

Using palynological evidence from royal jelly to mediate the spread of *Paenibacillus larvae* in Brazil

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ABSTRACT - (Using palynological evidence from royal jelly to mediate the spread of *Paenibacillus larvae* in Brazil). Royal jelly may contain pollen grains and their presence can be used to determine the phytogeographical origin of the product. This study analyzed the phytogeographical origin of commercial royal jelly samples from São Paulo State, tested as part of the Brazilian Federal Inspection System (SIF), found to be contaminated with spores of the bacterium *Paenibacillus larvae*, that causes the American Foulbrood Disease. The pollen grains of *Castanea* had the highest total percentage, with lower percentages of *Cirsium/Carduus*, *Cistus*, *Parthenocissus*, *Prunus*, *Quercus*, *Robinia*, Scrophulariaceae, *Taraxacum*, *Tilia*, among others. This pollen spectrum is incompatible with royal jelly samples produced in Brazil. The pollen spectrum resembled that of an imported product, compatible with the Northern Hemisphere origin. Brazilian legislation does not require the phytogeographic origin of imported bee products to be analyzed by palynological procedures, but it is mandatory to have a certificate issued by the country of origin attesting the absence of pathogens, monitored with the objective of preventing the exotic diseases from entering Brazil. Palynology, therefore, proved to be fundamental in detecting imports of this contaminated batches.

Keywords: *Apis mellifera*, Forensic palynology, foulbrood disease, Melissopalynology, pollen analysis

RESUMO - (Usando evidências palinológicas em geléia real para intervir na disseminação de *Paenibacillus larvae* no Brasil). A geléia real pode conter grãos de pólen e sua presença pode ser usada para determinar a origem fitogeográfica do produto. Este estudo analisou a origem fitogeográfica de amostras comerciais de geléia real do Estado de São Paulo, com registro no Sistema de Inspeção Federal (SIF) brasileiro, que estavam contaminadas com os esporos da bactéria *Paenibacillus larvae*, causadora da doença Cria Pútrida Americana. Os grãos de pólen de *Castanea* apresentaram a maior porcentagem total, com menores percentuais de *Cirsium/Carduus*, *Cistus*, *Parthenocissus*, *Prunus*, *Quercus*, *Robinia*, Scrophulariaceae, *Taraxacum*, *Tilia*, dentre outros. Este espectro polínico é incompatível com amostras de geléia real produzidas no Brasil. O espectro polínico assemelhou-se ao de um produto importado, compatível com a origem do Hemisfério Norte. A legislação brasileira não exige que a origem fitogeográfica dos produtos apícolas importados seja analisada por procedimentos palinológicos, mas é obrigatório ter um certificado atestando a ausência de patógenos emitido pelo país de origem, monitorado com o objetivo de impedir que doenças exóticas penetrem no Brasil. A palinologia, portanto, provou ser fundamental na detecção de importações desses lotes contaminados.

Palavras-chave: Análise polínica, *Apis mellifera*, cria pútrida americana, Melissopalynologia, Palinologia forense

Introduction

A decline in the honey bee population has been reported in various countries over the past decade

by the FAO (United Nations Food and Agriculture Organization) and the NAS (U.S. National Academy of Sciences), including populations in Brazil. This is a matter of great concern due to the decline in

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pollination of important food crops (Klein *et al.* 2007). The honey bee mortality observed is not seasonal and there seems to be a complex interaction between various factors, including insecticide application by farmers as well as fragmentation and degradation of habitats (Johnson 2010). However, the data are still imprecise and the causes are undefined due to the absence of standards and/or weak monitoring (Pires *et al.* 2016).

Although honey bees are attacked by many parasites and pathogens (Chen *et al.* 2004), Africanized bees are more tolerant and resistant to several pathogens. However, in recent years there has been a sudden weakening of swarms in various places in the southeast of Brazil, with mortality of adult bees and the appearance of abnormal symptoms in broods (Message *et al.* 2012).

American foulbrood disease, caused by the bacterium *Paenibacillus larvae*, one of the main cause of losses of colonies in the world (Funfhaus *et al.* 2013). This pathogen contaminates colonies through spore ingestion by larvae (Garrido-Bailón *et al.* 2013), causing their death, consequently a decrease in the colony population. The bacterium *P. larvae* is exotic in Brazil and American foulbrood disease was only detected twice in the country. The disease was detected for the first time in 2001 in the municipality of Candelária, Rio Grande do Sul State, where samples of adult bees, honey, pollen and honeycombs were collected and analyzed (Schuch *et al.* 2003). Spores of the bacterium were detected in three groups of adult bees and in honeycombs, as well as in imported honey and pollen samples. In 2006, clinical signs of the disease were reported in samples from Quatro Barras, Paraná State. This bacterium was quickly controlled and extinguished in the country (MAPA 2006).

Exotic diseases can enter new territories by natural migration of bees, or by importing queen bees or bee products contaminated with pathogens (Hitchcock & Revell 1963, Gochnauer & Corner 1974, Hale & Menapace 1980, Flores *et al.* 2005, Higes *et al.* 2008, Pettis *et al.* 2013, Fries *et al.* 2013, OIE 2014, Guimarães-Cestaro *et al.* 2016). Diseases caused by microorganisms are of particular concern due to the easy transmission of resistant spores, which can remain viable for decades (Hornitzky 2010). For example, spores of *P. larvae* can remain viable for about 70 years in the environment (Shimanuki & Knox 1994, 2000).

Inside the bee colony, products such as honey, pollen, and royal jelly are susceptible to contamination

by these pathogens, mainly by being stored in comb cells by the bees and transferred through trophallaxis (OIE 2014). Due to the high risk of introducing exotic pathogens into the country by imported bees' products and queens, sanitary control is regulated by a specific resolution of the Brazilian Ministry of Agriculture. Among the requirements are documents from the country of origin that include the results of diagnostic tests.

Royal jelly is a product secreted by the hypopharyngeal glands of worker honeybees (*Apis mellifera*). It is derived mostly from the nutrients contained in the pollen grains ingested by the bees. Pollen is the main source of proteins, peptides and secondary metabolites in royal jelly. Therefore, pollen is the main precursor product that results in royal jelly and substantially influences its biological properties (Stocker 2005). Although it does not make sense to refer to the botanical origin of royal jelly, because it is a bee secretion, observing the pollen types and their percentages (pollen spectra) can provide some evidence regarding the phytogeographical origin of a royal jelly sample. Pollen flow into the product is an accidental process, introduced from the internal environment of the colony, during manipulation in the mouthparts of the adult bees when feeding queen larvae or adult queens. As the royal jelly is not stored in the hive, it can be found pollen grains that happened to be in the bees' bodies or in their nectariferous vesicles (Simpson 1955, Renner *et al.* 2003, Barth 2005).

Pollen grains analysis in bee products is an effective tool to support the certification of their phytogeographical origin because it shows the presence of pollen types that are characteristic of specific plants. However, according to Brazilian legislation, this analysis is not mandatory for imported or local bee products.

There are few palynological studies of royal jelly (Chauvin & Louveaux 1956, Ricciardelli D'Albore *et al.* 1977, Ricciardelli-D'Albore & Battaglini 1978, Biondi *et al.* 2003, Piana *et al.* 2006, Dimou *et al.* 2007, Dimou *et al.* 2013). In Brazil, only the studies carried out by (Barth 2005) and (Morgado & Barth 2011) can be found, which samples are rich in pollen grains of Asteraceae, *Cecropia*, *Eucalyptus*, Melastomataceae and *Mimosa pudica*/*M. scabrella*.

This study evaluated the phytogeographical origin of royal jelly samples sold in São Paulo State, Brazil, tested by the Federal Inspection System (Sistema de Inspeção Federal - SIF) in which positive signs of *Paenibacillus larvae* were previously detected based

on PCR results. The results of these analyses are useful for elucidating the possible dispersion of this pathogen in Brazil through the sale and distribution of bee products.

Material and methods

Eight royal jelly samples, which were inspected by the S.I.F., were analyzed using palynological techniques. They were purchased in São Paulo State, Brazil and were bottled from 2012 to 2014. Positive signs of *Paenibacillus larvae* were detected according to molecular analyses (Guimarães-Cestaro *et al.* 2016, Teixeira *et al.* 2018).

The palynological treatment of the royal jelly followed Barth (2005) using 1 g of the product for the analysis and preparation of the microscope slides which include sample dilution, centrifugation, resuspension in acetic acid for overnight dehydration, and subsequent acetolysis (Erdtman 1952). Three slides of each sample were prepared using standard uncolored and fuchsin stain colored (one slide) glycerine jelly (Barth 2005). Fuchsin stained pollen grains were not photographed.

The identification of the pollen types was based on the European and Asian pollen flora catalogs (Punt & Clarke 1974, Lieux 1975-1977-1978, Göschl 2008, Haiqing 2015), among many publications that were consulted and that are part of the collection from the Palynological Research Centre (NPP) at the Institute of Botany, as well as others obtained from the Internet. The “Northwest European Pollen Flora” (Punt & Clarke 1974) is the world’s most detailed pollen morphological study and covers around 130 families and ca. 600 species.

In the present study was adopted as counting methodology the observation of all pollen grains contained in the three slides. Pollen grains were identified as “pollen types” meaning that a single plant species, group of species or higher taxa could present with similar pollen morphologies.

In order to evaluate the phytogeographical origin of each sample, the percentage calculation representing all the pollen types per sample (Total Pollen Sum) was used. The pollen types were grouped according to their relative frequencies, following (Louveaux *et al.* 1978), corresponding to: Predominant pollen (P) = >45% of the total pollen grains counted; Secondary pollen (S) = 16% to 45%; Important Minor Pollen (I) = 3% to 15% and, Minor pollen (M) = <3%.

Although quantification by *Lycopodium* tracer spores provides a context for pollen spectra that is

both useful for supporting phytogeographic origins of bee products from botanical source (bee pollen and honey) and, for later comparisons with similar studies, they were not utilized in this study to collect pollen data, due to the fact that royal jelly is a question of bee secretion. The pollen concentration calculation in royal jelly has no use whatsoever, as it does not come from botanical excretions. In addition, pollen concentration calculations using *Lycopodium* tracer spores were only carried out in royal jelly in Barth (2005) and Morgado & Barth (2011) studies, and even so, without an important discussion about it. *Lycopodium* tracer spores were not used in royal jelly in the studies conducted by Chauvin & Louveaux (1956), Ricciardelli D’Albore *et al.* (1977), Ricciardelli-D’Albore & Battaglini (1978), Biondi *et al.* (2003), Piana *et al.* (2006), Dimou *et al.* (2007), Dimou *et al.* (2013). Another reason for not having done concentration calculation and saturation curve is that this is a product manipulated for commercialization, without possibility to control the field production, as observed in other studies, like *e.g.*, in Dimou *et al.* (2007), and there is no way of knowing if these samples suffered some type of filtering before being commercialized. Filtering could have artificially reduced the pollen quantity. Finally, in forensic terms, you never add something to a pollen sample and especially not other spores or pollen.

The similarity between the samples was analyzed using a principal component analysis (PCA) to examine the influence of quantitative and qualitative data on the ordination of the pollen types. The matrix included the absolute value of all pollen types counted in each sample. The FITOPAC software Shepherd (1996) was used for the transformation of the absolute count using the natural logarithm [$\log(x + 1)$] and the ordination was subsequently performed using the covariance matrix in the PC-ORD 5.0 software (McCune & Mefford 1999). The variability between samples was expressed using the first two axes of the PCA.

