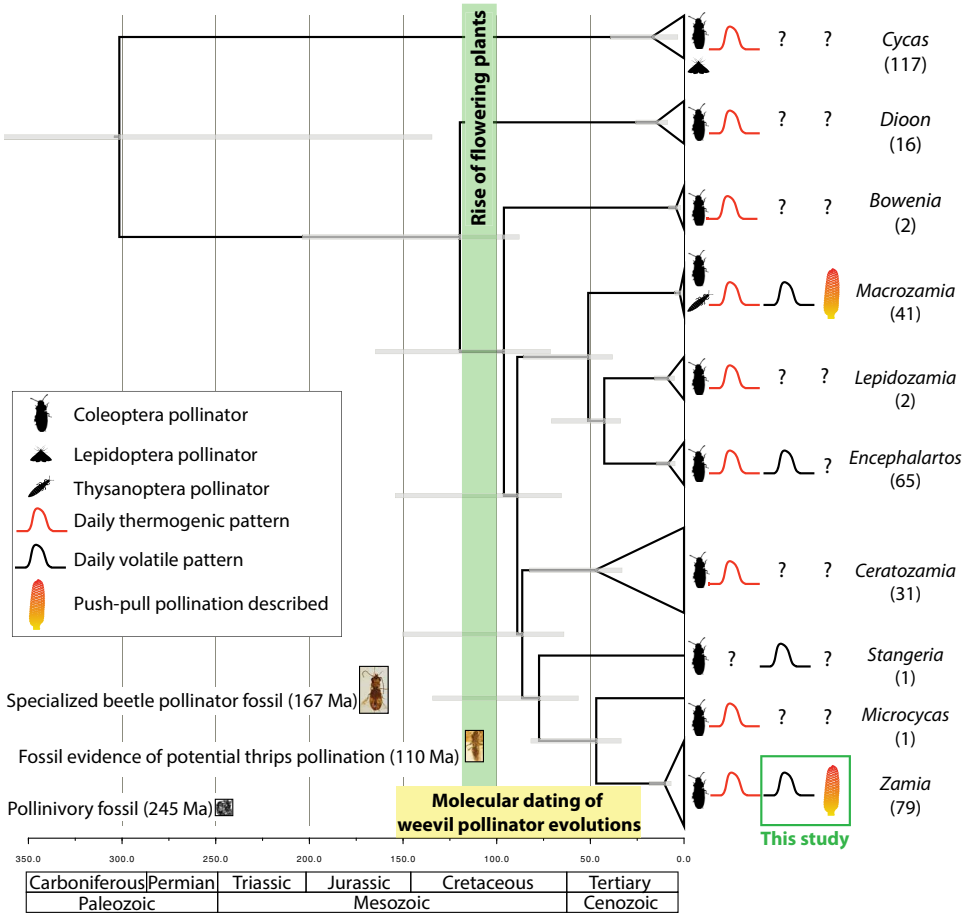


**Fig. 4. Plant behaviors responsible for push-pull pollination are widespread in Cycadales.** Bayesian molecular-dated phylogeny of Cycadales shows all known information on pollinators and thermogenic and volatile patterns. The number of species in each genus is noted in parentheses. Gray bars show the 95% confidence interval for dating at the nodes. Fossil evidence of cycad pollination, molecular dating of weevils pollinator lineages (*41*), and the estimated timing of the rise of flowering plants are noted. Fossil evidence is as follows: Recently reported fossil of a specialized Boganiidae beetle pollinator (*18*), the earliest evidence of thrips associated with cycad pollen (*20*) and the earliest evidence of cycad pollenivory in the form of pollen filled coprolites, likely of beetle origin (*25*). Citations for metadata are provided in table S3.

