Geographical matching of volatile signals and pollinator olfactory responses in a cycad brood-site mutualism

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Brood-site mutualisms represent extreme levels of reciprocal specialization between plants and insect pollinators, raising questions about whether these mutualisms are mediated by volatile signals and whether these signals and insect responses to them covary geographically in a manner expected from coevolution. Cycads are an ancient plant lineage in which almost all extant species are pollinated through brood-site mutualisms with insects. We investigated whether volatile emissions and insect olfactory responses are matched across the distribution range of the African cycad *Encephalartos villosus*. This cycad species is pollinated by the same beetle species across its distribution, but cone volatile emissions are dominated by alkenes in northern populations, and by monoterpenes and a pyrazine compound in southern populations. In reciprocal choice experiments, insects chose the scent of cones from the local region over that of cones from the other region. Antennae of beetles from northern populations responded mainly to alkenes, while those of beetles from southern populations responded mainly to pyrazine. In bioassay experiments, beetles were most strongly attracted to alkenes in northern populations and to the pyrazine compound in southern populations. Geographical matching of cone volatiles and pollinator olfactory preference is consistent with coevolution in this specialized mutualism.

1. Introduction

Coevolution is one of the major processes that drive diversification and speciation among plants and animals [1]. Obligate mutualisms represent some of the most significant coevolutionary interactions [2] and include various highly specialized brood-site pollination mutualisms [1] that are likely to be mediated by volatile signals [3]. Most research on coevolved mutualisms has focused on morphological co-adaptations rather than aspects of co-adaptation of plant chemical signals and animal perception and cognition. Floral volatiles that mediate plant–insect pollinator interactions are usually blends of compounds belonging to several chemical classes [4]. There is considerable variation among plant species in the number and relative amounts of the different constituents, and in their temporal and spatial emission patterns [5,6], all factors which can influence attractiveness to pollinators [7,8]. As for morphological floral traits, scent properties of a species can vary geographically [9] in a manner which can be clinal [10] or unstructured [11], continuous or discrete. Possible explanations for this geographical variation include phenotypic plasticity, local hybridization, neutral processes such as genetic drift, and adaptive processes such as coevolution or pollinator shifts [4,9,12].

In reciprocally specialized plant–pollinator interactions that are mediated by floral volatiles, both floral signals and olfactory responses in the pollinators
may be shaped by coevolution. Likely examples include the fig–fig wasp, yucca–yucca moth, Glochidion–Epicephala moth and Lithophragma–Grega moth pollination systems [13–16] in which there is already some evidence that volatiles mediate their extraordinary reciprocal specificity [17–19]. Variation in floral volatiles between species in these systems is usually associated with different pollinators. If coevolution, rather than unilateral pollinator shifts, has shaped these plant–pollination mutualisms, then one would predict that plant volatile signals and pollinator responses to those signals will also covary among populations in early stages of divergence.

Divergence in floral traits is often attributed to geographical mosaics or clines in pollinator composition, an idea known as the ‘pollinator-shift’ or ‘Grant–Stebbins’ model of divergence [20,21]. The rationale is that different pollinators often have different floral preferences, thus geographical variability in pollinator composition would be likely to result in divergent selection pressures and lead to the evolution of ‘pollination ecotypes’ [22]. These ecotypes may also evolve because pollinators exhibit geographical variation in their preferences due to differences in conditioning [21]. Yet another scenario is that both floral signals and pollinator preferences coevolve at the population or regional level, producing a pattern of geographically structured covariation in pollinator and plant traits [23,24].

The pollination system of cycads typically involves brood-site mutualisms in which the pollinators are insect herbivores whose larvae feed on male cone tissues and female cones are pollinated when visited by mistake [25–29]. Pollination in these dioecious gymnosperms is mediated by specific cone volatile compounds [30]. The brood-site mutualisms in cycads probably evolved from herbivore interactions in which herbivorous insects were attracted to specific cues associated with nutrient-rich cycad reproductive structures [31]. The obligate nature of these brood-site mutualisms makes it likely that cycads and their insect pollinators have subsequently coevolved [25,32,33]. A recent study of the African cycad Encephalartos villosus revealed remarkable intraspecific geographical variation in the profile of volatiles emitted from cones [34]. The main compounds emitted by cones of E. villosus from the southern distribution range were eucalyptol and 2-isopropyl-5-methoxypyrazine, whereas plants from the northern range emitted (Z)-1,3-octadiene and (E,Z)-1,3,5-octatriene. The pollinators Porthetes sp., Erotylidae sp., Nov., M. goodei and A. zamiae associated with E. villosus cones were tested for their electrophysiological responses to cone volatiles sampled from the SOUTH 1, SOUTH 2, NORTH 1 and NORTH 2 populations. For each analysis, the head was excised, and a few segments on the tip of the two antennae were cut off and mounted onto two silver metal electrodes using an electrical conductive gel (Spectra 360, Parker Inc, Orange, NJ, USA).