The MINITAB 15 software (Minitab Inc., State College, PA, 2003) was used to construct a dendrogram of the percent of similarity between the samples.

The samples were evaluated in two ways to identify its origin: 1) among samples, to verify its similarity, including all the pollen types and their absolute value and, 2) with those from other countries, to compare the pollen types found in each sample together with the presence or absence in royal jelly

samples from Brazil (Barth 2005, Morgado & Barth 2011), China (Piana *et al.* 2006), France (Stocker 2005), Greece (Dimou *et al.* 2013) and Italy (Piana *et al.* 2006) as well as Louisiana honey, United States (Lieux 1975-1977-1978). This allowed investigating if a possible illegal event occurred that could pose serious environmental risks due to the possibility of dispersing this exotic pathogen in Brazil.

The pollen types were grouped in a table showing their probable geographical origins, habitus (form) of the plant, vernacular names in Portuguese and in English, the presence or absence in the samples and a possible indication of the Brazilian extra origin.

The image captures of pollen grains were obtained using an OLYMPUS BX 50 light microscope with a video camera Olympus U-CMAD-2 and the program CellSens Standard® 1.5. for Windows.

Results and Discussion

We identified 78 pollen types representing 48 genera and 38 families of plants, in eight royal jelly samples (table 1). In addition, there were 16 unidentified pollen types. Some samples contained only very few pollen grains as observed also by Dimou *et al.* (2013) in Greece.

No samples showed pollen spectra compatible with Brazilian biomes or degraded areas by anthropic action of the country (table 2).

Several pollen types found in the samples were from cultivated plants for food, medicinal or ornamental purposes in many parts of the world (*Allium*, *Apium*, Brassicaceae, *Daucus*, *Lilium*, *Taraxacum*, *Zea mays*, among others). Pollen grains from these plants are not good indicators of phytogeographical origin as they can occur in various continents.

On the other hand, the pollen types *Acer negundo*, *Betula*, *Castanea*, *Cistus*, *Cytisus*, *Hypocoum*, *Larix*, *Nyssa*, *Quercus*, *Robinia*, *Sambucus*, *Saxifraga*, *Tilia* and *Ulmus* are excellent indicators of origin of samples outside Brazil.

The pollen spectra observed differed from those reported by Barth (2005) and Morgado & Barth (2011) for royal jelly samples produced in Brazil, which include a predominance of *Cecropia*, Asteraceae and *Eucalyptus* in the city of Viçosa (Minas Gerais State) and *Cecropia*, *Eucalyptus*, Melastomataceae and *Mimosa pudica*/*M. scabrella* in Pindamonhangaba (São Paulo State), among other pollen grains with lower frequencies. These groups are very distinct from

those of the Northern Hemisphere. The pollen spectra of the samples from Pindamonhangaba are similar to those from Vietnam (Piana *et al.* 2006) due to the presence of *Mimosa pudica* and *Eucalyptus*, but with different percentages. Another differentiating factor was the presence of other pollen types that only occur in either one of the countries.

The pollen spectra found in the analyzed samples indicate the presence of pollen grains from exotic plants such as thistles (*Cirsium/Carduus*), oak (*Quercus*), chestnut (*Castanea*), rockrose (*Cistus*), locusts (*Robinia*), woodbine or ivy (*Parthenocissus*), tilia (*Tilia*) and red clover (*Trifolium pratense*), among others. Several of these pollen grains are reported in European (Louveaux 1970, Ricciardelli-D'Albore & Intoppa 2000), American (Lieux 1975-1977-1978) or Chinese honey (Song *et al.* 2012), with some regional variations in frequencies.

The pollen variability among the samples comprised 69.68% of the two first axes in the PCA (figure 1). The first axis summed for 53.85% of the variance due to the high values of *Castanea*, Brassicaceae, *Daucus* and *Taraxacum* pollen types which were found in seven samples, as well as *Parthenocissus* and *Veronica*, in six and five samples, respectively (table 3).

Among the main pollen grains, those from *Castanea* were found in all analyzed samples and Predominant (P) in samples GR1, GR5, GR8 and GR10 whereas in samples GR2 and GR9 it was Secondary (S). However, in samples GR3 and GR4 it occurred as an Important minor pollen (I) (table 1).

The chestnut is a deciduous tree native to temperate regions of the Northern Hemisphere (North America, Asia and Europe), but it is cultivated in Brazil in some places in São Paulo State, where there are commercial orchards established as long as 20 years ago in Arapeí, Campinas, Campos de Jordão, Cunha, Itupeva, Itapeperica da Serra, Mogi Mirim, Piedade, Pindamonhangaba, Santo Antônio do Pinhal, São Bento do Sapucaí, São José dos Campos and Taubaté (Bueno & Pio 2014). In Minas Gerais State, the commercial orchards are located in the southern region, in Brazópolis, Conceição dos Ouros, Espírito Santo do Dourado, Pouso Alegre, Jacutinga and Gonçalves (Bueno & Pio 2014). However, as several other pollen types observed in all samples did not belong to Brazilian plants, it can be claimed that all samples analyzed were imported.

Other important pollen types for PCA ordination that did not occur frequently in the samples were

Table 1. Frequencies of pollen types in the royal jelly samples with *Paenibacillus larvae* spores purchased in São Paulo State, Brazil, inspected by the S.I.F. Predominant pollen (P), >45% of the total pollen grains counted; Secondary pollen (S) = 16% to 45%. Obs: values are in percentage. The designations after pollen type name indicate the code used in the PCA.

Samples	GR1	GR2	GR3	GR4	GR5	GR8	GR9	GR10
Pollen types/codes								
Gymnospermae								
Cupressaceae/Cupre	0	0	0	0	0	0	0.62	0
<i>Larix</i> (Pinaceae)/Larix	0	0	2.44	0	0	0	0	0
Angiospermae monocotiledons								
<i>Allium</i> (Amaryllidaceae)/Alliu	1.35	0	0	0	0	0	0	0
Iridaceae/Irida	0	0	2.44	0	0	0	0	0
<i>Lilium</i> (Liliaceae)/Lili	0	0	0	0	0	0	0	0.97
Monocotiledon rugulate/Monrug	0	0	0	0	0	5	0	0
Monocotiledon 2/Mon2	0	0	0	0	0	0	0.31	0
Monocotiledon reticulate/Monret	0	0	0	0	0	0	0.62	0
Monocotiledon rugulate 2/Monrug2	0	0	0	0	0	0	0	0.97
Poaceae/Poa	1.35	0	2.44	1.35	0.75	0	0	0
<i>Zea mays</i> (Poaceae)/Zea	0	0	0	0	0	0	0.93	0
Angiospermae eudicotiledons								
<i>Sambucus</i> (Adoxaceae)/Sam	1.35	0	0	0	0	0	0	0
Amaranthaceae-Chenopodiaceae/Amar	0	0	0	0	0	0	0.31	0.97
<i>Apium</i> (Apiaceae)/Apium	0	5.88	0	0	0	0	0	0
<i>Daucus</i> (Apiaceae)/Dauc	2.70	5.88	2.44	1.35	0.75	0	10.52	0.97
<i>Pleurospermum</i> (Apiaceae)/Pleur	0	5.88	0	0	0	0	0	0
<i>Ilex</i> (Aquifoliaceae)/Ilex	2.70	0	0	0	0	0	0	0
<i>Ambrosia</i> (Asteraceae)/Ambro	0	0	0	0	0	5	0	0
<i>Artemisia</i> (Asteraceae)/Arte	0	0	0	0	0	2.5	0	0
Asteraceae 1/Aster1	1.35	5.88	0	0	0	5	2.78	0
Asteraceae 2/Aster2	2.70	0	0	0	0	0	0	0
Asteraceae 3/Aster3	0	0	0	0	0	0	0.93	2.91
Asteraceae 4/Aster4	0	0	2.44	0	0	0	0.31	0
Asteraceae 5/Aster5	0	0	0	0	0	0	0	0.97
<i>Cirsium-Carduus</i> (Asteraceae)/Cardu	1.35	0	0	0	0	2.5	0	0
<i>Senecio</i> (Asteraceae)/Sene	2.70	0	0	1.35	0	0	0	0
<i>Taraxacum</i> (Asteraceae)/Tarax	1.35	5.88	0	1.35	0.75	5	1.54	0.97
<i>Betula</i> (Betulaceae)/Betu	1.35	0	0	0	0	2.5	1.54	0.97
Brassicaceae/Brassi	1.35	17.64 (S)	7.32	32.43 (S)	1.50	0	1.24	0.97
<i>Myosotis</i> (Boraginaceae)/Myos	0	5.88	0	0	0	0	1.24	0
Caryophyllaceae 1/Caryo	0	0	2.44	0	0	0	0.31	0
<i>Melandrium</i> (Caryophyllaceae)/Mela	0	0	0	0	0	0	0.62	0
<i>Cistus</i> (Cistaceae)/Cistus	1.35	0	7.32	0	1.50	0	0.93	0
<i>Helianthemum</i> (Cistaceae)/Helia	0	0	0	1.35	0	2.5	0.31	1.94
<i>Nyssa</i> (Cornaceae)/Nyssa	1.35	0	0	0	4.51	0	1.24	1.94
Euphorbiaceae/Eupho	0	0	0	0	0	0	0.31	0.97
<i>Cassia</i> (Fabaceae)/Cassia	1.35	0	0	0	0	0	0	0
<i>Cytisus</i> (Fabaceae)/Cytis	0	0	2.44	0	0	0	0	0
<i>Mimosa pudica</i> (Fabaceae)/Mimpu	0	0	0	0	0	2.5	0	0
<i>Robinia</i> (Fabaceae)/Robi	0	0	19.51 (S)	0	0	0	0	0
Fabaceae 1/Faba1	1.35	0	0	0	0	0	0.62	0
Fabaceae 2/Faba2	0	5.88	0	0	0	0	0	0
Fabaceae 3/Faba3	0	0	7.32	0	0	0	0	0
Fabaceae 4/Faba4	0	0	0	1.35	0	0	0	0
<i>Lotus corniculatus</i> (Fabaceae)/Lotus	0	0	2.44	16.21 (S)	0.75	0	0	0
<i>Trifolium</i> (Fabaceae)/Trifo	0	5.88	0	0	0	0	0	0
<i>Trifolium incarnatum</i> (Fabaceae)/Trifoin	0	0	0	0	0.75	0	1.86	0.97

continue

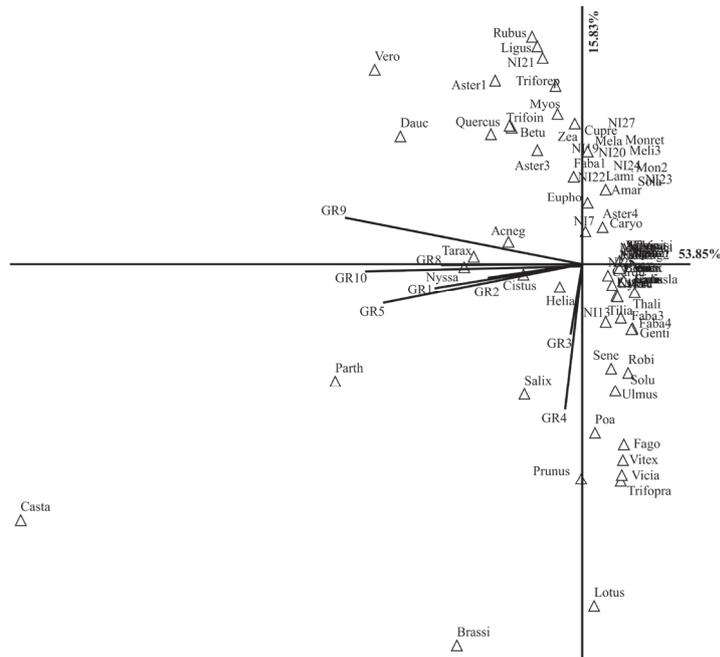


Figure 1. Principal Component Analysis performed with the pollen type counting variables in eight royal jelly samples with *Paenibacillus larvae* spores purchased in São Paulo State, Brazil. Codes for pollen types can be found in Table 1.

