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Variation in the chemical composition of cone volatiles within the African cycad genus *Encephalartos*

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ABSTRACT

Volatiles play a key role in attraction of pollinators to cycad cones, but the extent to which volatile chemistry varies among cycad species is still poorly documented. Volatile composition of male and female cones of nineteen African cycad species (*Encephalartos*; Zamiaceae) was analysed using headspace technique and gas chromatography–mass spectrometry (GC–MS). A total of 152 compounds were identified among the species included in this study, the most common of which were monoterpenes, nitrogen-containing compounds and unsaturated hydrocarbons. Male and female cones emitted similar volatile compounds which varied in relative amounts with two unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene present in the volatile profile of most species. In a multivariate analysis of volatile profiles using non-metric multidimensional scaling (NMDS), a number of species clusters were identified according to shared emission of unsaturated hydrocarbons, pyrazines, benzenoids, aldehydes, alkanes and terpenoids. In comparison, terpenoids are common in *Zamia* and dominant in *Macrozamia* species (both in the family Zamiaceae) while benzenoids, esters, and alcohols are dominant in *Cycas* (Cycadaceae) and in *Stangeria* (Stangeriaceae). It is likely that volatile variation among *Encephalartos* species reflects both phylogeny and adaptations to specific beetle pollinators.

1. Introduction

When different plant species are pollinated by the same group of pollinators, and especially by closely related pollinator taxa, they may be expected to have floral volatiles with similar chemical constituents (e.g. Knudsen and Tollsten, 1995). This expectation is based on data showing that floral volatiles influence the composition and behaviour of pollinators (Dodson et al., 1969; Pellmyr, 1986; Dobson et al., 1997; Raguso, 2008) and act as important cues in plant–pollinator interactions (Dobson, 2006; Knudsen et al., 2006; Raguso, 2008). However, floral odours can be complex blends that can influence pollinators in different ways (Raguso, 2008) and it is not always clear which compounds act as the main triggers for pollinator behaviour or how these compounds vary between species or populations (Raguso, 2004, 2008).

There are examples in which plant species that are pollinated by the same functional group of pollinators vary in their floral volatile compounds (e.g. Miyake et al., 1998). Such variation in floral volatiles may be due to adaptations to different pollinator species, environmental conditions such as climate and soil chemistry, which can have significant effects on floral scent (Jakobson and Olsen, 1994), or phylogenetic influences.

Variation in volatiles can occur even among populations of a plant species (e.g. Anderson et al., 2010). The recent discovery of geographic variation in the cone volatiles of *Encephalartos villosus* Lem. (Suinyuy et al., 2012) raised important questions about how pollination may be mediated by volatile compounds across different environments. Results from previous studies showed that in some parts of its range, *E. villosus* emitted the same biologically active compounds as other cycads in the area, e.g. (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene emitted by *E. villosus* plants in KwaZulu Natal (KZN) populations (Suinyuy et al., 2012) that is also emitted by *E. natalensis* R. A. Dyer & I. Verd (Suinyuy et al., 2010). This suggests that there could be local convergence in floral volatiles among unrelated species that share similar pollinators. In contrast, in other parts of its range *E. villosus* emitted compounds that have not yet been isolated from other cycads in the same area, e.g. 2-isopropyl-3-methoxypyrazine in the Eastern Cape (EC) plants (Suinyuy et al., 2012). It is thus not yet clear whether cycads in a given area show convergence or divergence in floral odours.

Southern Africa has a wide range of biomes (Rutherford et al., 2006) and a complex mosaic of vegetation types and is an ideal

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area in which to examine variation in volatile compounds across different environments. The 37 species of *Encephalartos* that occur in South Africa (Donaldson, 2003; Hill et al., 2007) are distributed across a range of different biomes and vegetation types. Although many *Encephalartos* species are restricted to a particular vegetation type (e.g. grassland, forest or savannah), and some are endemic to small areas comprising one vegetation type (Dyer, 1965; Donaldson, 1993; Jones, 1993; Goode, 2001), the majority of species were historically distributed across several vegetation types on different soils. This means that it is possible to determine whether the pattern of geographical differences observed in *E. villosus* is more widespread within the genus *Encephalartos.*

Comparisons of volatile compounds across species should take phylogenetic relatedness into consideration (e.g. Azuma et al., 1997; Williams and Whitten, 1999; Levin et al., 2003). Such an analysis is difficult for *Encephalartos* because the phylogeny for the genus is poorly resolved due to low genetic variation within the genus (Treutlein et al., 2005). Nevertheless, genetic analysis by Treutlein et al. (2005) has revealed several well defined clades and these authors even raised a question about whether these clades represent separate subspecies rather than well differentiated species groups. As a result, it should be possible to compare variation in volatile compounds within and between clades despite the absence of a complete phylogeny. One of the best supported clades is the group that includes *E. villosus* and comprises *E. aplanatus* Vorster, *E. caffer* (Thunb.) Lehm., *E. cerinus* Lavranos and D. L. Goode, *E. ngoyanus* I. Verd. and *E. umbeluziensis* R. A. Dyer that all occur within or close to the current distribution range of *E. villosus.* Although these species are phylogenetically related and grow geographically close to one another, they have diversified into forest, savannah and grassland species and therefore provide an opportunity to analyse several factors that may influence volatile profiles.