The GC column (DB-wax) was 30 m × 0.25 mm internal diameter × 0.25 μm film thickness (Alltech, Deerfield, IL, USA) and had a Y-tube connection installed at the end to split the stream of volatile compounds into two equal parts, one going to the FID and one to the EAD, where a prepared antenna had been inserted under a humidified air stream to prevent the antennae from drying out. The GC–EAD data were analysed using AUTOSPIKE v. 3.3 software (Syntech, Kirchzarten, Germany). A total of 24 GC–EAD analyses (six individuals per beetle species) were carried out to determine the responses of beetles. A compound was considered to be physiologically active when it consistently elicited antennal responses in at least three runs with three different antennae.

(d) Field bioassays with bucket traps

To test the effects of the electrophysiologically active compounds on cycad insect behaviour, bioassays were carried out in the field using bucket funnel traps obtained from Insect Science, South Africa. Because colour influences the behaviour of several insect species [38–40], we conducted trials to determine whether the colour of the bucket traps would affect the behaviour of cycad insects. Pollen-shedding cones were used as lures in five green and five yellow bucket traps. These were placed together with an equivalent

2. Experimental procedures

(a) Study area and plant material

Field bioassays were conducted between 2009 and 2010 in natural populations of E. villosus in the southern and northern parts of the distribution range in the Eastern Cape (EC) and KwaZulu-Natal (KZN) provinces of South Africa. The populations were Umtiza Nature Reserve (SOUTH 1) and Ocean View Guest Farm (SOUTH 2) near East London in EC, and Oribi Gorge Nature Reserve (NORTH 1) and at the Kranzkloof Nature Reserve (NORTH 2) in KZN (figure 1a–c).

(b) Collection, analysis and identification of volatile compounds

To confirm the geographical variation of cone volatile composition documented in a previous study [34], volatile odours were sampled from male and female cones of E. villosus in the SOUTH 1, SOUTH 2, NORTH 1 and NORTH 2 populations between 2012 and 2013. Volatile compounds were analysed and identified using the same collection methods and gas chromatography–mass spectrometry (GC–MS) protocols described previously [34].

(c) Gas chromatography–electroantennogram detection

We used combined gas chromatography–electroantennogram detection and a flame ionization detector (GC–EAD–FID) [37] to identify physiologically active compounds in headspace samples of cones of E. villosus. Porthetes sp., Erotylidae sp. nov., M. goodei and A. zamiae associated with E. villosus cones were tested for their electrophysiological responses to cone volatiles sampled from the SOUTH 1, SOUTH 2, NORTH 1 and NORTH 2 populations. For each analysis, the head was excised, and a few segments on the tip of the two antennae were cut off and mounted onto two silver metal electrodes using an electrical conductive gel (Spectra 360, Parker Inc, Orange, NJ, USA).

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number of empty bucket traps of the same colour serving as controls in the botanic garden of the University of KZN.

To test whether individual compounds attracted insects to traps, \(3E\)-1,3-octadiene and 2-methoxy-3-isopropylpyrazine, two electrophysiologically active compounds, and eucalyptol, a compound that consistently occurs in substantial concentration in almost all populations, were used as baits in bucket traps set-up in the SOUTH 1, SOUTH 2, NORTH 1 and NORTH 2 populations. The electrophysiologically active alkene \(3E,5Z\)-1,3,5-octatriene was omitted from this analysis as it is not commercially available. For each treatment, we used the same proportions (50 : 50 or as close to this as possible) of green and yellow bucket traps. Solutions of 50 \(\mu l\) of either \(3E\)-1,3-octadiene, 2-methoxy-3-isopropylpyrazine, or eucalyptol and 950 \(\mu l\) of liquid paraffin (Alpha Pharma, South Africa) used as baits were put into amber glass bottles with cotton wicks inserted through the cork to facilitate gradual release of volatile compound. Paraffin oil without the test compounds served as a control. Each bait bottle or control was placed into an individual bucket trap. The bucket traps were placed about 15 m apart in an area with \(E.\) villosus individuals. Each trial lasted 24 h (starting at 9.00 and ending at 9.00 the following day), after which the positions of the traps were randomized and the number and identity of insects were recorded. Each treatment and control had five replicates and each trap was monitored for five days, apart from those at NORTH 2, which were additionally sampled with seven replicates each on day 5 and 6, and those at NORTH 1, which were additionally sampled on days 6, 7 and 8 with nine, eleven and eight replicates each, respectively.