Table 2. Geographical origin, life form, vernacular names, economic data of plant usage, and the main occurring environment in Brazil of the plants identified at the genus level by means of the pollen types found in the royal jelly samples with *Paenibacillus larvae* spores purchased in São Paulo State, Brazil. Note: “Native” means a plant indigenous to a particular region, “Naturalized” means after being introduced into a region, develops without the need to be treated or cultivated under human intervention and “Exotic” means introduced from abroad, but not fully naturalized. Native and naturalized vegetation quoted by Flora do Brasil (Brazilian Flora).

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Ocurrence in Brazil
Gymnospermae					
Cupressaceae	tree	cypress, cedar, etc/ cipreste, cedro, etc	Native in the temperate regions with milder temperatures of both hemispheres. They are valuable for timber, production of gums or resins and with ornamental value.	GR9	Exotic. None is native to Brazil, but can be found sporadically as an ornamental plant in gardens and parks.
<i>Larix</i> (Pinaceae)	tree	larches/alerce, larício, lariço or larice	Native conifer of most of the Northern Hemisphere, Eurasia and North America, inhabited the cooler temperate climate, from the lowlands to high mountains. It is one of the dominant plants in the immense boreal forests of Russia, Canada and Scandinavia.	GR3	Exotic. None is native to Brazil.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Ocurrence in Brazil
Angiospermae monocotiledons					
<i>Allium</i> (Amaryllidaceae)	herb	onion, garlic, leek, etc/cebola, alho, alho-poró, etc	Are annual or biennial bulbous plants that grow in temperate climates of the northern hemisphere. except for a few species growing in Chile, Brazil or in tropical Africa. Cultivated worldwide for food purposes.	GR1	Few native species in Brazil.
<i>Lilium</i> (Liliaceae)	herb	lily/lírio	Native of the northern hemisphere with occurrences in Europe, Asia, North America and South America. More than half of the species are found in China and Japan. Cultivated worldwide for ornamental value.	GR10	Exotic. <i>Lilium formosanum</i> A. Wallace is an exotic naturalized and <i>Lilium longiflorum</i> Thunb. is cultivated in Brazil.
<i>Zea mays</i> (Poaceae)	herb	corn/milho	Native to Central America where it was domesticated for thousands of years.	GR9	Exotic. It is one of the largest plantations in the world, together with wheat and rice, also in Brazil.
Angiospermae eudicotiledons					
<i>Sambucus</i> (Adoxaceae)	tree, shrub	elder/sabugueiro	Native in the temperate and subtropical regions of both hemispheres, with highest significance in the Northern Hemisphere. Cultivated worldwide for medicinal purposes.	GR1	Only <i>S. australis</i> Cham. & Schldtl. is native in Brazil. <i>S. nigra</i> L. is an exotic naturalized.
<i>Apium</i> (Apiaceae)	herb	celery/aipo, salsão, etc	Subcosmopolita distribution in Europe, Asia, Africa, South America and Australia.	GR2	Two species are exotic naturalized and several species cultivated for food purposes in Brazil.
<i>Daucus</i> (Apiaceae)	herb	carrot, coriander, etc/cenoura, coentro, etc	<i>Daucus carota</i> is native to North America, Asia, Australia and Europe, <i>Coriandrum sativum</i> , is native to Africa, Asia and Europe.	GR1, GR2, GR3, GR4, GR5, GR9, GR10	Exotic. Cultivated worldwide for food purposes, also in Brazil.
<i>Ilex</i> (Aquifoliaceae)	tree, shrub, climber	holly, maté/azevinho, erva mate	Distributed in all temperate and subtropical regions of the world.	GR1	Several native species in Brazil.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Ocurrence in Brazil
<i>Ambrosia</i> (Asteraceae)	herb	ragweed/ambrósia	Distributed in tropical and subtropical regions of the New World, especially in North America where the origin and genus diversity center are in the southwestern United States and northwestern Mexico.	GR8	Few native species in Brazil.
<i>Artemisia</i> (Asteraceae)	herb, shrub	mugwort, common wormwood/artemisia	They grow in temperate climates in both hemispheres, usually in arid habitats or semi-arid.	GR8	Only <i>Artemisia verlotorum</i> Lamotte is an exotic naturalized in Brazil.
<i>Cirsium/Carduus</i> (Asteraceae)	herb	thistle/cardo	Native to Eurasia and North Africa, with about 60 species in North America.	GR1, GR8	Several species have been introduced worldwide for its ornamental value, also in Brazil.
<i>Senecio</i> (Asteraceae)	herb, shrub	Ragworts, groundsels / tasneira, tasna, erva-de-São-Tiago	Worldwide distribution.	GR1, GR4	Several native species in Brazil.
<i>Taraxacum</i> (Asteraceae)	herb	dandelion/dente-de-leão	Weeds of fields in Eurasia and North America.	GR1, GR2, GR4, GR5, GR8, GR9, GR10	Exotic. None is native to Brazil. Cultivated worldwide for its medicinal properties and ornamental value, also in Brazil.
<i>Betula</i> (Betulaceae)	tree, shrub	birch/bétula	Characteristic of temperate climate and has distribution as much in Asia as in Europe and North America.	GR1, GR8, GR9, GR10	Exotic. None is native to Brazil, but can be found sporadically as an ornamental plant in gardens and parks.
Brassicaceae	herb, shrub, climber	broccoli, cabbage, cauliflower, kale, etc/brócolis, repolho, couve-flor, couve, etc	Native to the northern coast of the Mediterranean and Asia, having expanded later throughout Europe, as well as other continents.	GR1, GR2, GR3, GR4, GR5, GR9, GR10	Few native species and several naturalized species in Brazil. Cultivated worldwide for food purposes, also in Brazil.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Occurrence in Brazil
<i>Myosotis</i> (Boraginaceae)	herb	forget-me-nots/ não-me-esqueças	Restricted to Eurasia and New Zealand. One or two European species, especially <i>Myosotis sylvatica</i> , were introduced in most temperate regions of Europe, Asia and the Americas. Very popular in gardens.	GR2, GR9	Exotic. None is native to Brazil, but can be found sporadically as an ornamental plant in gardens and parks.
<i>Cistus</i> (Cistaceae)	shrub	rockrose, laudanum/estevas, ládano	Native of the Mediterranean, from the Canary Islands, off the coast of Morocco to the Middle East coast.	GR1, GR3, GR5, GR9	Exotic. Cultivated worldwide as ornamental plant, medicinal properties and to obtaining Labdanum, an aromatic resin used in perfumery and in homeopathy.
<i>Helianthemum</i> (Cistaceae)	herb, shrub	rockrose, /estevas, rosa-da-pedra	Widely distributed throughout the Northern Hemisphere, especially in the Mediterranean where it is the greatest diversity. They are distributed in America, Europe, and North Africa to Asia Minor and Central Asia.	GR4, GR8, GR9, GR10	Only <i>Helianthemum brasiliense</i> (Lam.) Pers. is native in Brazil. Cultivated worldwide as ornamental plant in gardens.
<i>Nyssa</i> (Cornaceae)	tree	tupelo/tupelo	It occurs spontaneously in North and Central America, from Mexico to Canada. Other species of <i>Nyssa</i> occur naturally in Southeast Asia, from China to the southwest of the Himalayas.	GR1, GR5, GR9, GR10	No species of <i>Nyssa</i> occurs spontaneously in Europe or in Brazil.
<i>Cassia</i> (Fabaceae)	tree	canafistula, golden rain tree, cassia/ canafistula, canudo de pito, cássia	Pantropical distribution. The fruits are used in food.	GR1	Several native species in Brazil. The plant has ornamental value and also used in ayurvedic medicine.
<i>Cytisus</i> (Fabaceae)	shrub	booms/chamiça, giesteira	Native in open areas (typically brushwood and heathland) of Europe, western Asia and North Africa.	GR3	Exotic. None is native to Brazil.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Ocurrence in Brazil
<i>Mimosa pudica</i> (Fabaceae)	shrub	sleepy plant, shy plant, sensitive plant, touch-me-not /dormideira, malícia, sensitiva	Tropical origin. It is a native species of Central and South America. Nowadays is invasive, with pantropical distribution, occurring in Africa, North America, Asia and Australia.	GR8	Native.
<i>Robinia</i> (Fabaceae)	tree, shrub	locusts/falsa acácia	Endemic of North America and northern Mexico. <i>Robinia pseudoacacia</i> was introduced in the seventeenth century in Europe for ornamental and medicinal purposes and for soil stabilization, becoming invasive in sunny locations and disturbed areas of Iberian countries, France, Greece, Hungary, Germany, Netherlands, United Kingdom and Cyprus. It has also become invasive in Asia (Israel, Turkey), South Africa, South America (Argentina), Australia and New Zealand. It is a cultivated species in China.	GR3	Only <i>Robinia ferruginea</i> Kunth is native in Brazil.
<i>Lotus corniculatus</i> (Fabaceae)	herb	bird's-foot trefoil/cornichão	Native of temperate grassland in Eurasia and North Africa.	GR3, GR4, GR5	Exotic. Used as a forage legume in southern Brazil.
<i>Trifolium incarnatum</i> (Fabaceae)	herb	crimson clover, italian clover / trevo-encarnado erva-do-amor, trevo-vermelho	Native to southwestern Europe, but widely used as fodder for cattle in various parts of the world, primarily in North America and Australia.	GR5, GR9, GR10	Exotic. Cultivated for livestock feed in southern Brazil.
<i>Trifolium pratense</i> (Fabaceae)	herb	red clover/trevo vermelho	Native to Europe, western Asia and northwest Africa. Widely used as fodder for cattle in various parts of the world.	GR3, GR4	Exotic. Cultivated for livestock feed in southern Brazil.
<i>Trifolium repens</i> (Fabaceae)	herb	white clover/trevo branco	Natural distribution in Europe, North Africa and West Asia. It was introduced in most temperate regions as fodder production plant and as to enhancing natural and semi natural pasture lands, being common nowadays in most of North America and New Zealand.	GR9	In Brazil there is the native <i>T. argentinense</i> Speg. whose pollen is similar.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Occurrence in Brazil
<i>Vicia faba</i> (Fabaceae)	herb	broad bean, fava bean, bell bean, horse bean /fava, feijão-fava, fava cavalinha	The origin of this vegetable is undetermined, but has been cultivated in the Middle East in the last 8000 years before spreading to Western Europe. Great importance in Europe, particularly in Italy.	GR4	Exotic. In Brazil it is rarely cultivated.
<i>Castanea</i> (Fagaceae)	tree	chestnut/castanheira	Native to temperate Northern Hemisphere region (North America, Asia and Europe), but it is cultivated in many parts of the world.	GR1, GR2, GR3, GR4, GR5, GR8, GR9, GR10	Exotic. In Brazil it is rarely cultivated.
<i>Quercus</i> (Fagaceae)	tree	oak/carvalho	Native to Northern Hemisphere, and includes deciduous and evergreen species distributed from colder temperate regions to the tropical latitudes of Eurasia and the Americas.	GR4, GR9, GR10	Exotic. In Brazil, the cultivation is restricted to Paraná State, for the “shitake” production.
<i>Hypocoum</i> (Fumariaceae)	herb		Native to the Northern Hemisphere and Southern Africa.	GR5	Exotic.
<i>Salvia</i> (Lamiaceae)	herb, shrub	common sage, mint/sálvia, menta	Distributed throughout the Old World and the Americas, with three distinct regions of diversity: Central and South America, Central Asia and the Mediterranean and East Asia.	GR8	Several native species in Brazil. Some species are used as a condiment, medicinal herb and as ornamental plants worldwide, also in Brazil.
<i>Vitex</i> (Lamiaceae)	tree, shrub	chastetree/árvore da castidade, pau de angola	They are native to the tropics and subtropics, with some species in temperate Eurasia.	GR4	Several native species in Brazil.
<i>Tilia</i> (Malvaceae)	tree	lime trees, linden/tília	Native to Northern Hemisphere, naturally occurring in temperate regions of Eurasia and North America. It is used for ornamental purposes and for infusions and medicine. It is pollinated by insects. Its greatest use is in beekeeping because it provides unifloral honey of excellent quality.	GR1, GR3	Exotic.
<i>Ligustrum</i> (Oleaceae)	tree, shrub	privet/ligustro	Native to Europe, North Africa, Asia and Australia. Some species have become widely naturalized or invasive. Its pollen produces respiratory allergies.	GR9	Three species are exotic naturalized and cultivated for ornamental purposes in Brazil.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Ocurrence in Brazil
<i>Fagopyrum</i> (Polygonaceae)	herb	buckwheat/trigo sarraceno, trigo-mourisco	Native to Central Asia and Northern Europe.	GR4	Exotic. Cultivated worldwide for human and animal consumption, and as an ornamental plant. Its cultivation is restricted to the southern of Brazil.
<i>Rumex</i> (Polygonaceae)	herb	docks, common sorrel/labaça, cata-cruz, regalo-da-horta	Common in the northern hemisphere where often become weeds.	GR1	Few native species in Brazil. Several species have been introduced almost everywhere to be edible, providing tannin and by its purgative qualities.
<i>Veronica</i> (Plantaginaceae)	herb	speedwell, bird's eye, gypsyweed/verônica-dos-campos, morrião-da-água, verônica-da-pérsia	Cosmopolitan dispersion, although the largest concentration is in the temperate regions of the Northern Hemisphere. It is widely used in land cover and medicine as an expectorant.	GR1, GR5, GR8, GR9, GR10	Few species are exotic naturalized in southeastern and southern of Brazil.
<i>Caltha</i> (Ranunculaceae)	herb	marsh-marigold, kingcup/calta, malmequer dos brejos	Occurs in moist environments in temperate and cold regions of both northern and southern hemispheres. Used in homeopathic medicines.	GR5	Exotic
<i>Thalictrum</i> (Ranunculaceae)	herb	meadow-rue/ruibarbo-dos-pobres, taliestro	Native to temperate regions.	GR1, GR3	Exotic
<i>Prunus</i> (Rosaceae)	tree, shrub	plums, cherries, peaches, apricots, etc/ameixeiras, cerejeiras, pessegueiros, damasqueiros, etc	By all temperate regions of the Northern Hemisphere.	GR1, GR3, GR4, GR5	Few native species in Brazil. Many species are cultivated worldwide for food purposes and ornamental value, also in Brazil.
<i>Rubus</i> (Rosaceae)	herb, shrub, climber	raspberries, blackberries, dewberries, etc/framboesa, amora, mirtilo, etc	Native to the Americas, Europe, Africa and Asia. Use for consumption and for medicinal properties.	GR9	Few native species in Brazil. Many species are cultivated worldwide for food purposes, also in Brazil.
<i>Borreria</i> (Rubiaceae)	herb, shrub	false buttonweed/vassourinha, vassourinha de botão, etc	In tropical and subtropical regions. His greatest diversity is found in the Americas, followed by Africa, Australia and Asia.	GR1	Several native species in Brazil.

