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Original article

Discriminating unifloral honey from a dioecious mass flowering tree of Brazilian seasonally dry tropical forest through pollen spectra: consequences of honeybee preference for staminate flowers

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Abstract – *Myracrodruon urundeuva* ("aroeira") is a dioecious tree of the seasonally dry tropical forest (SDTF) of Brazil and source of a unique unifloral honey. To discriminate this honey by its pollen spectra, we compared melissopalynological analysis of reference honey samples with those of other samples collected in the SDTF belt. Reference honeys had on average 99% of aroeira pollen, while the other honey samples averaged 84% of this pollen. We used the receiver operating characteristic (ROC) curve, applied here for the first time in honey analysis, for determining the cut-off value of at least 93% of *Myracrodruon* pollen in a sample for classifying unifloral aroeira honey. The over-representation of aroeira pollen in this honey reflects that honeybees visited ten times as many staminate flowers as pistillate flowers. We conclude that unifloral aroeira honey has uniform pollen spectra, as a byproduct of the preference of honeybees for staminate flowers.

Myracrodruon urundeuva* / receivers operating characteristic (ROC) curve / melissopalynology / *Apis mellifera

1. INTRODUCTION

Pollen analysis of honey provides a microscopic fingerprint of the environment from which the honey came and can serve as criteria for quality and origin control (Louveaux et al. 1978; Barth 1989; Barth 2004; Von Der Ohe et al. 2004; Marquele-Oliveira et al. 2017). Mixed with nectar, pollen grains enter honey in varying quantities depending on flower morphology, flower size, and pollen production per anther (Todd and Vansell 1942; Maurizio and Hodges 1951; Faegri et al. 1989). Moreover, small pollen grains adhering to the body surface of honeybees more frequently contaminate honey than large ones (Maurizio and Hodges 1951; Bryant Jr and Jones 2001). Pollen from flowers with easily accessible anthers and numerous small pollen grains is generally over-represented in honey, such as *Eucalyptus* and *Castanea* (Louveaux et al. 1978; Von Der Ohe et al. 2004; Nedic et al. 2013). In contrast, those with large pollen grains or with nectar flowers that produce small quantities of pollen and deposit pollen on specific body parts of bees may be underrepresented, such as *Citrus* and Lamiaceae species (Louveaux et al. 1978; Von Der Ohe et al. 2004; Westerkamp and Claßen-Bockhoff 2007; Nedic et al. 2013).

Floral honey produced mainly from one plant species is referred to as unifloral. Unifloral honey

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has a pollen spectrum where one species is predominant; that is, it accounts for more than 45% of the pollen grains in the honey (Maurizio and Hodges 1951; Louveaux et al. 1978; Anklam 1998; Von Der Ohe et al. 2004). Unifloral honeys have, in general, greater commercial value than heterofloral honeys because they possess specific physicochemical and organoleptic characteristics, and specific tastes and flavors (Oddo et al. 1995; Anklam 1998; Terrab et al. 2014; Makhloufi et al. 2015; Fagúndez 2016; Kadri et al. 2016; Tette et al. 2017).

Unifloral honeys originate in environments that experience mass flowering of a single melliferous plant species. This can occur in non-natural environments such as monoculture plantations of rapeseed (*Brassica napus* L., Brassicaceae), sunflowers (*Helianthus annuus* L., Asteraceae), lavender (*Lavandula angustifolia* Mill., Lamiaceae), orange (*Citrus* spp., Rutaceae), eucalypt (*Eucalyptus* spp., Myrtaceae), among others (Louveaux and Vergeron 1964; Louveaux et al. 1978; Goplen 1980; Moar 1985; Oddo et al. 1995; Bryant Jr and Jones 2001; Nedic et al. 2013; Consonni and Cagliani 2015; Makhloufi et al. 2015; Fagúndez 2016; Kadri et al. 2016;).