plants (*19*) from at least the mid-Mesozoic (*18*) and perhaps as early as 245 Ma (Fig. 4) (*25*). Stereotypical sequential timing of thermogenesis in pollen and ovulate cones and specialized brood-site pollination have been documented to occur across the Cycadales (table S3). These observations, in combination with our discovery here of convergence in the behavioral responses of both thrips and weevils to volatiles released by the cones of Old World (*Macrozamia*) and New World (*Zamia*) lineages, support the hypothesis that the push-pull system found in cycads represents the most ancient insect/plant pollination mechanism yet documented. Plant thermogenic behaviors appear to have remained consistent over evolutionary time although extant insect pollinators may represent more recent shifts (*26*, *27*). These dioecious plants give us insight into the earliest forms of insect pollination before the prolific diversification of visual floral signaling and reward systems used by angiosperms. Odor attraction has been thought to precede color attraction in angiosperms (*28*), and the coupling of thermogenesis with strong odors as seen in cycads has been suggested as an exaptation, leading to early angiosperm pollination syndromes (*29*). Plant volatiles attractive to pollinators have been hypothesized to be derived from herbivore deterrent secondary compounds (*30*), and here, we find that attractive volatile compounds in cycad push-pull pollination maintain a repellent component for their specialized mutualists. Early insects are thought to have found rewards such as high concentrations of starch in cone tissues (*4*) and pollination drops (*4*, *31*), ovular exudates found in cycads and other gymnosperms that predate compositionally similar angiosperm nectar. If early cycad herbivores overcame any deterrent quality of host plant compounds and found brood sites in plant reproductive tissues, then plant volatiles may have become the basis of chemical plant-insect communication, with signals for attraction and repulsion mediated, in part, by the daily cycle of thermogenesis.

## MATERIALS AND METHODS

### Experimental design

The purpose of this study was to determine the insect and plant behaviors responsible for the specific brood-site pollination mutualism seen in *Z. furfuracea* cycads and their weevil pollinators, *R. furfuracea*, and to contrast that with the behaviors seen in other cycad pollination systems, including *M. lucida* cycads and their thrips pollinators, *Cycadothrips chadwicki* (*5*). Plant volatile analysis was used to

identify the volatile components produced by pollen and ovulate cones. Physiological analysis of *R. furfuracea* weevils was used to identify the electro-antennally active plant compounds and behavioral responses of *R. furfuracea* to plant compounds. Field experiments matched plant volatiles corresponding to weevil behavior to confirm laboratory experiments. Last, thermogenic and volatile patterns across cycad genera were mapped onto a dated phylogeny to provide an evolutionary context.

### Study species

*Z. furfuracea* is an endangered dioecious gymnosperm native to Veracruz, Mexico (*32*). Both pollen and ovulate cones are known to undergo a daily synchronous thermogenic peak at the time that they are reproductive, with a higher temperature spike in pollen cones (2.5°C above ambient; ovulate cones, 0.8°C above ambient) (*6*). Pollen cones produce two main volatile components, the hydrocarbon 1,3-octadiene and the alcohol linalool (*11*).

*R. furfuracea* is an obligate pollination mutualist with *Z. furfuracea* (*10*, *17*). It is required for *Z. furfuracea* reproduction (*17*) and its entire life cycle is tied to that of its host plant (*10*). *R. furfuracea* adults feed, breed, and lay eggs in *Z. furfuracea* pollen cones and larvae feed and develop inside of microsporphylls, pupating inside of the microsporophyll stalk (*10*). *R. furfuracea* visit ovulate cones of *Z. furfuracea* but do not feed or lay eggs on them. The life cycle is 7 to 8 days, with many generations occurring during the plant reproductive season (*10*). At the end of the plant reproductive season, late instar larvae go into diapause for the 10 months of the year when plants are not reproductive, remaining inside of the stalks of the dried, dead microsporphylls (*10*).

