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## Pollen analysis as a means to determine the geographical origin of royal jelly

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### Abstract

In this study we investigated the use of pollen analysis as a method to determine the geographical origin of royal jelly. We recorded the pollen flora sampled by bees using pollen traps for two consecutive years and we also collected and examined royal jelly samples from the same apiary. For royal jelly production, bees mainly used the freshly collected pollen. All major pollen types that were recorded in the area using pollen traps were also detected in the royal jelly samples. Thus, pollen analysis can be used as a method to determine the geographical origin of royal jelly.

**Keywords:** *royal jelly, melissopalynology, pollen trap, bee flora, Apis mellifera L.*

Royal jelly is a product secreted by the hypopharyngeal and mandibular glands of nurse bees (*Apis mellifera* L.). It is produced by partial digestion of honey and pollen (Witherell, 1978). Royal jelly is mainly fed to queens and queen larvae, as well as to worker and drone larvae (Free, 1957; Haydak, 1970; Witherell, 1978; Crailsheim, 1991, 1992). The most important compounds of royal jelly are water, protein, lipids and carbohydrates (Witherell, 1978; Karaali et al., 1988).

Royal jelly has a great nutrient value and offers important financial profits to beekeepers. Like honey, the determination of the geographical origin of royal jelly is important for its marketing. Although the physicochemical properties and composition of royal jelly have been studied (Chen & Chen, 1995; Nagai et al., 2001; Simúth, 2001; Boselli et al., 2003; Sesta, 2006), only a few melissopalynological studies of royal jelly have been reported (Chauvin & Louveaux, 1956; Ricciardelli d'Albore & Battaglini Bernardini, 1978; Barth, 2005). Royal jelly can be enriched by pollen grains that fall from bees, or from the pollen contained in the honey stomach (Simpson, 1955; Renner et al., 2003). Thus, pollen

analysis could be used to determine its geographical origin.

Pollen from pollen traps is the most widely used method to record the flora sampled by bees in an area (Severson & Parry, 1981; Pearson & Braiden, 1990; Coffey & Breen, 1997; Nabors, 1997; Barth & Da Luz, 1998; Webby, 2004; Andrada & Telleria, 2005). In this study we recorded the bee flora of an area in Thessaloniki, Greece, using pollen traps in four colonies for two consecutive years. We also compared the pollen spectra from the bee pellets to the pollen spectra that we recorded through the melissopalynological analysis of royal jelly samples produced from colonies at the same area. This was done in order to investigate if melissopalynological techniques could be used to determine the geographical origin of royal jelly.

### Material and methods

The apiary where the work was carried out was located in the farm of the Aristotle University of Thessaloniki (Greece). For the purpose of this study colonies of *Apis mellifera* L. were used.

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### Sampling

**Pollen traps.** Pollen traps were fitted in the entrance of four colonies during August 2003 and 2004. According to Dimou et al. (2006), the use of four colonies for two consecutive years can inform us sufficiently about the pollen flora of an area. We collected and analysed 10% of the total weights of the pollen loads from each trap every third day totalling 40 samples per year. The collected pollen loads were sorted by colour and two - three slides of each colour-class were prepared to identify the botanical origin of each pollen type (Louveaux et al., 1978). Pollen loads from the same taxon were weighed together in order to evaluate the contribution of each pollen source. The following frequency classes were defined: "I": detected (<1%), "II": very few (1-3%), "III": few (3-15%), "IV": frequent (>15%).

**Royal jelly.** The queens from four colonies were removed, and 60 artificial cells were put in each hive. Each artificial cell contained a 12-24 hs old worker larva. Three days later the cells were removed, the larvae discarded and the royal jelly was collected from each colony. This technique was repeated five times totalling 24 samples of royal jelly during August.