Unifloral honeys from natural or near-natural plant cover are rare and can only be obtained in homogenous vegetation types with low plant diversity or where mass flowering species temporarily dominate the floral resources under highly seasonal climatic conditions. The seasonally dry tropical forest (SDTF) of Brazil experiences such high seasonality with well-defined dry and rainy seasons (Santos et al. 2012). The majority of species flower in the rainy season, while during the severe dry season woody plants shed their leaves, the herbaceous plant layer disappears, and only a few trees set blossoms (Santos et al. 2012).

Myracrodruon urundeuva Freire Alemao (Anacardiaceae), commonly referred to as "aroeira" in Brazil, is a common deciduous tree in the SDTF, and which intensely blooms during the dry season (Santin and Leitão 1991). Bees are cited as the main pollinators of the species (Kiill et al. 2010), and beekeepers know this species as an excellent nectar and pollen source for honeybees and stingless bees in the region. The results of the first pollen analysis of honey collected in the Brazilian SDTF during the dry season showed an extraordinary high content of aroeira pollen (Bastos et al. 2016). Differing from most other mass flowering food resources for honeybees, *M. urundeuva* is dioecious (Santin and Leitão 1991) and both pistillate and staminate flowers produce nectar. Thus, the unisexual flowers have different displays and resources and may have different attractiveness to bees.

Aiming to know if honeybees have preference to flowers of one of the genders and to discriminate unifloral aroeira honey by pollen analysis, we determined the frequency of visits to staminate and pistillate flowers and established reference honey samples produced in monitored experimental hives in a preserved SDTF fragment. We compared the monitored samples with numerous other honey samples produced within the Brazilian SDTF belt during the flowering period of aroeira. To establish a cut-off value to discriminate aroeira honey by its pollen spectra, we used the receiver operating characteristic (ROC) curves as tool. So, we asked: How does aroeira pollen content differ among the samples? Do honeybees visit staminate and pistillate flowers with the same frequency, and do plants of both genders make the same contribution to honey?

2. MATERIAL AND METHODS

2.1. Study species and sites

Myracrodruon urundeuva is native to the Gran Chaco domain in Paraguay, Argentina, and Bolivia, while in Brazil, the species occurs in the Caatinga, Cerrado, Atlantic Forest, and the belt of seasonally dry tropical forest, where it is particularly abundant (Santin and Leitão 1991; Caetano et al. 2008; Santos et al. 2012). This vegetation neighbors the Caatinga domain to the north and northeast, with transition to the Cerrado to the south and west (Brandão 1994; Santos et al. 2012). According to Pennington et al. (2009), SDTF is comprised of tall forest on moister sites, to scrub rich in succulent plants on the driest sites. Water availability is erratic and, in general, substrates are mineral rich (Pennington et al. 2009). The vegetation is dominated by trees, most of which are deciduous during the dry season (Santos et al. 2012). The dry period, from April to September, is pronounced. The vegetation at the studied site is composed of widespread characteristic SDTF species, which include *Anadenanthera peregrina* (L.) Speg. (Fabaceae), *Cordia trichotoma* (Vell.) Arráb. ex Steud. (Boraginaceae), *Chaetocalyx acutifolia* (Vogel) Benth. (Fabaceae), *Ceiba speciosa* (A. St.-Hil.) Ravenna (Malvaceae), *Commiphora leptophloeos* (Mart.) J.B. Gillett (Burseraceae), *Cnidoscolus pubescens* Pohl (Euphorbiaceae), *Neoglaziovia variegara* (Arruda) Mez (Bromeliaceae), *Schinopsis brasiliensis* Engl., *Spondias tuberosa* Engl. (Anacardiaceae), *Triplaris gardneriana* Wedd. (Polygonaceae). The common name "aroeira" is also used for other tree species of Anacardiaceae in Brazil, including other species of the genus *Myracrodruon*, and species from *Schinus* and *Astronium*. Individuals of *M. urundeuva* can reach 25 m in height, and several studies have reported that it is one of the most frequent and widespread species in the region of the SDTF, where the present study was undertaken (Andrade-Lima 1981; Prado and Gibbs 1993; Santos et al. 2007; Caetano et al. 2008; Santos et al. 2012; Calvo-Rodriguez et al. 2017). *Myracrodruon urundeuva* is widely used in popular therapeutic treatments (Monteiro et al. 2012), due to the high concentration of secondary metabolites present in vegetative parts (Viana et al. 1995; Queiroz et al. 2002).