### Volatile collection

*Z. furfuracea* plant volatile collections were performed using headspace collection methods (*33*) on eight pollen dehiscing male cones and seven receptive female cones to determine the volatile profile. Three pollen cones and two ovulate cones were further sampled at hourly time points throughout the day to assess daily variation. All volatile collections were done in situ at Montgomery Botanical Center in Miami, Florida. Accession information for these plants is found in table S1. Cones remained attached to the plant and covered in oven bags (Reynolds Consumer Products, Lake Forest, IL) that were tied shut at the bottom of the cone. Low-flow vacuum air samplers (Gilian model LFS-113DC) calibrated to 0.1-liter/min pulled headspace air and volatiles for 45 min through filters made with 300-mg Porapak Q adsorbent mesh 80-100 75 $cm^3$ (Sigma-Aldrich, Saint Louis, MO, part no. 20331) held into glass Pasteur pipettes size 14.6 cm (VWR International, Radnor, PA, part no. 14672-200) with plugs of glass wool (Sigma-Aldrich, Saint Louis, MO, part no. 20411). Before volatile collection, filters were soaked in high-performance liquid chromatography (HPLC)–grade dichlormethane (Sigma-Aldrich, Saint Louis, MO, part no. 650463) for 48 hours and then prewashed 10 times with full flow-throughs of dichloromethane and dried with charcoal-purified air pushed through at 0.4 liter/min. After volatile collection, samples were eluted with 500 µl of HPLC-grade dichloromethane (Sigma-Aldrich, Saint Louis, MO, part no. 650463) pushed through with charcoal-purified air.

### Gas chromatography–mass spectrometry

Initial chemical analyses were conducted using a combined Agilent Technologies 6890 network gas chromatograph (GC) and 5973 mass-selective detector. The GC was equipped with a DB-5 column [J&W Scientific Inc., Folsom CA; 30 m by 0.25 mm inside diameter (ID); film thickness, 0.25 µm; splitless mode]. Helium was the carrier gas at a constant flow rate of 0.7 ml/min. The injector temperature was 250°C. Oven temperature was held at 40°C for 1 min, programmed to 170°C at 10°C/min, and held for 5 min. Volatile peaks were manually integrated. Volatiles were identified on the basis of their mass spectra (NIST version 2.0, 2002), Kovats indices (*34*, *35*), and by comparison of the retention indices and mass spectra with those of available authentic synthetic compounds and a computerized data library (NIST version 2.0,2002). An authentic standard of 1,3-octadiene was obtained from ChemSampco Inc. (Dallas, TX, catalog no. 7015.90). Authentic standards of (±) linalool were obtained from Sigma-Aldrich Co. (Saint Louis, MO, catalog no. L2602-5G).

### Electrophysiological analysis: GC-EAD

The physiological response of *R. furfuracea* weevils to host plant *Z. furfuracea* volatile compounds was determined using GC-EAD. The coupled GC-EAD system used was as previously described by Crook *et al.* (*36*), with a few modifications. Samples of aerations or standards were injected (2 µl), splitless, onto a Hewlett Packard (Agilent Technologies, Santa Clara, CA) 6890 GC with a DB-5MS-DG column (J&W Scientific Inc., Folsom, CA; 0 m by 0.25 mm ID, 0.25-µm film thickness) and a 1:1 effluent splitter that allowed simultaneous flame ionization detector (FID) and EAD detection of the separated volatile compounds. Helium was the carrier gas (2.5 ml/min). Oven temperature was held at 50°C for 1 min, programmed to 280°C at 10°C/min, and held for 15 min. Injector temperature was 280°C. The GC outlets for the EAD and FID were 300°C. The column outlet for the EAD was held in a water-cooled humidified air stream (20°C) flowing at 20 ml/min over the antennal preparation of adult *R. furfuracea* attached to an EAG probe (Syntech, Hilversum, the Netherlands). Whole heads of an adult beetle were removed so that both antennae could be used for recording. Tips of both antennae were cut off to make a clean opening for conducting gel (Spectra 360, Parker Laboratories, Fairfield, NJ) to form an uninterrupted connection to the EAG probe. The EAG probe was connected to an IDAC-232 serial data acquisition controller (Syntech). Signals were stored and analyzed on a PC equipped with the program EAD (version 2.6, Syntech). GC-EADs using whole *Z. furfuracea* plant volatile collections were performed on six males and four females, and GC-EADs of the 1,3-octadiene standard were performed on three males and three females.