continue

Table 2 (continuation)

Pollen types	Life form	English and Portuguese vernacular names (when available)	Geographical origin and economical use of the correspondent plants	Presence on samples	Ocurrence in Brazil
<i>Salix</i> (Salicaceae)	herb, shrub, tree	willows/salgueiro, chorão	Mainly inhabit moist soils of the cold and temperate regions of the Northern Hemisphere. The trees are grown in various parts of the world: Australia, were considered invasive weed pests; in China, it has been grown in order to protect agricultural areas, serving as a barrier to the desert winds. Its bark is used in the manufacture of wicker and the production of aspirin.	GR4, GR5, GR9, GR10	Only two native species in Brazil. <i>Salix babylonica</i> L. was introduced as ornamental plant in the southern States of Brazil.
<i>Acer negundo</i> (Sapindaceae)	tree	box elder, boxelder maple, ash-leaved maple, maple ash / bôrdo	North and Central America, occurring naturally from Canada to Guatemala. It was introduced in Europe, China and Australia, becoming invasive. In Australia is listed as a pest.	GR3, GR5, GR9, GR10	Exotic.
<i>Saxifraga</i> (Saxifragaceae)	herb	saxifrages, rockfoils/coroa do rei, manto dourado, saxifraga	They are typical inhabitants of the Arctic-alpine ecosystems and subarctic in glacial habitat, and are almost never found outside the temperate parts of the Northern Hemisphere (Europe, Asia and North Africa). Used worldwide as an ornamental plant and with medicinal purpose.	GR3	Exotic.
<i>Solanum</i> (Solanaceae)	herb, shrub	potato, aubergine, pepper, tomato/batata, berinjala, pimenta, tomate	Cultivated in various parts of the world.	GR1, GR3, GR4	Several native species in Brazil. Several species are cultivated worldwide to food purposes, also in Brazil.
<i>Ulmus</i> (Ulmaceae)	tree	elms/olmo	Constitute natural forests in temperate regions of Eurasia and North America, but with tropical distribution in Indonesia. It is cropped for various purposes: its wood is used in carpentry and furniture in the shipbuilding industry; in Australasia is used with ornamental purposes, both in parks and in bonsai; its foliage are widely used in animal feed in Asia; as a source of biomass in Europe and for medicinal purposes.	GR1, GR3, GR4	Exotic.
<i>Parthenocissus</i> (Vitaceae)	climber	virginia creeper, five-finger, thicket creeper, grape ivy/ hera	Native to the Himalayas, East Asia and North America. Often used as ornamental plants in Europe and elsewhere, including in the tropics.	GR1, GR3, GR5, GR8, GR9, GR10	Exotic. Few species are cultivated worldwide to ornamental purposes, also in Brazil.

Acer negundo, *Betula*, *Cistus*, *Ligustrum*, *Myosotis*, *Nyssa*, *Prunus*, *Quercus*, *Salix*, *Trifolium incarnatum*, *Zea mays*, some Asteraceae and Fabaceae and some unidentified pollen types (table 1). Many of these are indicators of the Northern Hemisphere origin of the samples (table 2).

Some pollen types that had less importance in the PCA ordination of the samples, but that can be good indicators of a Northern Hemisphere origin were *Larix*, *Rumex*, *Sambucus*, *Tilia* and *Ulmus* (figure 1).

The pollen types *Nyssa* and *Mimosa pudica* are good indicators of phytogeographical origins recovered in the present study. The observations of these pollen types demonstrate an Asian or North American origin as opposed to a European origin (Piana *et al.* 2006, Dimou *et al.* 2013).

The pollen detected from *Nyssa* (“tupelo”) may be explained because trees of this genus are highly nectariferous and are valued by beekeepers due to the high quality of honey produced by bees that explore them. Nevertheless, pollen from species of this genus are generally underrepresented in honey samples (Lieux 1978). Some of the *Nyssa* species are native to eastern North America to Central America. Other species also occur naturally in Southeast Asia from China to the southeastern Himalaya Mountains, but they are lacking in Europe. The pollen grains of these two groups can be differentiated through scanning electron microscopy by the presence of tiny elongated sexine elements (rugulae) in the margins of the colpi of the Asian species, while the American ones do not show them (Göschl 2008). In light microscopy, it is not easy to distinguish them. This pollen type was found in samples GR1, GR5, GR9 and GR10 (table 2). Therefore, according to the total pollen spectrum of each sample, this pollen type can be an

excellent indicator of the Asian or Northern American phytogeographical origin of royal jelly.

The presence of the *Mimosa pudica* pollen grains indicates tropical origin. This species is native to Central and South America, but because it is an invasive weed, it now also occurs in Africa, North America, Asia and Australia.

According to Piana *et al.* (2006), *Mimosa pudica* is one of the pollen types that occur in Asian royal jelly (specifically from Vietnam) but is absent in 28 samples from Italy, as well as in 82 royal jelly samples from Greece analyzed by Dimou *et al.* (2013). In the samples sold in São Paulo, this pollen type was only found in GR8, as an Important minor pollen (I) (table 1). This sample had 97.33% similarity with the group formed by samples GR1, GR5, GR9, and GR10, which are characterized by the presence of *Nyssa* pollen type and probably originated from Asia or North America (figure 2).

Sample GR4, with Apiaceae, Brassicaceae, *Fagopyrum esculentum*, *Helianthemum*, *Lotus corniculatus*, *Quercus*, *Salix*, *Taraxacum*, *Trifolium*, *Vicia faba* and *Vitex*, was more similar to royal jelly from China (figure 3), based on seven samples analyzed by Piana *et al.* (2006). Several of these pollen types were also reported in Chinese honey by Song *et al.* (2012).

Among the frequent pollen types in sample GR4, *Quercus* and *Fagopyrum esculentum* occur in Brazil. *Quercus* is restricted to Paraná State, used for the cultivation of Shiitake mushrooms on oak logs (Bett & Perondi 2011). Buckwheat (*Fagopyrum esculentum*) was introduced in the south of the country during the 1920s by the Slavic immigrants (Gorgen 2013). However, the pollen spectrum of sample GR4 has no similarity with the pollen spectra of Brazilian

Table 3. Pearson and Kendall correlation coefficients for pollen grains counting variables of the first and the second axis of PCA ordination in eight royal jelly samples with *Paenibacillus larvae* spores purchased in São Paulo State, Brazil.

Variables/samples	(Principal components)	
	Axis 1	Axis 2
GR1	-0.2926	-0.2179
GR2	-0.1359	-0.0942
GR3	-0.0685	-0.3167
GR4	-0.1162	-0.6241
GR5	-0.4369	-0.3575
GR8	-0.2479	-0.0338
GR9	-0.6814	0.5587
GR10	-0.4013	-0.1131

royal jelly, honey, or bee pollen (Barth 2004-2005, Morgado & Barth 2011). Therefore, the probable phytogeographical origin of this sample is Asia.