2.2. Honey samples

The study analyzed 190 honey samples, which were divided into two groups. One group, the reference group, is composed of 10 samples collected in a monitored SDTF site, where the above-cited plant species are common. The second group, the evaluation group, is composed of 180 honey samples from numerous locations within the SDTF belt (see below).

*Reference group*The ten samples that compose the reference group came from a reference site (43° 31′ 06″ W, 15° 40′ 34″ S) about 30 km west of the town of Janaúba (Figure 1a). One sample was collected in June 2014, three in July 2016, and six in June 2017. The samples came from different experimental hives. In the dry season, previously to flowering of *M. urundeuva*, honey was harvested from the hives so that no honey from the rainy season remained. Honey samples were then collected soon after the blooming period of *M. urundeuva*, with the honey of each hive being centrifuged and stored separate from those of other hives. The site for the collection of the reference honey samples is part of a larger well-preserved fragment of SDTF, with an area of approximately 5.9 km^2, average annual rainfall of 730.46 mm and temperatures ranging from 19.1 to 31.6 °C (INMET 2018)

*Evaluation group of honey samples*The 180 samples that compose the evaluation group were collected at 180 apiaries among 53 municipalities within the SDTF belt. The collection of samples was made possible through the cooperation of *Companhia de Desenvolvimento dos Vales do São Francisco e do Parnaíba* (CODEVASF), which is a public Brazilian development company that, among other activities, provides support to beekeepers in the semi-arid region of Brazil. This cooperation aimed to establish a protected denomination of origin for the honey produced in this region. This enabled the collection of honey samples directly from the registered local apiaries within the SDTF (Figure 2). The samples were obtained between April and September 2014, and the beekeepers were instructed to collect honey under the same conditions as cited above for the reference honey samples.

2.3. Honey analysis and identification of pollen content

For microscopic analysis, 10 g of honey from each sample was acetolyzed (Erdtman 1960) following Louveaux et al. (1978). Pollen grains were examined using an Olympus BX50 light microscope and identified by comparison with reference slides from the collection of Fundação Ezequiel Dias, Belo Horizonte, Brazil, and by consulting specific literature. Pollen grains were identified as specifically as possible to type, genus, or family. Two slides were prepared from each acetolyzed honey sample and 500 pollen grains were counted in each and summed together to calculate relative frequencies expressed in percentages. Pollen types were expressed in

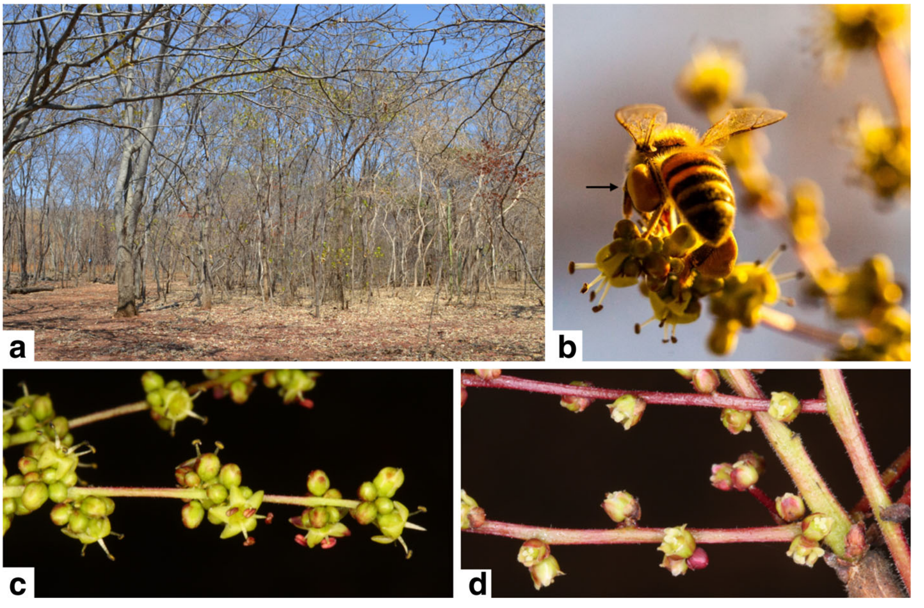

Figure 1. a Study site with typical seasonally dry tropical forest (SDTF), where reference honey samples were obtained. The trees of *Myracrodruon urundeuva* already shed their leaves. **b** Honeybee worker with full corbicula pollen load (arrow) visiting a staminate flower. **c** Staminate and **d** pistillate flowers of *M. urundeuva*.