### Behavioral analysis: Pitfall tests of 1,3-octadiene concentrations

Because the GC-EAD is only able to determine a physiological response, the behavioral response (attraction or repulsion) of *R. furfuracea* weevils to the *Z. furfuracea* plant volatile compound 1,3-octadiene was determined in pitfall tests (fig. S2) using differing amounts of the compound, along with a control of dichloromethane carrier solvent. Pits were constructed of 100 mm by 15 mm petri dishes with 1.5-cm circular holes cut into them and hot glued onto 5-cm-deep plastic cups. These were set into larger glass cups so that all dishes were completely level. Ten microliters of 1,3-octadiene standards diluted in dichloromethane (1, 10, 100, 1, 10, and 65.5 µg/µl) was applied to 1 cm by 4 cm pieces of filter paper (Whatman plc, no. 4) (GE Healthcare Life Sciences, Chicago, IL) handled with forceps and dropped into the pits. For each trial, 1,3-octadiene

concentrations and controls of 10 µl of dichloromethane were run simultaneously. The number of trials for each treatment is as follows: dichloromethane control, 28 trials; 10 ng, 26 trials; 100 ng, 26 trials; 1000 ng, 27 trials; 10,000 ng, 27 trials; 100,000 ng, 26 trials; and 650,000 ng, 22 trials. *R. furfuracea* weevils were taken directly from *Z. furfuracea* pollen cones so as to be well fed. Five to 10 weevils were placed inside the edge of the petri dish, which was then closed and placed in the dark at room temperature (22°C) for 30 min, at which time the number of weevils inside of the pits was counted and transformed into proportions (weevils in pits/total weevils). Dead weevils or copulating weevils were not counted.

### Behavioral analysis: Weevil movement in relation to 1,3-octadiene concentrations

To determine whether the high concentration of 1,3-octadiene acts as a repellant, arenas were constructed in which 80 to 100 *R. furfuracea* weevils were introduced, and their behavior was recorded for 31 min in a series of five trials to either 10,000 ng ($n$ = 2) or 650,000 ng of 1,3-octadiene ($n$ = 3) suspended in dichloromethane. Arenas were constructed of white acrylic sides and bottom with a clear acrylic top (fig. S3). Dimensions were 14.5 cm by 9.5 cm and 3.5 cm tall. To allow airflow and the dissipation of volatile compounds, windows were cut into the sides and covered with white mesh fabric (13.5 cm by 1 cm holes in the long sides, and 8.2 cm by 1 cm holes in the short sides). Openings (2 cm by 5 cm) were cut in the bottom of the arena for sample and control. Weevils for each trial were inserted through these openings, which were then covered with fine white mesh fabric from the bottom. Ten microliters of 1,3-octadiene dilution (1 or 65.5 µg/µl) in dichloromethane was placed onto 1 cm by 4 cm filter paper (Whatman plc, no. 4) (GE Healthcare Life Sciences, Chicago, IL) and set under the opening in the bottom of the arena. To account for any visual attraction to the openings in the arena floor, a blank filter paper piece was placed under the other opening as a control. This entire setup was placed on a clear acrylic panel suspended over a 29.6 cm by 29.6 cm light-emitting diode (LED) light panel (no. BK3301, U.S. Solid State LLC, Shreveport, LA) and encased in a black box covered with black fabric so as to be backlit. Weevils were allowed to feed freely on *Z. furfuracea* pollen cones before trials so as to be well fed.

Raw videos were recorded using a Sony Handycam HDR-CX260V (Sony, Tokyo, Japan) and subsequently processed in MATLAB using custom scripts. The locations of the outer arena edges and the left and right bait locations were manually located for each trial. Next, a background image was computed separately for each trial by calculating the median intensity at each pixel. This median-averaging method meant that weevils that were mostly immobile are considered part of the background and excluded from further analysis.

For each 31-min trial, where 80 to 100 weevils were introduced to either amount of 1,3-octadiene, we analyzed a subset of 930 frames (video frame rate, 29.97 frames/s; subsampling, 1 frame every 2 s). For each analyzed frame, weevils were separated from the background by subtracting the frame's pixel intensity values from the computed background image. This intensity-differential image was then turned into a binary map of weevils using a manually calibrated intensity threshold. After this segmentation step (and digital erosion and dilation to reduce noise), image regions that were within a size threshold (i.e., between 150 and 800 pixels, manually calibrated to exclude digital noise) and located within the arena were considered separate weevils (average weevil pixel area = 300 pixels). For each identified weevil, distance was calculated to the nearest point on each bait. If weevils were located within the edges of the baits, then the distance to that bait was set to zero. For each sampled frame, a mean distance to sample and to control was calculated across all the weevils tracked for that frame, giving one mean value for the group.

