Spatial variation in the fatty acid composition of elaiosomes in an ant-dispersed plant: Differences within and between individuals and populations

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\textbf{A B S T R A C T}

\textit{Euphorbia characias} is a common myrmecochorous plant of the western Mediterranean Basin whose seeds are dispersed by ants following fruit explosion. The variation in elaiosomes’ fatty acid composition of this species was studied at three hierarchical levels (sub-individual, individual and population) in four populations from the Iberian Peninsula. We found that differences in fruit location on the inflorescence do not seem to influence the fatty acid composition of elaiosomes, providing to each propagule an equal chance of being dispersed. However, significant differences in elaiosome fatty acid composition between individuals and populations were found for most of the compounds identified. The content of oleic acid, a key mediator in the ant–seed interaction, differed widely between populations, probably reflecting geographic variations in co-adaptation between plants and their dispersers. The finding that the fatty acid composition of \textit{E. characias} elaiosomes is distinct from that of the seed itself, but very similar to that of elaiosomes from unrelated species, reinforces the idea of convergent evolution in the chemical composition of these structures.

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\textbf{Introduction}

Dispersores are biological structures subjected to strong natural selection by both animal dispersers and predators (Fenner and Thompson, 2005; Harper et al., 1970; Howe and Smallwood, 1982). In myrmecochorous plants, seeds of which present specific adaptations for dispersal by ants, the seeds have a specialized external appendage named elaiosome. The elaiosome is a lipid rich structure that functions by luring ants to retrieve the seeds to their nests (Beattie, 1985; Sernander, 1906). This ant–plant relationship has been generally labelled as a facultative mutualism, with plants benefitting from having their seeds dispersed away from the parent plant, having lower losses to post-dispersal seed predators and eventually having the possibility to reach better microsites for germination and establishment (Beattie, 1985; Giladi, 2006), and ants getting benefits by feeding on the nutrient rich elaiosomes (Bono and Heithaus, 2002; Fischer et al., 2008; Gammans et al., 2005; Morales and Heithaus, 1998). The elaiosomes are particularly rich in lipids, aminoacids and monosaccharides, and provide various nutrients that are essential for insect reproduction and development (Bresinsky, 1963; Brew et al., 1989; Fischer et al., 2008). Among lipids, fatty acids are major components of elaiosomes and a few of these compounds (e.g., oleic or oleyl-based glycerides) have been identified as mainly responsible for elaiosome attractiveness by ants (Brew et al., 1989; Marshall et al., 1979; Skidmore and Heithaus, 1988). Consequently, some studies have focused in assessing the fatty acid composition of elaiosomes of a plant species, occasionally drawing interspecific comparisons on fatty acid profiles (Lanza et al., 1992; Mackay and Whalen, 1998; Pfeiffer et al., 2010; Soukup and Holman, 1987), but only a few of them have assessed the effects of natural variation in the fatty acid profile within species (Boulay et al., 2006, 2007).

Biological characteristics are subjected to variation, which can be wide even within the same spatial and temporal scale. By incorporating natural variation in ecological studies, we may achieve a better interpretation of the phenomena under study and provide more accurate predictions. In \textit{Euphorbia characias}, seed set, seed quality and seedling survival were demonstrated to be intimately related with variation due to plant architectural effects (Espadaler and Gómez, 2001): distal fruits produced more and bigger seeds than basal ones. Moreover, seedlings issued from distal seeds have enhanced emergence and survival than seedlings from basal fruits. Therefore, the question arises whether elaiosomes of distal fruits have a different quality from those of basal fruits and how large is that variation between individuals and populations. The aim of this study is to identify the fatty acid profile of \textit{Euphorbia characias}...
elaiosomes and evaluate variations in their composition across three hierarchical levels: sub-individual, individual and population levels. We specifically investigate if: (a) fruit position affects elaiosome fatty acid composition, (b) elaiosome composition shows variation between individuals within a population, and (c) elaiosome composition shows variation between populations in the Iberian Peninsula.