Pollen analysis was a simplified version of Ricciardelli d'Albore & Battaglini Bernardini's (1978) methodology. For each sample, 0.5 g of royal jelly was diluted in 10 ml KOH 2.5% (w/w). The samples were centrifuged for 10 min to 3000 rpm/min in a Centra CL2 centrifuge, with a radius of 7.8 cm from the centre of the rotor to the sample (Pendleton, 2006). The sediment was re-suspended in 10 ml distilled water and was centrifuged again. Then, the sediment was spread onto a 22 × 22 mm area on a slide and at least ten fields of view and 300 pollen grains distributed uniformly over the area were counted at a magnification of 200 X using light microscopy (except from three samples that contained very few pollen grains). The following frequency classes were defined: "I": detected (<1%), "II": very few (1-3%), "III": few (3-15%), "IV": frequent (>15%).

### Reference material

In order to identify the pollen types in the collected samples, pollen reference slides were prepared from flowers around the apiary (approximately 1 km radius) (Louveaux et al., 1978).

### Statistics

Regression Analysis (Legendre & Legendre, 1998) was used to find the correlation between the weight

of the produced royal jelly, the number of pollen types, and the absolute number of pollen grains on the prepared slides.

Nonparametric Kruskal-Wallis and Mann-Whitney tests were used to compare the abundance of pollen types in royal jelly samples among colonies. Nonparametric tests were used since the assumptions of normality and homogeneity of variance were not met. The normality assumption was tested using the Kolmogorov-Smirnov test and the homogeneity of variance was tested using Levene's test. The observed significance level (*p* value) of the nonparametric tests was estimated by Monte Carlo simulations (Mehta & Patel, 1996).

The statistical analyses were carried out using SPSS v.12. The significance level of the tests was  $\alpha=0.05$ .

### Results

The amounts of the royal jelly stored in the queen cells differed among the colonies and ranged from 1.92 to 7.76 g ( $4.85 \pm 1.76$  g). The total number of pollen grains per sample ranged widely ( $9\,994 \pm 8\,617$  pollen grains/0.5 g of royal jelly). We did not observe any correlation between the quantity of the produced royal jelly per colony and the absolute number of pollen grains that we counted in the slides ( $p=0.327$ ); or the number of pollen types ( $p=0.426$ ).

A total of 17 pollen types were identified in royal jelly samples (Table I). The number of pollen types

Table I. Number of samples per frequency type per taxon, and their frequency classes in royal jelly analysed [classes: I: detected (<1%), II: very few (1-3%), III: few (3-15%), IV: frequent (>15%)].

| Pollen type                    | Frequencies (n=24) |    |     |    |
|--------------------------------|--------------------|----|-----|----|
|                                | I                  | II | III | IV |
| <i>Carduus</i> sp.             | 2                  | 2  | 1   | –  |
| <i>Chenopodium</i> -Type       | 1                  | 2  | 14  | 3  |
| <i>Cyperus</i> sp.             | 5                  | 3  | 2   | –  |
| <i>Daucus carota</i> L.        | 5                  | 3  | 2   | –  |
| <i>Lavandula</i> sp.           | 2                  | –  | –   | –  |
| <i>Ligustrum lucidum</i> Aiton | 1                  | 4  | 13  | –  |
| <i>Lilium</i> -Type            | –                  | –  | 10  | 14 |
| <i>Limonium</i> sp.            | 2                  | 2  | –   | –  |
| <i>Lotus</i> sp.               | 6                  | 6  | –   | –  |
| <i>Lythrum salicaria</i> L.    | 2                  | 2  | –   | –  |
| <i>Phoenix</i> -Type           | 1                  | 3  | 2   | 15 |
| <i>Polygonum aviculare</i> L.  | 1                  | 7  | 9   | 4  |
| <i>Portulaca oleracea</i> L.   | –                  | 2  | –   | –  |
| <i>Rubus ulmifolius</i> Schott | –                  | 1  | 11  | 12 |
| <i>Sonchus</i> sp.             | –                  | 2  | –   | –  |
| <i>Tribulus terrestris</i> L.  | –                  | 6  | 7   | –  |
| <i>Zea mays</i> L.             | 4                  | 6  | 8   | –  |
| Unidentified                   | –                  | 8  | 14  | 2  |