(e) Olfactometer tests with cycad cones

Olfactometer assays were carried out in the laboratory between 2009 and 2013 using cycad cones at the pollen-shedding stage.
to determine how individuals of the known pollinator *Porthetes* sp. collected from the southern and northern range of *E. villosus* responded to cone volatiles of *E. villosus* from these two regions. Two types of experiment were undertaken under laboratory conditions (temperatures between 25.0°C and 28.0°C) using male cones from plants in the southern and northern populations. In the first, pollen-shedding cones of plants from the southern and northern regions were offered to *Porthetes* sp. individuals from these two regions in a design where beetles had a choice between an arm containing cycad cone sporophylls and an empty arm containing pure air. In the second, pollen-shedding cones of *E. villosus* from both regions were simultaneously offered, each in a different arm of the olfactometer, to insects from northern and southern populations, respectively. Each experiment was replicated 12 times using 10–20 beetles per replicate, and for each replicate in the different experiments fresh cone samples and a new batch of insects were used.

The olfactometer used was a simple Y-tube (stem 10 cm, arms 8 cm at 45° angle, internal diameter 2 cm) and was placed on a light table so as to have homogeneous lighting and one arm connected to a polyacetate bag (Nalo Bratfolie Kalle GmbH) containing an odour source (cone sporophylls) and the other arm connected to an empty polyacetate bag with pure air. Air was drawn through silicon tubing by a portable battery-operated pump (Spectrex Personal Air Sampler PAS 500) set at 200 ml min⁻¹ and was split at the Y-junction to create equal air streams. Beetle pollinators were introduced to the base of the stem of the olfactometer using an insect holder. The response from the beetle was determined when they (a) entered one of the arms and stayed there, and (b) left the olfactometer arm and crawled into the polyacetate bag. Beetles that remained in the holder and the stem of the Y-maze were considered to have not made a choice. Ventiing of each odour field continued for an hour and the number of beetles collected in the different arms of the olfactometer was counted. After each experiment, the olfactometer was cleaned with 70% EtOH, rinsed with acetone and baked at 250°C for 2 h. On running the next experiment, the control arm of the olfactometer was switched from left to right or vice versa to avoid bias.

(f) Data analysis

Statistical analyses for bucket trap data were analysed using restricted maximum-likelihood (REML) and generalized linear mixed models implemented in GenStat v. 12.1. These models used a negative binomial error distribution with a log-link function and the aggregation factor was set to 1. Comparisons among means were conducted using Tukey tests. Models had a logit-link function and significance of effects was assessed by using likelihood ratio statistics. Means and standard errors were back-transformed from the logit scale.

3. Results

(a) Volatile emissions

The volatile compounds emitted by cones from different populations of *E. villosus* conformed to the same overall geographical pattern as described in a previous study [34] (figure 1a). The cone odour of *E. villosus* from SOUTH 1 and SOUTH 2 populations was dominated by the monoterpene 2-isopropyl-3-methoxypyrazine and the monoterpenes eucalyptol, with α-terpineene, α-pinene and β-pinene contributing substantial amounts. The unsaturated hydrocarbons (3E)-1,3-octadiene and (3E,5Z)-1,3,5-octatriene characterized the cone odour of NORTH 1 and NORTH 2 plants, together with eucalyptol and other monoterpenes also found in SOUTH 1 and SOUTH 2 plants (figure 1a).

(b) Electrophysiological response to cone volatiles

Three compounds in the odour profile of *E. villosus* were consistently electrophysiologically active in repeated GC–EAD experiments (figure 1d–f). Two compounds—(3E)-1,5-octadiene and (3E,5Z)-1,3,5-octatriene emitted by *E. villosus* in its northern distribution range—elicited electrophysiological responses in Erotylidae sp. nov. and *Porthetes* sp. collected from the northern region (figure 1d,e). There were no responses from *M. goodei* and *A. zamiae* from the northern populations. The compound 2-isopropyl-3-methoxypyrazine emitted by *E. villosus* from the southern region elicited electrophysiological responses in *Porthetes* sp. from the southern region (figure 1f). Erotylidae sp. nov., *M. goodei* and *A. zamiae* from the southern populations did not respond to any compounds.