Materials and methods

**Plant and study sites**

*Euphorbia characias* L. is a common woody perennial spurge with a western Mediterranean distribution and presents distinctive morphological features making its discrimination from related congeners relatively easy (Benedit et al., 1997). This species has a variable number of floral stems where flowers are arranged in inflorescences. The floral stems have a long leaf-free basal part, a leafy medial zone, and a distal elongated inflorescence, which is somewhat cylindrical in shape and has a terminal pseudumbel on top (Fig. 1). Flowers can be found in the upper part of the floral stem, where they occur sparsely distributed around the stem or more aggregated in the terminal pseudumbel (Fig. 1). The flowering period takes place between January and April and fruit maturation lasts until early June. The fruit is a green capsule enclosing three seeds. Seeds are light grey, ellipsoid in form and have a yellowish elaiosome. *Euphorbia characias* has a diplochorous dispersal system where seeds are first ballistically ejected getting scattered on soil at short distances (up to 4 m; unpublished data). Then, some ant species (e.g., *Aphaenogaster senilis, Pheidole pallidula, Tapinoma nigerrimum*) retrieve the diaspores to their nests where they consume the elaiosome, leaving the intact seeds inside the galleries or discarding them outside, where they may germinate (Bas et al., 2007; Espadaler and Gómez, 1996).

The samples for analysis were collected from four populations of the Iberian Peninsula: (1) Can Llevallo (41°24′N, 2°05′E; elevation 315 m), within the Collserola Natural Park, a preserved area close to Barcelona (eastern Spain), (2) Montejunto (39°10′N, 9°03′W; elevation 510 m) included in the Serra de Montejunto protected area (western Portugal), (3) Tesoureira (38°56′N, 9°08′W; elevation 240 m) near Lisbon (western Portugal), and (4) Ares (38°26′N, 9°04′W) in the western coast of Portugal, within the Arrábida Natural Park. Seed samples of each population were held in reference collections in the Faculty of Sciences, University of Lisbon (Portugal) and in the Centre for Ecological Research and Forestry Applications, Autonomous University of Barcelona (Spain).

**Elaiosome extraction and chemical analysis**

In each population, a single randomly selected mature floral stem was collected from different individuals (seven at Can Llevallo and five from the other populations) and then transported to the laboratory. There, the floral stems were cut and the distal (the terminal pseudumbel) and basal parts of the inflorescence were kept separated inside carton boxes, until seeds were ejected from the drying capsules. Then, a sample of seeds from each carton box was collected at random for subsequent analysis. Elaiosomes were carefully detached from the seeds with the aid of forceps and their air dry weight was measured to the nearest 0.1 mg. For chemical analysis, each sample contained around ten elaiosomes (mean ± SD = 10.34 ± 1.86) weighting approximately 6 mg (mean ± SD = 5.86 ± 0.80 mg). The fatty acid profile of elaiosomes was obtained via gas–liquid chromatographic analyses of the fatty acids methyl esters (FAME). The FAME were prepared by trans-methylation using the following procedure: 6 mg of elaiosomes was weighed and transferred to a flask, then we added 500 μl of boron trifluoride in 20% methanol (for synthesis, Merck) and 2 ml of 25 ppm nonadecanoic acid (internal standard – 99%, Sigma–Aldrich) in methanol. The trans-methylation was carried out by heating to reflux at 95 °C for 15 min. 5 ml of 200 g/l NaCl (p.a., Merck) and 1 ml of hexane was added to the reaction mixture and the FAME were extracted for 2 min. The mixture was centrifuged at 3300 rpm for 3 min and the hexane layer was transferred to a vial to be analysed. Analyses were performed employing a 6890 Series II Agilent GC equipped with FID as detector and an HP-23 cis/trans FAME column (60 m × 250 μm × 0.25 μm, Agilent). Operating conditions: injector temperature 280 °C, detector temperature 275 °C, splitless during 0.5 min, carrier gas helium at a flow of 1.8 ml/min; oven temperature was initially set at 130 °C for 1 min with programmed increases (130–160 °C at 1.5 °C/min, 160–170 °C at 0.5 °C/min, 170–230 °C at 7 °C/min and held for 12 min). Two microlitres of the hexane solution was injected onto the column. The FAME were identified by retention time comparison to a FAME standard mixture (37 FAME mix, Supelco) and a vaccenic methyl ester solution in hexane, obtained by applying the same experimental procedure to vaccenic acid (99%, Sigma–Aldrich). Quantitative results were obtained using nonadecanoic acid as internal standard, which was added at the beginning of the experimental procedure. Two injections onto the column were performed from each sample and the data were treated as means.