According to the PCA analysis, sample GR4, typically Asian, was similar to sample GR3. They were grouped based on the presence of *Castanea*, Brassicaceae, *Daucus*, *Lotus corniculatus*, *Trifolium pratense*, Poaceae, *Prunus*, *Solanum* and *Ulmus*. These pollen types are common in honey from Europe, Asia and North America (Lieux 1975-1977-1978, Louveaux 1970, Song *et al.* 2012).

The pollen spectra showing the presence of cultivated plants Brassicaceae, *Castanea*, *Daucus* and *Trifolium pratense* in samples GR3 and GR4 were also reported by Diaz-Losada *et al.* (1998) in Italian honey. These authors stated that the location of the apiary near a temperate forest is reflected in Italian honey by the presence of *Quercus* and *Ulmus* pollen grains or by the abundance of pollen from prairie herbs such as *Trifolium pratense* and Lamiaceae or also near areas of moist soil due to the presence of pollen of plants adapted to that environment, *e.g.*, *Salix*.

The royal jelly sample GR2 had a high percentage of pollen of cultivated plants (Brassicaceae and *Castanea*), similar to many others, but it was the only one with pollen of several species of cultivated plants as celery (*Apium*, Apiaceae) and *Pleurospermum* (Apiaceae), two pollen types of Fabaceae (*Trifolium* and Fabaceae 2), as well as Primulaceae, a family with several ornamental herbaceous plant species widely cultivated around the world. This sample had 88.42% similarity with the group formed by samples with pollen of *Nyssa* (GR1, GR5, GR9 and GR10) and *Mimosa pudica* (GR8) (figure 2), supposedly Asian or North American. As pointed out previously, these pollen types do not occur in European royal jelly.

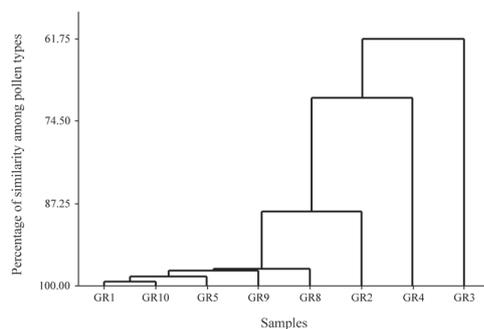


Figure 2. Dendrogram of similarity among the eight royal jelly samples with *Paenibacillus larvae* spores purchased in São Paulo State, Brazil.

The set with various pollen grains of exotic plants in the samples was similar to the group of European and Chinese royal jelly having 49.54% similarity with those of Brazilian origin (figures 3-12). This percentage was due to the presence of seven pollen types (Amaranthaceae/Chenopodiaceae, *Borreria*, Brassicaceae, *Mimosa pudica*/*M. scabrella*, Poaceae, *Senecio* and *Solanum*) reported in the Brazilian samples analyzed by Barth (2005) and by Morgado and Barth (2011) as well as in some of the samples analyzed in the present study.

Palynological evidence indicates that royal jelly samples contaminated with spores of *P. larvae* are sold in São Paulo State as Brazilian products, however, they were produced in the Northern Hemisphere. The fact that royal jelly samples from Europe analyzed by other authors have been found similar to those from China, due to the presence of some pollen types, suggests that palynology alone cannot accurately identify the continent or country of origin. No scientific specialty (plant anatomy, palynology, physicochemical) alone can solve problems of the origin of bee products and illegal imports of bee products. Thus, only field observations of bees' visits to flowers cannot define the botanical origin of the floral resources used by bees. Beekeepers' empirical observations should be followed up by physicochemical, anatomical and palynological analyses of bee products, as these analyses broaden the knowledge about bee visits to flowers. In order to be sure of the geographical origin of these contaminated royal jelly batches, public inspection agents should audit in the warehouses that packed the royal jelly about the origin of the contaminated product that is being marketed in São Paulo.

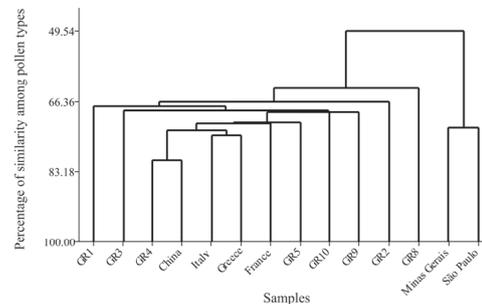


Figure 3. Dendrogram of similarity among the eight royal jelly samples with *Paenibacillus larvae* spores purchased in São Paulo State (Brazil) and those ones from Minas Gerais and São Paulo States (Brazil), China, France, Greece and Italy.

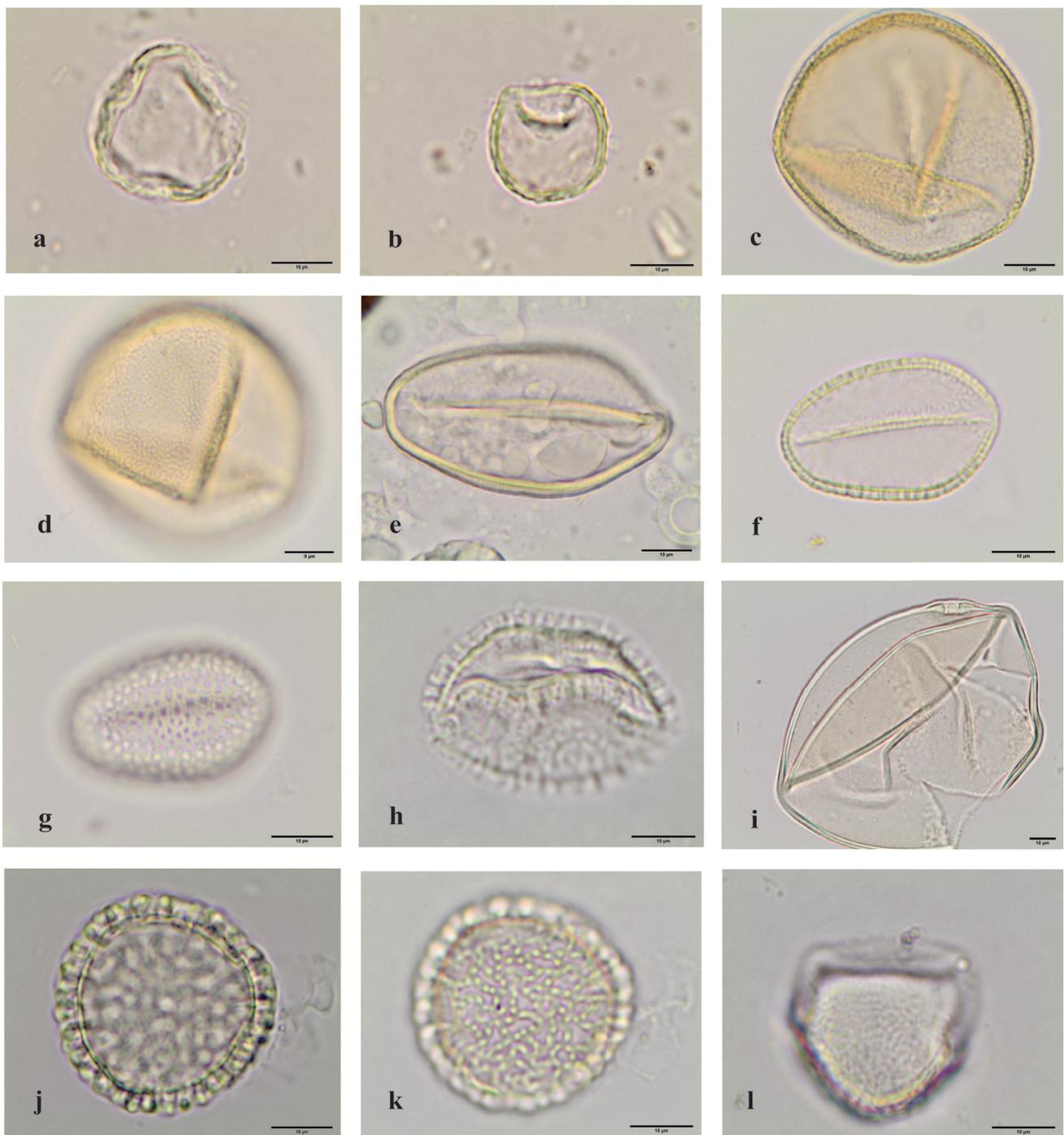


Figure 4. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-d. Gymnospermae. a-b. Cupressaceae. c-d. Pinaceae, *Larix*. e-l. Angiospermae monocolyledons. e. Amaryllidaceae, *Allium*. f-g. Iridaceae. h. Liliaceae, *Lilium*. i. Poaceae, *Zea mays*. j-k. Unknown reticulate monocolyledon. l. Unknown rugulate monocolyledon. Scale bars: 10 μm .