A second well supported clade is the so-called 'woolly-coned' cycads comprising *E. cycadifolius* (Jacq.) Lehm., *E. friderici-guilielmi* Lehm., *E. ghellinckii* Lem., *E. humilis* I. Verd., *E. lanatus* Stapf & Burtt Davy and *E. laevifolius* Stapf & Burtt Davy. This group includes relatively widespread species (e.g. *E. friderici-guilielmi*) and localised endemics (*E. cycadifolius*) within grassland vegetation types so that it should be possible to determine whether differences occur even within a group that is phylogenetically related and restricted to a single broad vegetation type.

A key part of an analysis of variation in cone volatiles is a comparison of species with closely related pollinators relative to species with different pollinators. Pollination studies on three species of South African *Encephalartos* have shown that there are at least two pollination systems within *Encephalartos*, one involving the weevil genus *Porthetes* (Coleoptera: Curculionoidea) (Donaldson, 1997; Suinyuy et al., 2009) and another involving species of cucujid beetles (Coleoptera) belonging to the genus *Metacucujus* (Boganiidae) or the family Erotylidae (Cucujoidea) (Donaldson et al., 1995; Donaldson, 1997; Suinyuy et al., 2009). In some cycad species, e.g. *E. friderici-guilielmi*, both systems are present whereas in species such as *E. cycadifolius* only one system has been found. Although the pollination biology of other *Encephalartos* species is not known, the distribution of insects on cones of *Encephalartos* is relatively well studied (Donaldson, 1991, 1999; Oberprieler, 1995a,b; Downie et al., 2008; J. Donaldson unpublished data) and it is possible to examine whether variation in cone volatiles is associated with different pollination systems.

The first aim of this study was to document variation in volatile compounds from both male and female cones across a relatively large number of *Encephalartos* species that represent different life histories, phylogenetic clades, pollination systems, geographical distributions and associations with different vegetation types (Table 1). A second aim was to investigate whether there are differences in the volatile compounds of male and female cones and whether the observed variation in volatile composition was correlated with geographical distribution, phylogenetic clades or pollination systems. This is the first study that provides such a comprehensive breakdown of cone odour composition in a relatively large number of *Encephalartos* species (ca. 30% of all taxa, 51% of South African taxa). Previous studies have analysed scent mostly in a few single species of *Encephalartos* (e.g. *E. altensteinii* Lehm., Pellmyr et al., 1991; Tang, 1993; *E. natalensis*, Suinyuy et al., 2010) or comparing single *Encephalartos* species with species from other cycad genera (e.g. Pellmyr et al., 1991; Tang, 1993) and comparing scent within a few species of *Zamia* L. (Pellmyr et al., 1991; Tang, 1993) and *Macrozamia* Miq. cycads (Terry et al., 2004a,b).

2. Results and discussion

The chemical composition of cone odours of the 19 sampled *Encephalartos* taxa (including two *E. villosus* chemotypes) consisted of a variety of volatile compounds identified by common names and CAS (Chemical Abstract Service) registry numbers and listed according to the estimated Kovats Retention Index (KRI) (Kovats, 1958) and in chemical classes (Table S1). A total of 152 compounds were detected and identified, which comprised 74 terpenoids (56 monoterpenes and 18 sesquiterpenes), 52 fatty acid derivatives or aliphatics (14 alcohols, 10 aldehydes, nine ketones, six unsaturated hydrocarbons, five aliphatic acids, four alkanes and four esters), 22 benzenoids and four nitrogen-containing compounds. All four nitrogen-containing compounds consisted of pyrazines. The most frequently occurring compounds were benzaldehyde and limonene in 18 species each, heptanal, benzyl alcohol and phenol in 12 species, β-caryophyllene in 11 species and (3*E*)-1,3-octadiene and methyl benzoate in nine species. Most of the compounds were detected in small relative amounts (between trace amounts and <10%) as only 27 compounds (three alkanes, two unsaturated hydrocarbons, one aliphatic acid, three aldehydes, one ketone, two alcohols, three benzenoids, nine monoterpenes, one sesquiterpene and two nitrogen-containing compounds) reached a relative amount of ⩾10% (Table S1). When both sexes were analysed, the mean number of compounds emitted was significantly higher in male cones (13.52 ± 0.42, mean ± SE) than female cones (10.02 ± 0.59) (ANOVA, $F_{1,258}$=23.29; $P < 0.001$). Out of 12 species where both sexes were tested, significant differences in the number of compounds between sexes were found in six species (*E. altensteinii* and *E. villosus* from the EC, $P < 0.01$; *E. friderici-guilielmi*, $P < 0.05$; *E. ghellinckii*, *E. humilis* and *E. natalensis*, $P < 0.01$).

Comparison of odour profiles between taxa from the Eastern Cape (9 taxa) and KwaZulu Natal (8 taxa) showed that there was an overrepresentation of hydrocarbons in the odour profiles of KZN taxa (50%) compared to EC taxa (22%) and an overrepresentation of 2-methoxy-3-methylpyrazine in EC taxa (44%) compared to KZN taxa (12.5%) but they were not statistically significant (χ^2 = 2.73, $P > 0.05$). These patterns were consistent with those found in *E. villosus* (Suinyuy et al., 2012).

Compounds emitted in small relative amounts have been suggested to contribute to species isolation in co-occurring plants (Okamoto et al., 2007) and play a critical role in plant–pollinator interactions (Schlumpberger and Raguso, 2008). This may be the case in *Encephalartos* in which the majority of compounds recorded from the taxa were emitted in small relative amounts ranging from trace amounts to just above 9% (Table S1). Terpenoids were the most numerous compounds in the volatile profiles of *Encephalartos*, but only nine monoterpenes and a sesquiterpene occurred in high relative amounts (⩾10%). The majority of terpenoids occurred in small relative amounts and their possible influence on pollinator