**Statistical analysis**

For any given sample, the area of each of the nine resulting peaks was divided by the total area of all peaks, thus assessing the relative composition of each compound to allow direct comparisons across samples. However, the nine ratios now represented a compositional data set subjected to a unit-sum constraint and could not be used in standard multivariate analyses. For this reason, following the recommendations by Aitchison (1986), log contrasts need to be calculated before the performance of exploratory multivariate analyses. Log contrasts were calculated by dividing all relative
amounts by an arbitrarily chosen one (in this case, the peak corresponding to vaccenic acid) and taking the log of the eight remaining new variables (for similar analyses see Neems and Butlin, 1995; Simmons et al., 2003). A multivariate approach using principal component analysis (PCA) was then run to reveal patterns in the data. Multivariate analysis of variance (MANOVA) was used to analyse overall patterns of variation in fatty acid profile due to differences in plant architecture and between populations. Then, we tested for differences in the relative composition of each fatty acid (μg compound/mg elaiosome) by performing two-way ANOVAs, with population identity and fruit position as fixed factors. Significant interpopulation differences in fatty acids’ composition were subsequently assessed by multiple comparison tests (Unequal N HSD post hoc test) to identify which treatments were significantly different. Among plant variation in elaiosome composition was assessed in one study population (Collserola) by conducting one-way ANOVAs on the relative composition of each compound (μg compound/mg elaiosome). All analyses were run under Statistica®.

Results

The chromatogram produced nine peaks corresponding to the following fatty acids: myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1, 9c), vaccenic (18:1, 11c), linoleic (18:2), linolenic (18:3) and an unidentified component. The major component was clearly oleic acid followed by palmitic acid, while the other fatty acids occurred in a lower proportion, usually around 5% or less (Fig. 2). Exploratory multivariate analysis (PCA) made evident the occurrence of patterns on the fatty acid profile of individuals from the four study populations (Fig. 3). The two first principal components explained 41.3% and 28.6% of the variance; the third component added 13.3%. The three components had eigenvalues greater than one (PC1: 3.30; PC2: 2.29; PC3: 1.07) and explained over 80% of the total variance. Some ordination of individuals was apparent by their segregation along the x-axis (PC1). The Spanish samples from Collserola were well separated from the Portuguese samples, with Ares, Montejunto and Tesoureira grouping together. In general, the two data points (basal and distal) for each plant individual were found close to each other suggesting similarity in elaiosome’s fatty acid composition at the intra-plant level. However, some inter-individual variation within populations can also be depicted (Fig. 3).

A multivariate analysis of variance confirmed that the population factor had a significant effect on the fatty acid profile of elaiosomes (F = 10.22, p < 0.0001), while no differences due to plant architectural effects (fruit position) were detected (F = 0.30, p = 0.96). For most of the compounds, there were significant differences between individuals from different populations and this was mostly due to the lowest content of fatty acids in the elaiosomes of individuals from Collserola (Table 1 and Fig. 2). On the other hand, the position of the fruit on the stem (basal vs. distal) did not influence the chemical composition of elaiosomes for any of the compounds (Fig. 2), neither did the interaction between the two main factors (population x fruit position; Table 1). For Collserola, we found significant differences among plant individuals in the fatty acid profile of elaiosomes. All components, except linolenic acid (F = 2.19, p = 0.07), showed significant variation in their relative amount in elaiosomes at the individual level and in most cases that variation was highly significant (p < 0.0001 for myristic, palmitic, palmitoleic, stearic, oleic and vaccenic acids).