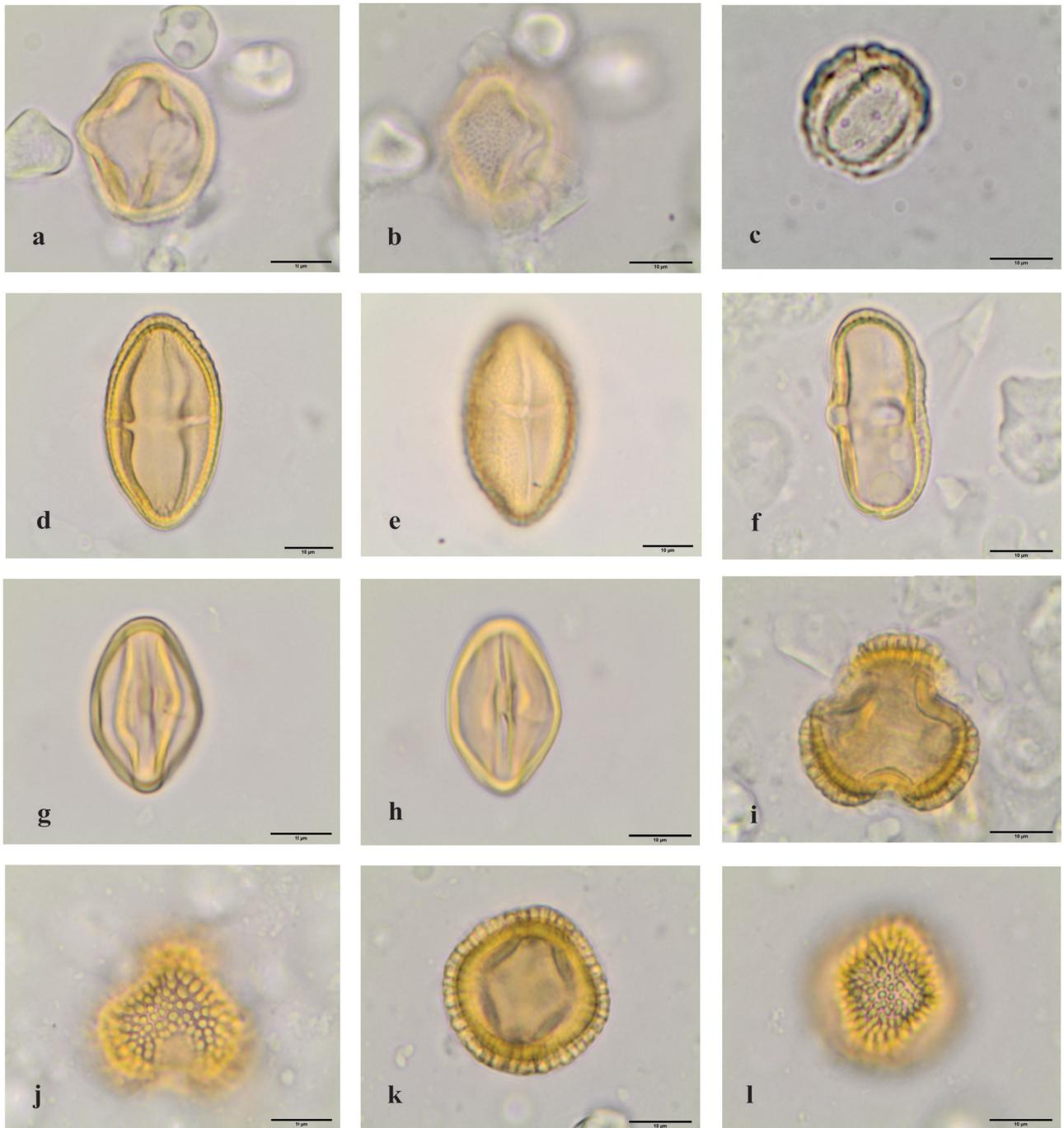


Figure 5. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons. a-b. Adoxaceae, *Sabuncus*. c. Amaranthaceae. d-h. Apiaceae. d-e. *Apium*. f. *Daucus*. g-h. *Pleurospermum*. i-l. Aquifoliaceae, *Ilex*. Scale bars: 10 µm.

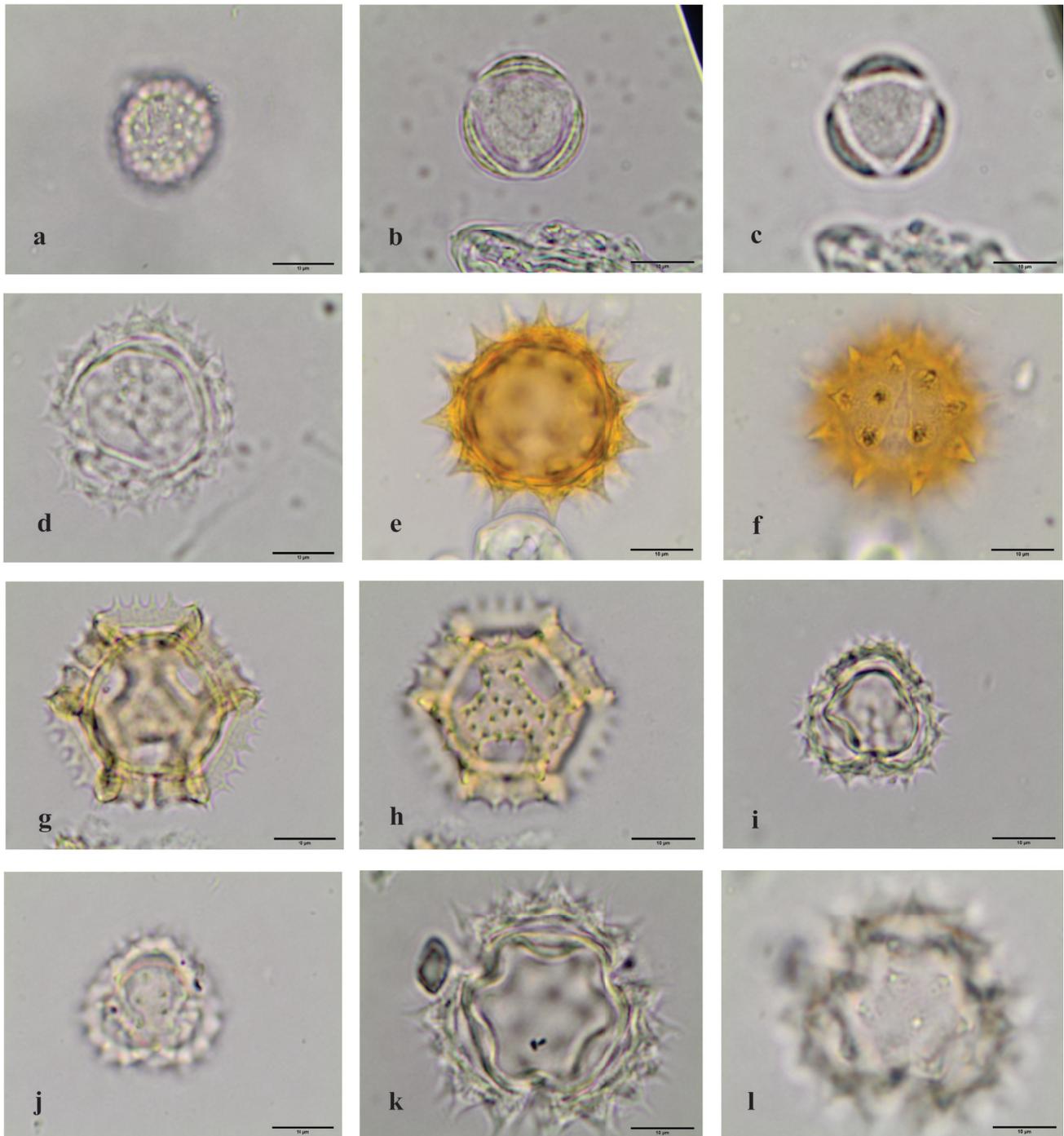


Figure 6. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons, Asteraceae. a. *Ambrosia*. b-c. *Artemisia*. d. *Cirsium/Carduus*. e-f. *Senecio*. g-h. *Taraxacum*. i-j. Asteraceae 1. k-l. Asteraceae 3. Scale bars: 10 µm.

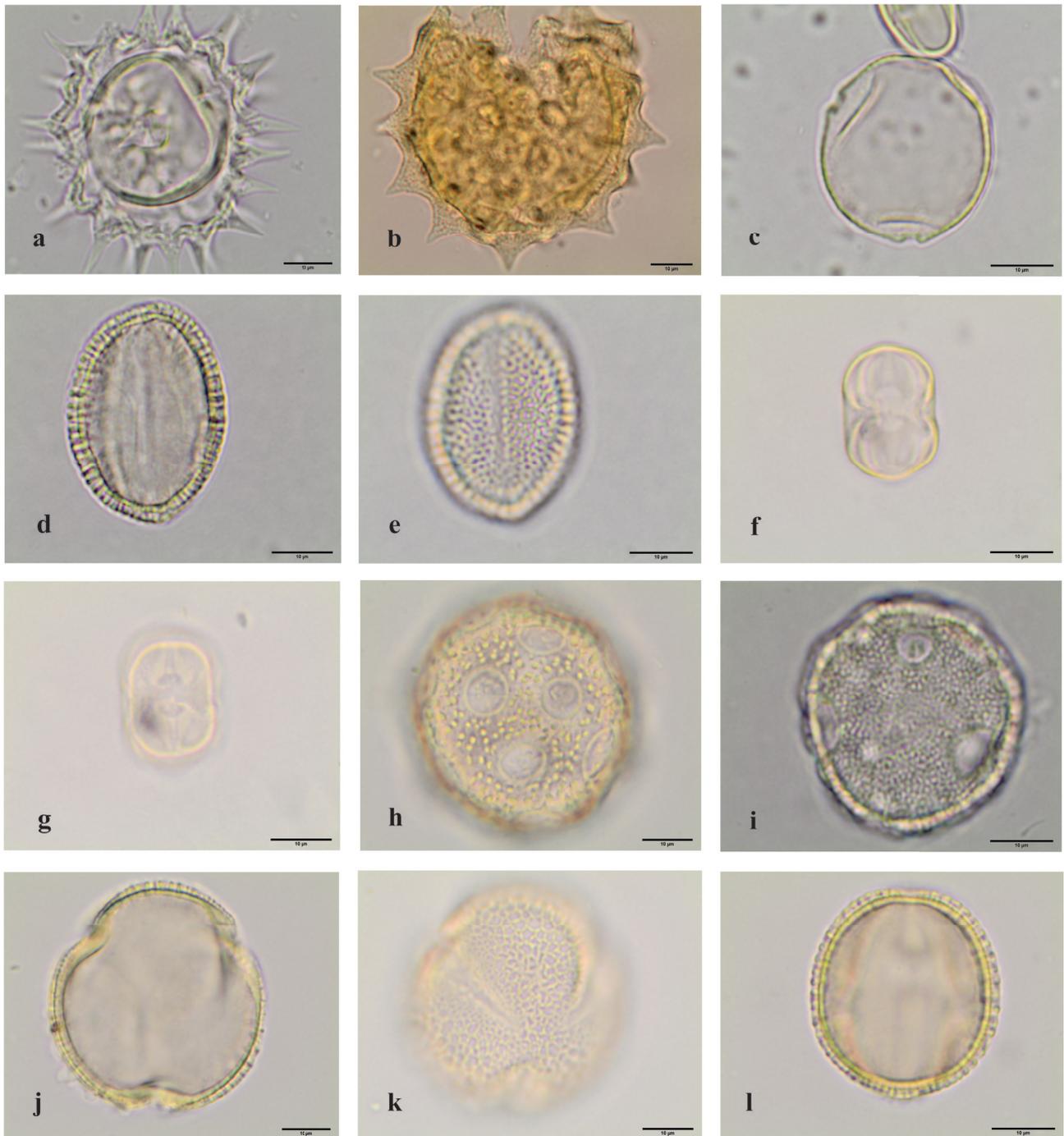


Figure 7. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons. a. Asteraceae 4. b. Asteraceae 5. c. Betulaceae, *Betula*. d-e. Brassicaceae. f-g. Boraginaceae, *Myosotis*. h-i. Caryophyllaceae. h. Caryophyllaceae 1. i. *Melandrium*. j-l Cistaceae, *Cistus*. Scale bars: 10 µm.