Weevil movement in relation to 1,3-octadiene amount was determined by the change in distance to the sample from the start of the trial to the end. The "starting" distance to the sample box was determined for each trial by creating one mean value of weevil location over the first 5 min of video. Weevils were then given ~18 min to make a choice, and before the "ending," asymmetry toward sample was determined, beginning at ~23 min (sampled frame, no. 700). On the basis of personal observations, 18 min provides sufficient time for weevil activity to begin, and the results were not sensitive to this specific cutoff. Autocorrelation of the change in distance to the sample was analyzed using the acf function in R separately for each trial. We then computed the average autocorrelation function, which showed a substantial diminishment in autocorrelation (correlation, <0.1) at a lag of 2 min (60 sampled frames) across all videos. We therefore subsampled our raw tracking data by taking a 1-min (30 sampled frames) mean value for weevil location every 3.33 min (100 sampled frames) to minimize the impacts of autocorrelation. The ending distance to sample was then subtracted from the starting distance to sample ($n$ = 6 for 10,000 ng and $n$ = 9 for 650,000 ng of 1,3-octadiene).

### Behavioral analysis: Weevil attraction to micro- and megasprophylls

Weevil attraction to pollen and ovulate cones over the course of a day was determined through video analysis of small arenas containing four weevils and either a pollen cone microsporophyll or an ovulate cone megasporophyll (pollen and ovulate cone scales). Pollen and ovulate cone scales were collected from pollination-stage cones at Montgomery Botanical Center in Miami, Florida. Four ovulate and two pollen cones were stored at 16°C overnight beginning at 21:30 hours. They were removed at 8:00 hours and slowly brought up to 82°C over the course of 1 hour. Video analysis began 1 hour later at 10:00 hours. Thirty-two trials were conducted simultaneously with 16 replications of pollen cone scales and 16 replications of ovulate cone scales arranged so as to have as much separation between individual cones and sexes as possible. Sample map is shown in fig. S5. Arenas were set up as follows: 60 mm by 15 mm petri dishes were place upside down on top of filter paper (Whatman plc, no. 4) (GE Healthcare Life Sciences, Chicago, IL), creating an enclosed environment. This setup rested on top of wide plastic mesh to allow for airflow and dissipation of any plant volatile compounds from the arena. Cone scales were broken off of the cone at 9:30 hours and placed in the middle of the designated arena. Four *R. furfuracea* weevils were taken directly from pollen cones so as to be well fed and placed into each arena. The entire setup was placed into a white plastic box that was illuminated from all four sides with fluorescent lighting and covered with black cloth so as to be evenly lit. Videos were recorded using a Sony HandyCam HDR-CX260V (Sony, Tokyo, Japan) and began at 10:01 hours and ended at 21:30 hours. Weevils' attraction to cone scales was manually counted from the resulting video at 30-min intervals. Weevils were counted as attracted to the cone if they were within 1 mm of the cone.

### Cone thermogenesis

*Z. furfuracea* thermogenesis patterns were tested on in situ cones at Montgomery Botanical Center in Miami, Florida. One pollination-stage ovulate cone, one pollination-stage pollen cone, and one prepollination-stage pollen cone were tested and compared with published data (*6*). Temperature probes from Onset UX120-014M (Onset, Bourne, MA) data loggers were inserted into the cone peduncles between mega- or microsporophylls and recorded from 8:00 until 21:00. Ambient temperature was collected adjacent to the plants. The plants, including the ambient temperature probe, were covered with shade cloth to prevent direct heating from the sun.

### Weevil expulsion from male cones

Ten pollen dehiscing male cones were collected and videotaped throughout the course of the day in conjunction with hourly plant volatile collections to determine the correlation between plant volatile emission and weevil expulsion from male cones. Cones were placed into 20.5 cm by 17 cm by 17 cm clear plastic containers. One square side of the container was mesh to allow airflow and volatile dissipation, and the cone was placed closest to this side. Cones used in video analysis were the same stage as those used for volatile collection, open and releasing pollen. These boxes were placed on top of a white sheet of paper set on a clear plastic sheet that was suspended over a 29.6 cm by 29.6 cm LED light panel (no. BK3301, U.S. Solid State LLC, Shreveport, LA). This entire setup was covered with black cloth so that the only source of light was the even backlit LED panel. Cones were videotaped for 11 hours using a Sony Handycam HDR-CX260V (Sony, Tokyo, Japan). Weevil expulsion from the cone was manually counted at 30-min intervals from the resulting videos.