Discussion

This study identified oleic acid as a major component of the elaiosomes of E. characias reinforcing previous findings on the fatty acid composition of elaiosomes from other plant families where oleic acid has been identified as one of their major components (Fischer et al., 2008; Hughes et al., 1994; Kusmenoglu et al., 1989; Lanza et al., 1992; Soukop and Holman, 1987). Oleic acid, either in its free form or as a glyceride (e.g., 1,2-diolein and triolein), has been shown to be a major elicitor of carrying behaviour in ants, playing a key role in myrmecochory (Brew et al., 1989; Marshall et al., 1979; Skidmore and Heithaus, 1988). Previous studies showed experimentally the role played by the elaiosome of E. characias in ant attraction and effective seed dispersal (Espadaler and Gómez, 1997; Gómez et al., 2005). While both, the elaiosome and the whole diaspor, are avidly collected by mutualistic ants, the seed alone presents no interest for these insects (Baiges et al., 1992). In fact, the fatty acid composition of E. characias seeds is different from the one reported for the elaiosomes and this is probably the main reason for the observed differences in removal rates. In seeds, linoleic acid is the dominant component (with over 50% of the fatty acid content) and stearic acid occurs in a much higher proportion (around 13%) than in the elaiosomes (Carriere et al., 1992). None of these two fatty acids seems to play a key role in ant attractiveness of diaspores for most of the plant species studied to date, though it has been suggested that in Trillium linoleic acid may, together with oleic acid, act as a chemical cue for ants (Gunther and Lanza, 1989; Lanza et al., 1992). These findings suggest some segregation of specific compounds into the elaiosomes making the diaspores more attractive to ants, thus enhancing the probability of being dispersed. An alternative non-conflicting hypothesis suggests that the elaiosome chemical composition differs from the seed itself, as a result of an evolutionary process that has been tuned to provide a rich and easily accessible source of nutrients to ants (see Fischer et al., 2008, and references therein). Fischer et al. (2008) showed that elaiosomes are particularly rich in amino acids and monosaccharides, nutrients that are essential for larval development. In fact, elaiosomes are preferentially fed to larvae and colonies supplemented with elaiosomes produced a higher number and heavier progeny or female-biased progeny (Fokuhl et al., 2007; Gammans et al., 2005; Morales and Heithaus, 1998). The fatty acid profile of elaiosomes of E. characias showed strong similarities with that of other plant species even from different families (e.g., Fischer et al., 2008; Hughes et al., 1994; Pfieffer et al., 2010). These similarities in the fatty acid composition of elaiosomes have probably evolved to attract ants with behaviours that may benefit seed survival (Hughes et al., 1994), making elaiosomes “one of the most dramatic example of convergent evolution in biology” (Lengyel et al., 2010).

To our knowledge this is the first study where the variation in elaiosome fatty acid composition was studied at three different hierarchical levels (sub-individual, individual, population). In ecological literature there is still a lack of studies dealing with the variation in chemical composition of plant traits across

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### Table 1

Differences in the relative amount of fatty acids between populations (Pop), according to fruit position (Pos) and considering the interaction of the two factors (Pos x Pop). F-values of the two-way ANOVAs are presented followed by their significance level and the results of Unequal N HSD post hoc tests are also shown (Populations: A – Ares; C – Collserola; M – Montejunto; T – Tesoureira). Populations are ordered according to the average content of each fatty acid.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Pop1</th>
<th>Pop2</th>
<th>Pop3</th>
<th>Pos1</th>
<th>Pos2</th>
<th>Pos3</th>
<th>Pos x Pop1</th>
<th>Pos x Pop2</th>
<th>Pos x Pop3</th>
<th>Post hoc test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic</td>
<td>10.59</td>
<td>1.07</td>
<td>0.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; A &lt; T &lt; M</td>
</tr>
<tr>
<td>Palmitic</td>
<td>51.23</td>
<td>1.45</td>
<td>1.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; A &lt; M &lt; T</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>40.50</td>
<td>0.74</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; AMT</td>
</tr>
<tr>
<td>Searic</td>
<td>21.02</td>
<td>0.78</td>
<td>1.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; AMT</td>
</tr>
<tr>
<td>Oleic</td>
<td>19.40</td>
<td>0.72</td>
<td>0.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; A &lt; M &lt; T</td>
</tr>
<tr>
<td>Vaccenic</td>
<td>20.71</td>
<td>0.49</td>
<td>1.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; A &lt; M &lt; T</td>
</tr>
<tr>
<td>Linoleic</td>
<td>4.36</td>
<td>0.45</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C &lt; LMAT</td>
</tr>
<tr>
<td>Linolenic</td>
<td>0.27</td>
<td>0.70</td>
<td>0.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Levels of significance: **p < 0.05, *p < 0.01, *0.01 < p < 0.05, *0.001 < p < 0.01, **p < 0.001.
Fig. 2. Fatty acid profiles of distal (white bars) and basal (black bars) elaiosomes from individuals of each of the four study populations. Results are presented as mean ± SD. The number of individuals sampled was five, except in Collserola (n = 7). (a) Ares; (b) Collserola; (c) Montejunto; (d) Tesoureira.