(c) Field trapping experiments with volatile compounds

In field trapping experiments, *A. zamiae*, Erotylidae sp. nov., *M. goodei* and *Porthetes* sp. were attracted to traps containing...
volatile compounds. *Porthetes* sp. at the northern sites were attracted mainly to (3\textit{E})-1,3-octadiene (figure 2a), whereas those at the southern sites were attracted mainly to 2-isopropyl-3-methoxypyrazine (figure 2b). \textit{Erotylidae} sp. nov. and \textit{A. zamiae} showed a similar pattern but were attracted in lower numbers (figure 2a, b). Relatively few beetles were recovered from traps baited with eucalyptol (figure 2a, b). Overall, the interaction of region and compound type had a highly significant (\(p < 0.001\); n.s., not significant) effect on the abundance of beetles attracted to the traps (electronic supplementary material, table S1). For the individual beetle species, the interaction of region and volatile compound was a strongly significant factor explaining the abundance of trapped *Porthetes* sp., \textit{A. zamiae}, \textit{Erotylidae} sp. nov. and \textit{M. goodei} (electronic supplementary material, table S1).

**Figure 2.** Number (mean ± s.e) of beetle individuals of different species attracted to (3\textit{E})-1,3-octadiene, eucalyptol or 2-isopropyl-3-methoxypyrazine in trapping experiments in different \textit{E. villosus} populations. The means and standard errors are back-transformed from the log scale. Significance values (****\(p < 0.001\); n.s., not significant) are given for predictors and their interactions. Letters above bars denote homogeneous groups (based on Tukey tests) for each beetle species across the different compounds. The key shows beetle species and sexual dimorphism for one species. Scale bar, 10 mm. (Online version in colour.)

(d) Behavioural response of *Porthetes* sp. to cone volatile compounds

In the experiments, a high proportion of beetles entered the olfactometer and chose either the scented or control arms (figure 3a). In olfactometer choices between a cone scent and an unscented control, responses of beetles differed significantly according to whether cones were from the same region as the beetles or from a different region. When beetles from the southern and northern populations were separately offered cones from corresponding populations and vice versa, they were more attracted to cones from the same region (\(\chi^2 = 105.65, \ p < 0.0001, \ n = 48\) trials; figure 3b). When pollen-dehiscent cones from both regions were simultaneously
offered to *Porthetes* sp., responses of individuals from the southern and northern regions differed ($\chi^2 = 39.95$, $p < 0.0001, n = 24$ trials), with each showing a significant preference for local cones (figure 3c).

### 4. Discussion

This study provides evidence for geographical matching of plant volatiles and insect physiological and behavioural responses. Olfactometer experiments showed that the weevil pollinator *Porthetes* sp. is attracted to *E. villosus* cones from its local region, rather than to *E. villosus* cones from a different region (figure 3b,c). The chemical basis for this pattern is revealed in the electrophysiological experiments in which antennal responses by beetles were detected in response to volatiles emitted by cones from the same region as the beetles. This was confirmed by the field bioassays, which showed that the main pollinators in the northern populations responded most strongly to (3E)-1,3-octadiene (figure 2a) while those in the southern populations responded most strongly to 2-isopropyl-3-methoxypyrazine (figure 2b). These patterns are consistent with the idea of coevolutionary divergence in obligate brood-site mutualisms [16,19,41].