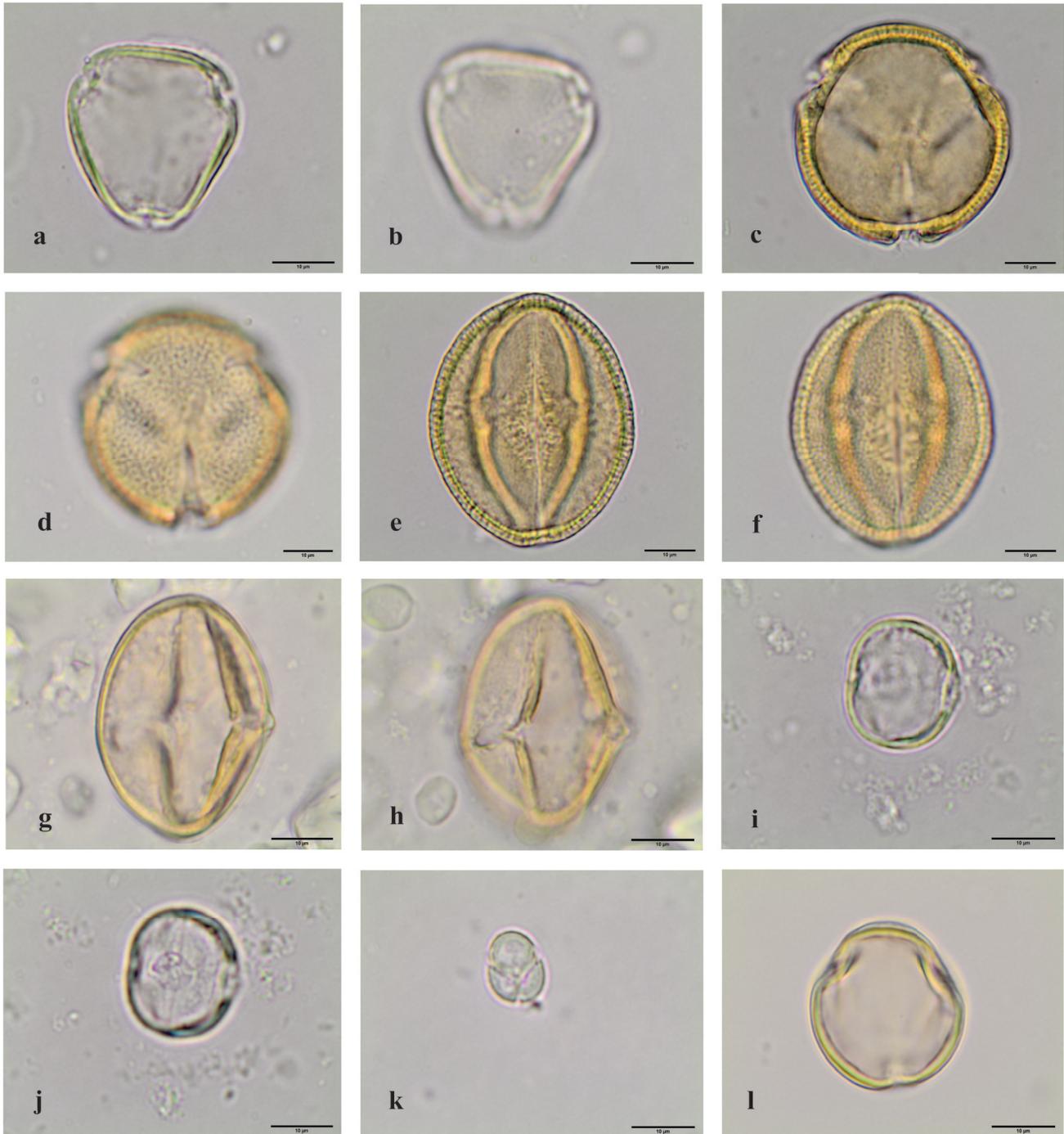


Figure 8. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons. a-b. Cornaceae, *Nyssa*. c-f. Euphorbiaceae. g-l. Fabaceae. g-h. *Cassia*. i-j. *Lotus corniculatus*. k. *Mimosa pudica*. l. *Robinia*. Scale bars: 10 µm.

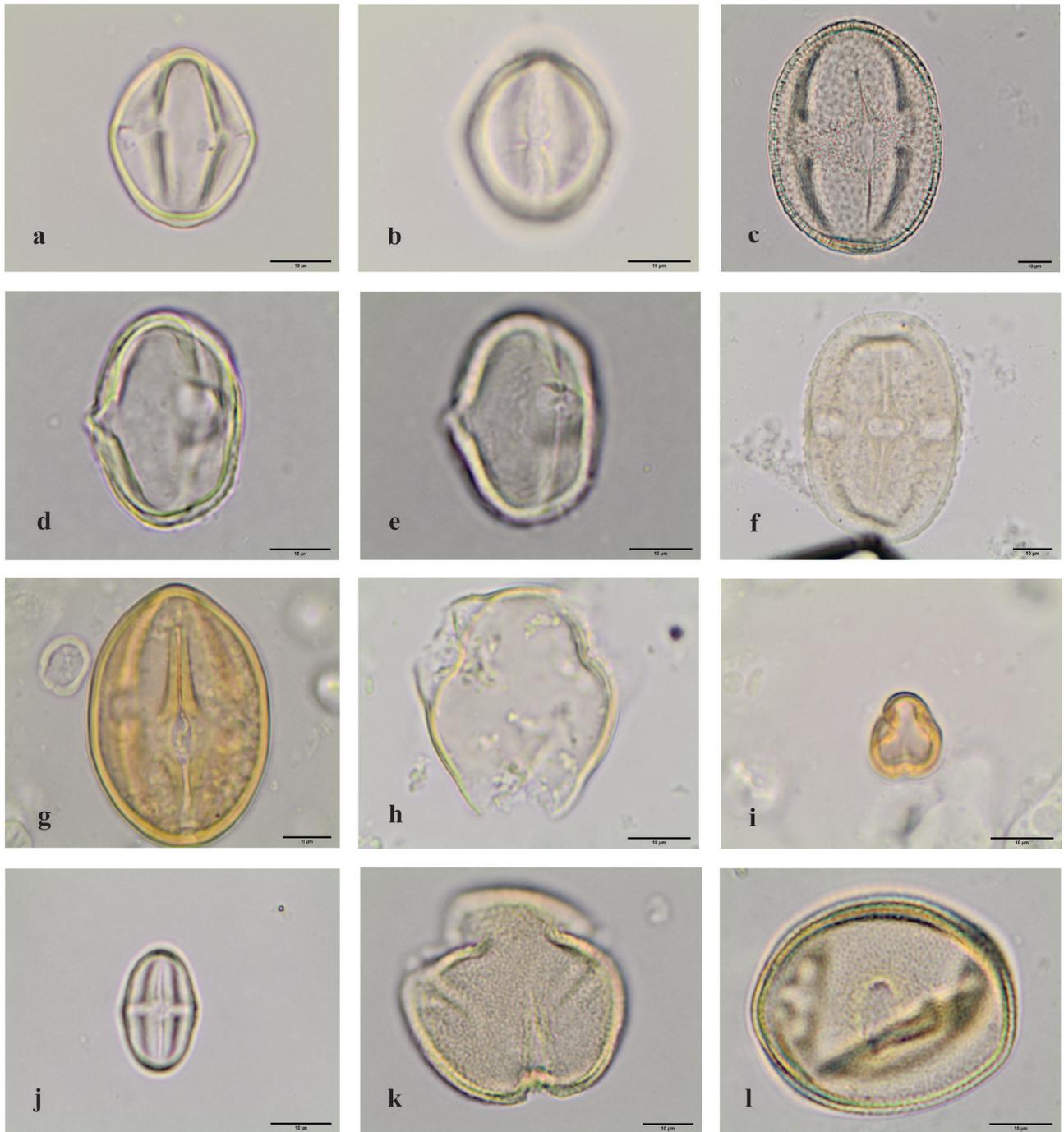


Figure 9. Light microscopy photographs of pollen grains observed in the royal jelly. a-l. Angiospermae eudicotyledons. a-h. Fabaceae. a-b. *Robinia*. c. *Trifolium incarnatum*. d-e. *Trifolium repens*. f. *Vicia faba*. g. Fabaceae 1. h. Fabaceae 4. i-l. Fagaceae. i-j. *Castanea*. k-l. *Quercus*. Scale bars: 10 µm.

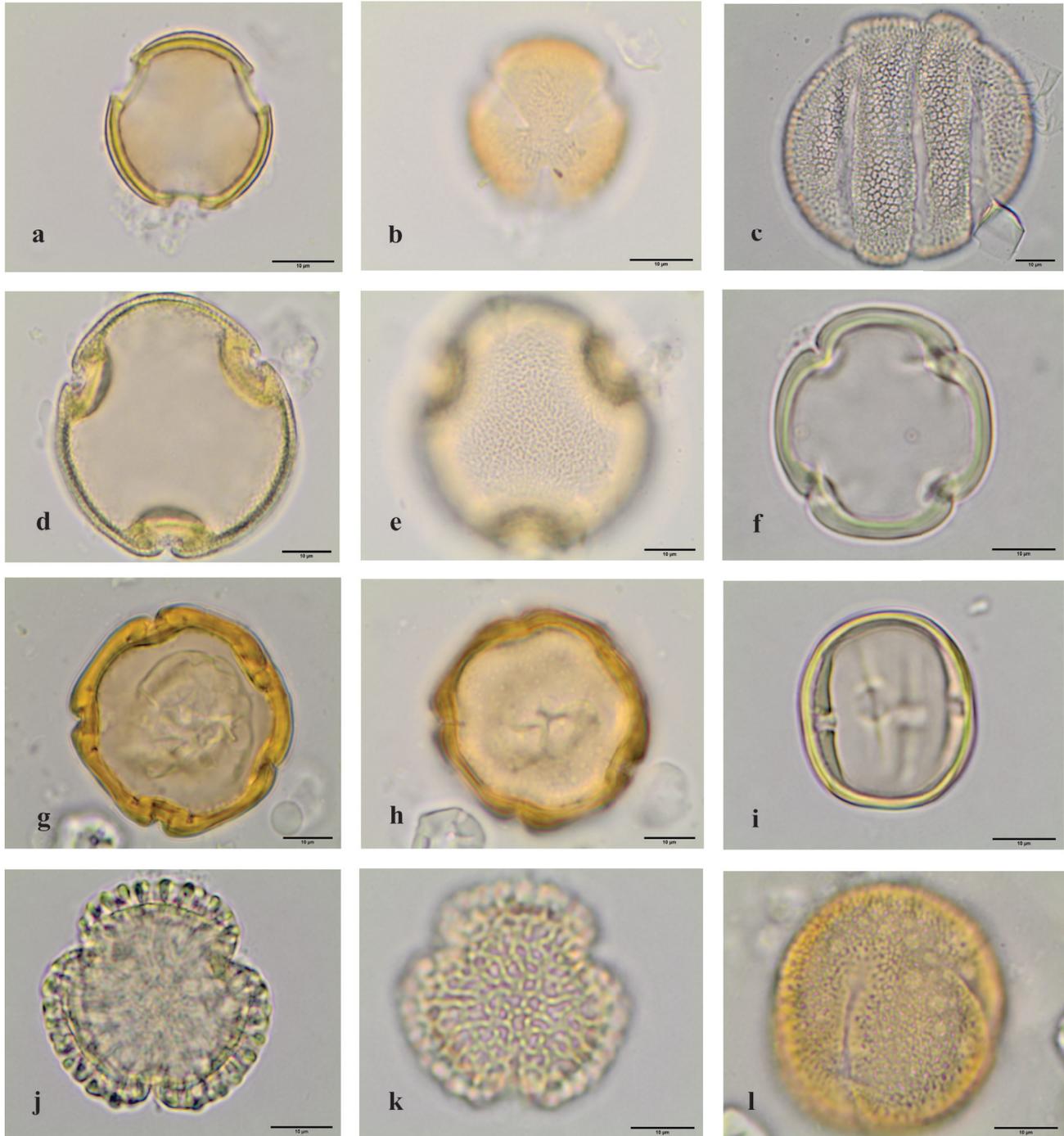


Figure 10. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons. a-b. Gentianaceae. c. Lamiaceae. d-e. Malvaceae, *Tilia*. f-i. Meliaceae. f. Meliaceae 1. g-h. Meliaceae 2. i. Meliaceae 3. j-k. Oleaceae, *Ligustrum*. l. Polygonaceae, *Rumex*. Scale bars: 10 µm.