Fig. 3. Fatty acid profile similarity of elaiosomes’ samples based on the first two principal components (PCs) extracted from the PCA. Each sample has a three-letter code meaning: population (A – Ares, C – Collserola, M – Montejunto, T – Tesoureira), individual (from 1 to 7 for Collserola, from 1 to 5 for the other populations) and position on plant (b – basal, d – distal).
various levels of organization and, proportionally, little attention has been devoted to study variation in plant traits within individuals. Although significant differences due to architectural effects were found in seed production, seed size, seedling emergence and survival in E. characias (Espadaler and Gómez, 2001), elaiosome chemical composition seems not to be influenced by the position of the fruit on the stem (basal vs. distal). This finding suggests that despite the differential probability of survival between basal and distal seeds, each diaspore has, from the point of view of its chemical composition, an equal chance of being collected by seed-dispersing ants. In this case, other characteristics, such as elaiosome and seed size, may then play a key role in selection of seeds by ants (Bas et al., 2009; Mark and Olesen, 1996; Peters et al., 2003).

Interplant variation in the relative concentration of elaiosomes’ fatty acids was in general significant for most of the compounds. Oleic acid showed the largest variation in relative concentration within populations, but this finding also reflects the fact that it is by far the main component in elaiosomes. Nevertheless, variation in the relative concentration of fatty acids between individuals may have important ecological consequences, like has recently been proposed for Helleborus foetidus. In that plant species, Boulay et al. (2007) found that the frequency of visits to plants by ant dispersers (and consequently seed removal) was highly correlated with interindividual differences in oleic acid content of elaiosomes. Thus, the chemical composition of elaiosomes seems to be under strong selection by ants and may indirectly play an important role in determining local plant demography. Geographic variation in the fatty acid composition of elaiosomes has seldom been studied, but the few studies on this subject provide evidence for significant variations along distance gradients (Boulay et al., 2006; Mackay and Whalen, 1998; Soukup and Holman, 1987). In our study, the Spanish population presented significant differences in elaiosome fatty acid composition from the Portuguese populations (located >1000 km apart), for most of the constituents. Although the differences were more evident between the Spanish and the Portuguese populations, within the latter it was also possible to detect significant geographic differences for some compounds. This finding reflects the effect of local selective pressures, and genetic and environmental constraints in shaping the chemical composition of elaiosomes. The role played by ant seed dispersers as selective forces on elaiosome chemical composition was highlighted by Boulay et al. (2006) after studying ant–seed interactions in Helleborus foetidus in two separated localities. These authors have illustrated how geographic variations in plant traits related with dispersal may reflect selection by local dispersal agents, thus potentially generating geographic mosaics of co-evolution. Other studies, however, have suggested that other interactors (e.g., predators) may also play a role in driving the evolution of elaiosome chemical composition (Gammans et al., 2006; Hanzawa et al., 1985).

Our knowledge on the chemical composition of elaiosomes and their implications in ant attraction and nutrition is still scarce. Only recently, some work has been made to evaluate the spatial variation in elaiosome chemical composition and how quantitative differences in key compounds may lead to different seed fates. Further studies are needed to elucidate the chemical basis of ant–seed interactions and their consequences for seed survival, seedling establishment and plant demography.

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