Volatiles appear to play a central role in attraction of pollinators in specialized brood-site mutualisms. In the fig–fig wasp mutualism, species specificity appears to be maintained by responses of the pollinating wasps to specific fig volatiles, although there is still uncertainty about which compounds mediate these interactions [42,43]. There is also evidence that specific volatiles mediate the brood-site pollination mutualism between *Glochidion* trees and *Epicephala* moths, and between *Lithophragma* plants and *Greta* moths [16,17]. In such highly species-specific brood-site mutualisms, plants may evolve one or a few specific and unusual compounds that represent ‘private channels’ in the sense that they would not be detected by other pollinators in the same communities [4,44]. However, this idea has not received much empirical support as most compounds reported thus far in the floral scent of brood-site pollination mutualisms are common volatile constituents of leaves and flowers [6]. In the yucca–yucca moth mutualism, yucca moths are attracted to yucca floral scent [18] and the odour profiles of three allopatric species contain a physiologically active compound (E)-4,8-dimethyl-1,3,7-nonatriene together with similar odour compounds that are widespread in flowering plants [11,18]. The physiologically active compound may function as a private communication channel, but its role remains to be determined. In this study, we found that (3E)-1,3-octadiene and (3E,5Z)-1,3,5-octatriene emitted by *E. villosus* from the northern region and 2-isopropyl-3-methoxypyrazine emitted by *E. villosus* from the southern region elicit electrophysiological responses (figure 1d–f) and attract pollinators of *E. villosus* in their respective regions (figure 2a,b). These compounds are rare and have been recorded in only a few plants [45,46], and may thus function as private channels of communication in the communities in which these cycads occur. The only previous report of attraction of a pollinator to a pyrazine compound involves a sexually deceptive Australian orchid that mimics the pyrazine sex pheromone of female thynnine wasps [47].

What makes the *E. villosus* cycad pollination system particularly novel is the geographical variation in key attractive compounds and the preference of widespread pollinators for

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**Figure 3.** Mean (± 95% CI) proportional responses of *Porthetes* sp. during olfactometer choice trials involving *E. villosus* cone volatiles. (a) Proportions of beetles that responded in the trials when they entered any arm of the olfactometer. (b) Proportions of responding beetles that chose the scented arm of the olfactometer rather than the unscented arm when beetles were separately offered cones from corresponding region and vice versa. (c) Response of *Porthetes* sp. when *E. villosus* cone volatiles from EC and KZN were simultaneously offered to the beetles from each region in a series of olfactometer choice experiments. The means and CI values are back-transformed from the logit scale. Significance values (**p < 0.001; n.s., not significant) are given for predictors and their interactions. Means with confidence intervals that do not overlap the dotted lines of equal choice in (b,c) represent significant preference for the scented arm. (Online version in colour.)
local plant chemotypes. The close correspondence between chemical signals of *E. villosus* and the olfactory responses of its pollinators suggests that these interactions may have been shaped by coevolution [48]. Nevertheless, it is possible that there has been adaptation of the cycads to cryptic weevil species, which would be a case of divergence driven by shifts in pollinators, rather than coevolution [49,50]. However, a recent molecular study of phylogenetic relationships within *Porthetes* spp. showed that specimens from different *E. villosus* populations across the distribution range had identical sequences for the mitochondrial cytochrome oxidase 1 (CO1) gene [51], with no obvious morphological characters separating specimens from northern and southern *E. villosus* populations. It is also possible that *E. villosus* consists of two geographically separate species. Few molecular data are available for *Encephalartos* due to notoriously low genetic variation within the genus [52], and among cycads in general [53,54].

There is some morphological variation in *E. villosus*, with plants from the southern region having shorter, heavily spined leaflets and cones with toothed edges, and those from the northern region having longer, almost entire leaflets and cones with lightly toothed edges [55]. However, this morphological variation is largely clinal and has been regarded as insufficient to split *E. villosus* into different species. There is no difference in the beetle assemblages on cones in different populations of *E. villosus* [34], but the geographical differences we observed in beetle olfactory responses may influence the direction and intensity of selection on plant traits leading to divergence and hence formation of chemotypes.

The cognitive basis of pollinator attraction to floral volatiles can be innate [56] or learned [57]. The sensory preferences of insects involved in highly specialized brood-site mutualisms are expected to be innate [58,59], and may arise from mutual adaptations of the pollinators’ sensory systems and floral signals. This contention is supported by the differences in electrophysiological responses to particular compounds that we observed for beetles from different populations (figure 1d–f). An alternative explanation for the finding that beetles respond positively to scent of local cones when offered local and non-local cones simultaneously (figure 3c) is that beetles undergo a chemical imprinting process during their development on local cones [60–62]. Interestingly, we found some evidence of active avoidance of cones that are not from the local region (figure 3b), suggesting that there are volatiles in these cones that affect behaviour of local beetles, even though we did not detect antennal electrophysiological responses to volatiles from non-local cones. Absence of detectable electrophysiological responses of insects to volatiles can, however, be simply due to limitations of the recording equipment.