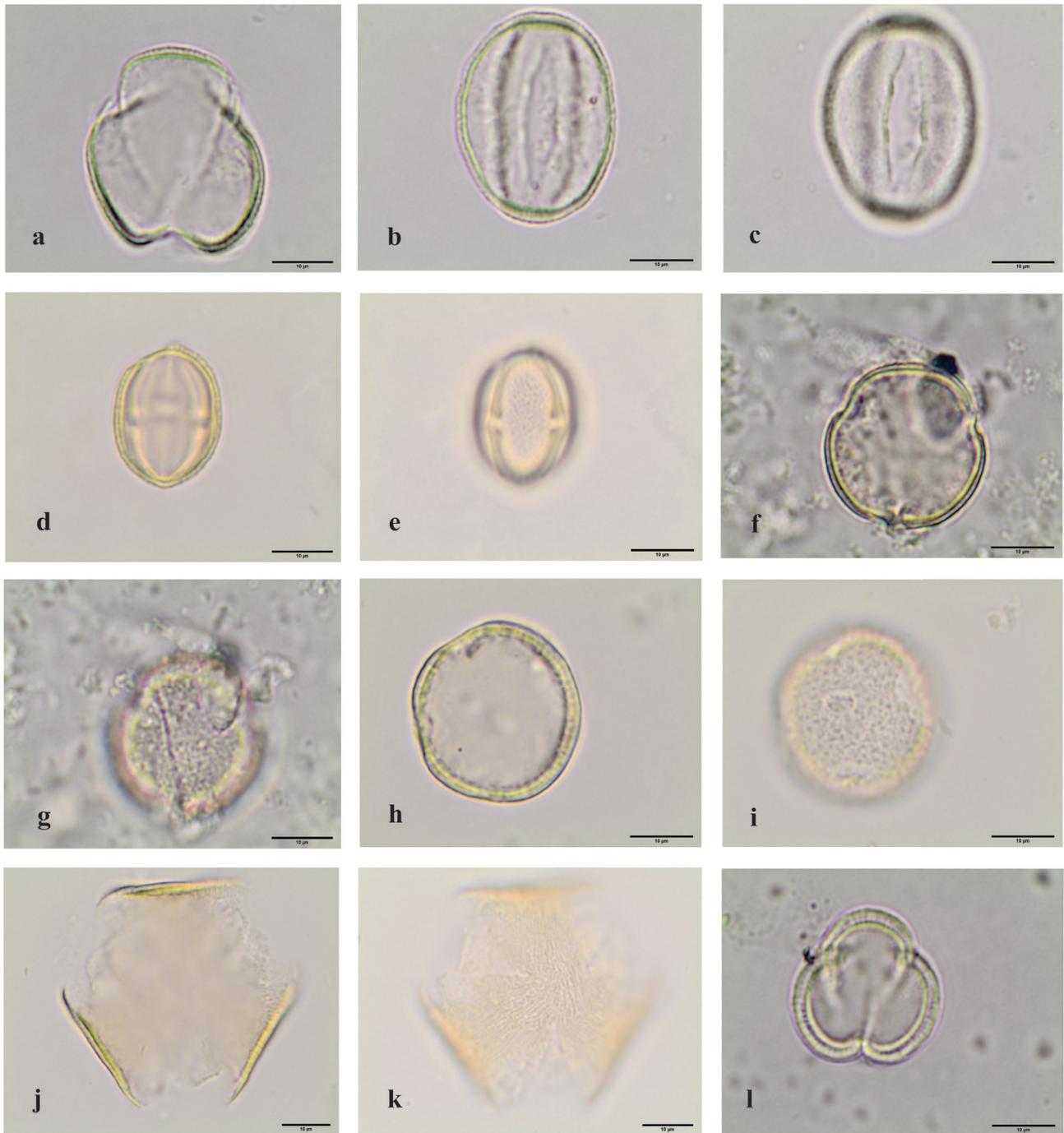


Figure 11. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons. a-c. Plantaginaceae, *Veronica*. d-e. Primulaceae, f-i. Ranunculaceae. f-g. *Caltha*. h-i. *Thalictrum*. j-k. Rosaceae, *Prunus*. l. Salicaceae, *Salix*. Scale bars: 10 µm.

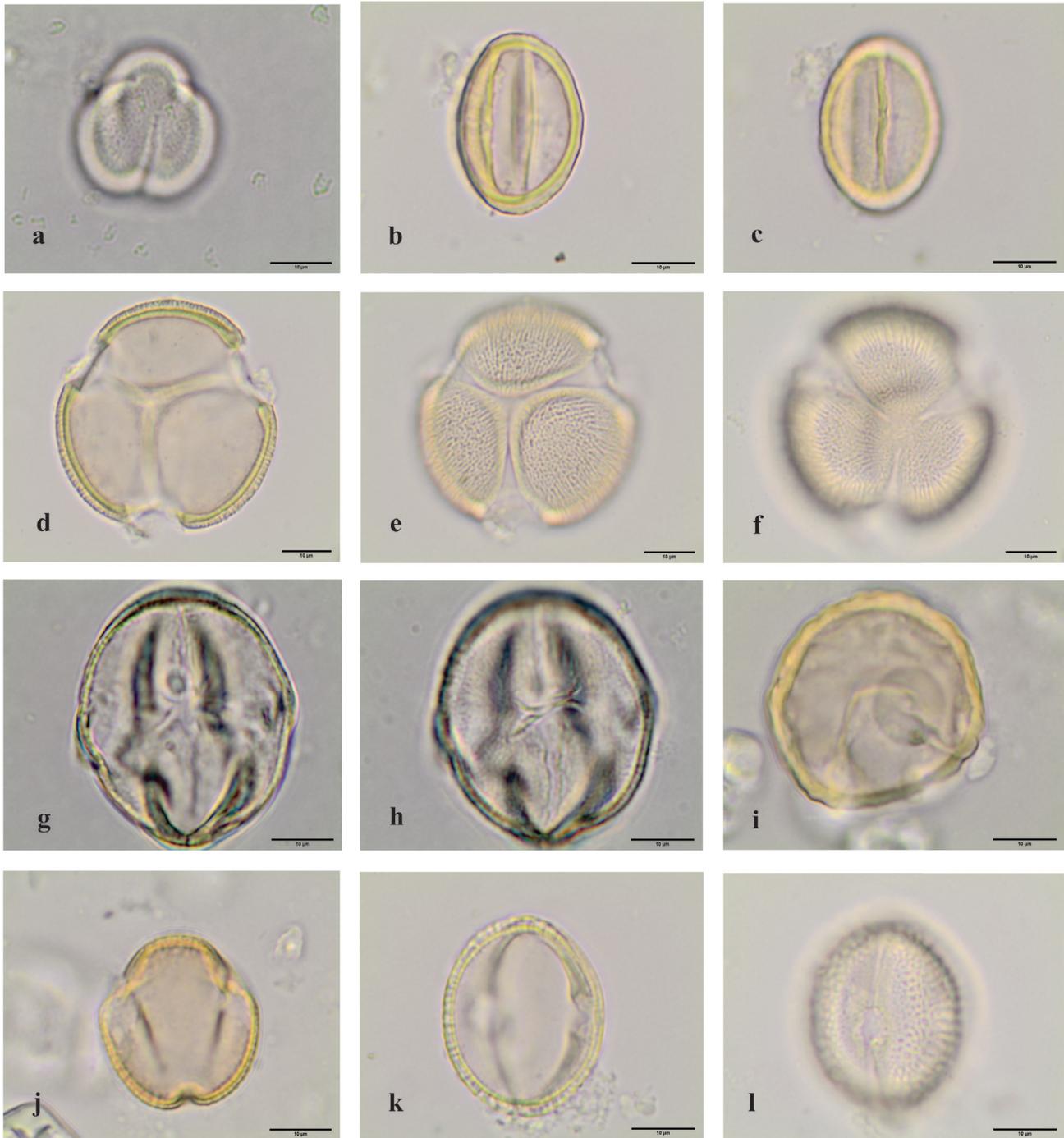


Figure 12. Light microscopy photographs of pollen grains observed in the royal jelly samples. a-l. Angiospermae eudicotyledons. a. Salicaceae, *Salix*. b-c. Sapindaceae, *Acer negundo*. d-f. Saxifragaceae, *Saxifraga*. g-h. Solanaceae. i. Ulmaceae, *Ulmus*. j-l. Vitaceae, *Parthenocissus*. Scale bars: 10 µm.

The Brazilian Ministry of Agriculture had no records of imported royal jelly from Europe, North America or Asia related to the period of the corresponding lots. Thus, the contaminated lots were probably illegally imported, threatening national beekeepers. As royal jelly production in Brazil is no longer economically viable due to the illicit entry of royal jelly over the past decade from China at low prices, we suggest that the analyzed products are original without the mixture of royal jelly produced locally in Brazil. This is a matter of concern because the dispersion of *P. larvae* spores from imported products without sanitary inspection is associated with the following contamination routes: using of contaminated royal jelly to feed queen larvae; manipulating contaminated products and/or utensils used to handle healthy colonies; using contaminated wax as comb substrate; access of bees to remains of contaminated imported products discarded near apiaries; cross-contamination in warehouses (*e.g.*, pollen dryers, where pollen grains of different origins are dehydrated) and supply of contaminated food supplements to healthy bees during the winter (especially bee pollen).

The possibility of *P. larvae* dispersion and contamination of healthy colonies is aggravated by traditional handling procedures such as transferring wax frames among colonies, using the same utensils in multiple bee hives and transferring colonies among apiaries, as well the natural trophallaxis among bees.

We stress the urgency of regulatory agencies taking appropriate measures at warehouses to verify the origin of the lots of royal jelly marketed and thus detect and avoid the illicit trade, as well as to implement risk analysis to identify critical points of the chain for traceability.

Conclusions

No royal jelly sample contaminated with *Paenibacillus larvae* had a pollen spectrum compatible with Brazilian vegetation, showing that the products analyzed were imported. The pollen types identified in the samples indicated probable phytogeographical origin from the Northern Hemisphere, either from Europe, Asia or North America.

By carrying out a palynological analysis, it was observed that the pollen grains in the royal jelly samples are from species occurring in temperate climates, such as boreal forests with conifers and milder climates, for example in the Mediterranean. Pollen grains from forage grasses, as well various crops were observed in the royal jelly samples showing

similarity with the samples produced by *Apis mellifera* bees in most parts of the world. Therefore, these pollen types are not appropriate for the characterization of the phytogeographical origin of royal jelly.

The presence of *P. larvae* spores in bee products from Brazilian apiaries is an unexpected finding because the country is officially considered free from the disease. However, a previous study (molecular analysis) done for pathogen detection in the same samples showed that in São Paulo State royal jelly has spores of this bacterium. The lack of effective control in this commercialization, as proven in this study, can ruin Brazilian beekeeping if this pathogen is spread throughout the country and applied palynology in forensic science is helping in this battle.

Overall, our findings show that palynology is a powerful tool in identifying the phytogeographic origins of royal jelly from the Northern Hemisphere. Although the current legislation does not require bee products to be examined palynologically to verify phytogeographic origins, this analysis can help to control pathogens from spreading. In addition, we show the fragility of actions regarding the control of imported products and thus the urgent need for more effective rules and actions by sanitary authorities.

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