per sample ranged from 6 to 12 ( $8.3 \pm 2.1$ ). Statistical analysis showed a positive correlation between the number of pollen types and the total number of pollen grains per sample ( $p=0.007$ , Pearson Correlation 0.572). *Rubus ulmifolius* Schott and *Lilium*-Type were located in all the samples. Most of the samples contained pollen grains from *Chenopodium*-Type, *Ligustrum lucidum* Aiton, *Phoenix*-Type, *Polygonum aviculare* L. and *Zea mays* L. (Table I). The most frequently occurring pollen types were *Chenopodium*-Type, *Lilium*-Type, *Lotus* sp., *Phoenix*-Type, *P. aviculare*, and *R. ulmifolius* (Table II). Pollen types such as *Lavandula* sp., *Portulaca oleracea* L. and *Sonchus* sp. occurred in only a few samples (average contribution  $<1\%$ ).

A wide range in the frequency of pollen types was detected among the samples (Table I). These differences were caused mainly by the changes in the pollen flow from the plants around the area of the apiary. The presence of each pollen type was less frequent in royal jelly samples at the beginning and the end of the blooming period of a taxon, and rose in the middle. There was a significant difference in the abundance of some pollen types among colonies ( $p < 0.001$ ). These differences were expected since

genetics can influence the pollen preferences and foraging behaviour of the bees (Nye & Mackensen, 1965, 1968; Page et al., 1995; Fewell & Page, 2000; Pankiw et al., 2002). However, these differences were restricted to a few cases, and thus, did not significantly change the average results. The taxa concerned were *Daucus carota* L., *Lilium*-Type and *P. aviculare* pollen and only one colony per case.

The analysis of pollen traps indicated 23 pollen sources in total in the two years (Table II). All pollen types that were found by the pollen trap analysis were also present in the royal jelly samples except *Lavandula* sp. (whose pollen production is scarce). The seven pollen types that were found only in the bee pellets occurred in small amounts and their contribution to the total pollen weight was less than 1% in both years. The most abundant pollen types in both years were *Lilium*-Type, *P. oleraceae*, *P. aviculare*, *Sonchus* sp. and *Z. mays*.

Several quantitative differences were seen in the pollen types that were present both in the pollen trap and the royal jelly samples (Table II). For example, the contribution of *Z. mays* pollen exceeded 15 % in traps, while it was significantly less in royal jelly samples ( $<3\%$ ). Conversely, the presence of *Phoenix*-Type pollen was high in royal jelly samples ( $>15\%$ ) and low in pollen trap samples ( $<1\%$ ). However, quantitative differences relative to the pollen abundance between royal jelly and pollen trap samples were expected since during the production of royal jelly both pollen and nectar are consumed.

Table II. Combined pollen types detected in royal jelly and pollen trap samples, and their average percentage [classes: I: detected ( $<1\%$ ), II: very few (1-3%), III: few (3-15%), IV: frequent ( $>15\%$ )].

| Pollen type                                     | Average abundance in |             |
|-------------------------------------------------|----------------------|-------------|
|                                                 | royal jelly          | bee pellets |
| <i>Carduus</i> sp.                              | I                    | I           |
| <i>Centaurea</i> sp.*                           | –                    | I           |
| <i>Chenopodium</i> -Type                        | III                  | II          |
| <i>Convolvulus arvensis</i> L.*                 | –                    | I           |
| <i>Cyperus</i> sp.                              | I                    | I           |
| <i>Daucus carota</i> L.                         | II                   | II          |
| <i>Erica malipuliflora</i> Salisb.*             | –                    | I           |
| <i>Gossypium hirsutum</i> L.*                   | –                    | I           |
| <i>Lagerstroemia indica</i> L.*                 | –                    | I           |
| <i>Lavandula</i> sp.**                          | I                    | –           |
| <i>Ligustrum lucidum</i> Aiton                  | II                   | II          |
| <i>Lilium</i> -Type                             | IV                   | III         |
| <i>Limonium</i> sp.                             | I                    | I           |
| <i>Lotus</i> sp.                                | III                  | I           |
| <i>Lythrum salicaria</i> L.                     | I                    | I           |
| <i>Phoenix</i> -Type                            | IV                   | I           |
| <i>Polygonum aviculare</i> L.                   | III                  | III         |
| <i>Portulaca oleracea</i> L.                    | I                    | III         |
| <i>Rubus ulmifolius</i> Schott                  | III                  | II          |
| <i>Senecio</i> -Type*                           | –                    | I           |
| <i>Sonchus</i> sp.                              | I                    | III         |
| <i>Torilis leptophylla</i> (L.) Reichenb. fil.* | –                    | I           |
| <i>Tribulus terrestris</i> L.                   | II                   | I           |
| <i>Zea mays</i> L.                              | II                   | IV          |
| Unidentified**                                  | III                  | –           |