As is the case for the fig–fig wasp mutualism [42,43], cycad brood-site mutualisms are not always completely reciprocally specialized. Some of the beetles that pollinate *E. villosus* also visit male and female cones of other *Encephalartos* cycads. The *Porthetes* sp. in this study also occurs on cones of *E. aplanatus* and *E. umbeluziensis* [51], which are phylogenetically related to [52] and out of the range of *E. villosus*. *Encephalartos aplanatus* emits the same volatile compounds as *E. villosus* in its northern distribution range, while *E. umbeluziensis* emits compounds that are different from those in any *E. villosus* populations [63]. Hence some cycad weevil species may have coevolved with more than one cycad species.

In mutualisms, coevolution may occur mainly between strongly interacting species, and the resulting trait evolution may be tracked by other species that interact less strongly [1,64]. Since *Porthetes* sp. is the major pollinator of *E. villosus* [26], we expect this species to have coevolved with *E. villosus*. By contrast, the responses of Erotylidae sp. nov., *M. goodei* and *A. zamiae* to the volatile odours of *E. villosus* may reflect tracking of chemical traits that have diverged between populations, as these beetles have a near-obligate dependence on its cones but do not contribute to pollination of *E. villosus* to the same extent as *Porthetes* sp. [26].

The beetles Erotylidae sp. nov. and *M. goodei*, which are attracted to *E. villosus* volatiles, also visit the cones of *E. natalensis* [65], which similarly emits (3E)-1,3-octadiene [63]. They play a small role in pollination and occur in low numbers in male and female cones of *E. villosus* [26,36], where their larvae develop in central axis and sporophyll tissues of both male and female cones in the case of entolittids, and in male cone tissues in the case of *M. goodei* [26]. Other entolittids and *Metacucujus* spp. occur on *E. natalensis* and other *Encephalartos* cycads [29,66,67] that overlap with *E. villosus* distribution and emit different compounds [63]. These beetles contribute to pollination of various *Encephalartos* species [26,29,67] and may have coevolved with the genus. The taxonomy of Erotylidae sp. nov. in South Africa has not been studied and there are no molecular studies of this beetle to provide any information on species-level variation. It is thus difficult to confirm for this secondary pollinator whether there is only one species that has coevolved with *E. villosus* across the distribution range, and whether it has been involved in coevolution with *E. villosus* or, alternatively, has either tracked evolutionary changes in the cycad or shows local chemical imprinting according to the brood site.

The beetle seed predator *A. zamiae* occurs in very low numbers in male cones and is a minor pollinator of *E. villosus* [26], but occurs in large numbers in female cones during the pollination stage, where they mate and oviposit, and their larvae develop in the seeds [26,36]. Though attracted in small numbers, there was some evidence that they could distinguish between the alkene and pyrazine compounds offered in traps at the southern sites (figure 2b; electronic supplementary material, table S1). These beetles also occur in other *Encephalartos* species [68] that overlap in distribution and volatile compound emission in *E. villosus*, as well as others that emit different compounds [63], suggesting that they may also use additional cues for host plant location. They have a rostrum length that varies according to the seed size of different cycad species [69]. This phenotypic trait matching suggests that they have coevolved with various cycads.

This study shows that plant volatiles and signal perception and cognition by insects involved in specialized brood-site mutualisms may reflect coevolutionary processes that drive divergence among populations, and could ultimately lead to speciation. The divergence in the odour profile observed among populations of *E. villosus* may be viewed in the light of the geographical mosaic theory of coevolution [23]. This theory predicts that geographical differences in selection pressures due to interactions not just between strongly interacting species pairs, but also with other species in local communities, as well as genetic drift and migration, create a range of possible outcomes [24]. These varying outcomes at the population level lead to a geographical mosaic of coevolutionary hotspots and cold spots [1,23,24]. Our study suggests that localized coevolution may have occurred between beetles and *E. villosus* despite the lack of variation in molecular markers among populations.
of these organisms. Further studies on chemical signal perception and cognition in the beetle pollinators together with efforts to resolve the detailed phylogeny of cycads and their pollinators will be likely to improve our understanding of cycad–insect coevolution.

Ethics. The experiments conducted herein comply with the ethical regulations of South Africa.

Data accessibility. These data are available on Dryad: http://dx.doi.org/10.5061/dryad.t6g5t.

Authors’ contributions. T.N.S., J.S.D. and S.D.J. conceived the study; T.N.S. carried out the research (designed the study field data and wrote the manuscript); T.N.S. and S.D.J. analysed data; T.N.S., J.S.D. and S.D.J. contributed substantially to the revision.

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among populations of the African cycad