### Phylogeny

Dated molecular phylogenetic analyses have been performed in the Cycadales with similar dates but conflicting generic relationships (*37*–*39*). The dataset from Salas-Leiva *et al.* (*38*) was used to construct a dated phylogeny using the methods and "traditional" fossil calibrations suggested in Condamine *et al.* (*37*). The Salas-Levia *et al.* dataset (*38*) was chosen over Condamine *et al.* (*37*) because of its use of markers from more gene regions (5 versus 3) and the completeness of its matrix (100% gene coverage for all taxa versus 51% coverage). The two datasets were not combined because the represented taxa did not overlap completely, and preference was given to a complete data matrix. Molecular dating was performed using BEAST v.1.10.0 (*40*). Fossil calibrations are based on Hermsen *et al.* (*24*) and were defined as uniform priors as follows: stem node of *Bowenia* (lower = 33.9 Ma, upper = 265.1 Ma), stem node of *Lepidozamia* (lower = 33.9 Ma, upper =265.1 Ma), and stem node of *Dioon* (lower = 56 Ma, upper = 265.1 Ma). Methods followed Condamine *et al.* (*37*) except for an unpartitioned analysis and the use of a random starting tree and a random local clock model for determining tree likelihood as suggested by Salas-Leiva *et al.* (*38*). Literature used to determine the presence of pollinators and thermogenic and volatile patterning can be found in table S3.

### Statistical analysis

For analysis of significance in pitfall tests, a likelihood ratio test was used to determine whether variances between the groups should be accounted for. No significant difference was found between a weighted least squares model that allowed for different variances between groups and an ordinary least squares model ($P = 0.1044$). Therefore, the ordinary least squares model with fewer parameters was used to determine significant differences in proportions of weevils attracted to baits between the different amounts of 1,3-octadiene [182 degrees of freedom, $t$ values = 0.148 (10 ng/μl), −0.0157 (100 ng/μl), 0.283 (1000 ng/μl), 3.789 (10,000 ng/μl), 2.172 (100,000 ng/μl), and 0.0458 (650,000 ng/μl)]. For behavioral arenas, a likelihood ratio test was used to determine whether variances between the groups should be accounted for, and no significant difference was found between a weighted least squares model that allowed for different variances between groups and an ordinary least squares model ($P = 0.5869$). Therefore, the ordinary least squares model with fewer parameters was used to determine the significance of change in distance to sample for the two amounts of 1,3-octadiene ($t$ value = −3.596, 15 degrees of freedom). For weevil attractiveness to cone scales, weighted least squares models were used to account for variation between groups in determining the significance between pollen and ovulate cone attractiveness ($t$ value = 2.379, 512 degrees of freedom). For attractiveness over time, no significant difference was found between a weighted least squares model and an ordinary least squares model ($P = 0.9853$ for pollen cone scales and $P = 0.9558$ for ovulate cone scales) so the ordinary least squares with fewer parameters was used to determine significance ($t$ value = −5.0315, 256 degrees of freedom for pollen cone scales; $t$ value = −2.256, 256 degrees of freedom for ovulate cone scales). Differences in the variances in attractiveness between pollen and ovulate cone scales were determined using a variance test. Last, differences in volatile production between pollen and ovulate cones were determined using a weighted least squares model to account for the variation between cone sexes ($t$ value = 3.595, 56 degrees of freedom), and differences in 1,3-octadiene increases over time in pollen cones were determined using an ordinary least squares model ($t$ value = 2.566, 22 degrees of freedom) when no significant difference was found between a weighted least squares model and an ordinary least squares model ($P = .6334$).

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/24/eaay6169/DC1

View/request a protocol for this paper from *Bio-protocol*.

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# An ancient push-pull pollination mechanism in cycads

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