\*: not found in royal jelly; \*\*: not found in bee pellets

## Discussion

The determination of the geographical origin of royal jelly is very important to ensure the quality of this food. In this study, we investigated the use of pollen analysis as a method for determining the geographical origin of royal jelly by comparing the pollen spectra in the royal jelly to the pollen spectra from the bee pellets. We also studied the similarities and differences among colonies relative to the pollen spectra of the produced royal jelly. Finally, we compared the total number of pollen grains and the number of pollen types to the produced amount of royal jelly per colony.

Barth (2005) examined six samples of royal jelly from corresponding number of colonies and reported that there were differences in the botanic origin of pollen sources among samples. We also found qualitative differences relative to pollen types among samples. The number of pollen types had a positive correlation to the absolute number of pollen grains per sample. However, the dominant pollen types were found in most cases. Moreover, commercial royal jelly is collected from several colonies

and lumped together. This collection of royal jelly from several colonies reduces differences among the colonies.

The production of royal jelly can be influenced by several factors such as; the season, weather conditions, genetics, available food, population of the colonies, etc. (Visscher, 1986; Huang & Otis, 1989; Vantoor & Littlejohn, 1994; Mouro & Toledo, 2004). There was no correlation between the produced amount of royal jelly and the absolute number of pollen grains or the number of pollen types per sample.

The pollen types in royal jelly came from basically the same taxa as the ones found in the bee pellets for the same time period. Thus, because of the similarity in the pollen spectra between the two types of samples (royal jelly and bee pellets) melissopalynological techniques can be used to determine the geographical origin of royal jelly.

The pollen detected in the royal jelly analysis is typical of the Mediterranean flora. Several pollen types (*D. carota*, *Lotus* spp., *Lavandula* spp., *Rubus* spp., *Z. mays*) located in the royal jelly samples have also been reported in Italian royal jelly (Ricciardelli d'Albore & Battaglini Bernardini, 1978) as well as in Greek honeys (Thrasivoulou & Manikis, 1995; Tsigouri et al., 2004). Although the pollen spectrum from royal jelly pollen analysis was not as diverse as the one from the bee pellets, it is representative of the taxa in the area where the royal jelly was produced.

Bees seemed to prefer the consumption of "fresh" pollen. Pollen sources whose blooming period ended in the first days of August (i.e. the period before the collection of royal jelly samples started) were located in two cases (*Carduus* sp. and *L. lucidum*) in the royal jelly samples. However, pollen grains from these taxa were not frequently present in the samples. The consumption of freshly collected pollen for royal jelly production shows that pollen analysis could be also used to reveal the current diet "preferences" of the bees.

## Conclusions

In this paper we investigated the use of pollen analysis as a method for determining the geographical origin of royal jelly. Although there were significant differences relative to the contribution of the pollen sources between the royal jelly and pollen trap samples, all major pollen types recorded in the bee pellets with the use of pollen traps were also found in the royal jelly samples. Thus, the pollen analysis of royal jelly can inform us about the pollen sources of an area and consequently, the geographical determination of royal jelly.

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