standard frequency classes according to Louveaux et al. (1978) as follows: "Predominant Pollen" (PP), more than 45% of the pollen grains counted; "Secondary Pollen" (SP), 16–45%; "Important Minor Pollen" (IMP), 3–15%; and "Minor Pollen" (MP), less than 3%. Pollen grains whose frequency was 1% or less were considered as "present" (Louveaux et al. 1978), and excluded

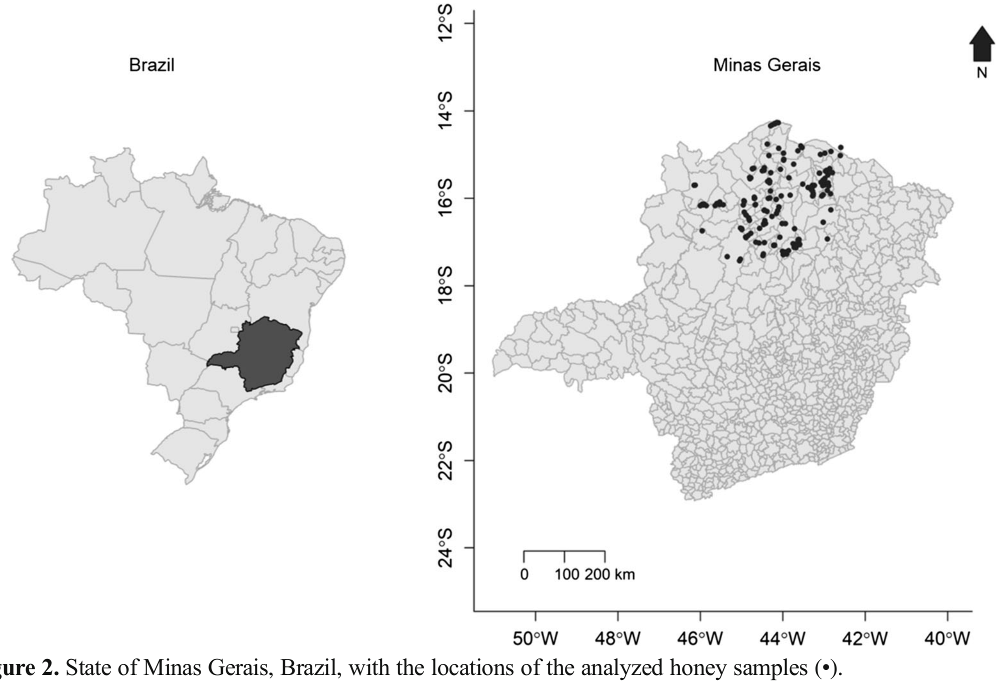

Figure 2. State of Minas Gerais, Brazil, with the locations of the analyzed honey samples (•).

from statistical analysis of our quantitative dataset. The variable "number of pollen types per sample" was created and added to the data set.

2.4. Honeybee flower visiting frequency

The frequency of honeybee visits to staminate and pistillate flowers (Figure 1b–d) was recorded in seven trees with pistillate flowers (pf) and nine trees with staminate flowers (sf). Three sf and two pf individuals were studied at the reference site and five sf and pf individuals in the municipality of Lagoa Santa (43° 52′ 11.0″ W, 19° 36′ 53.7″ S) between May and July 2016/2017. The number of visits was recorded by direct observation made during 5 min each hour of the day (Pierrot and Schlindwein 2003) on each tree. Observations of flower visits were performed using a 3-m long ladder placed in the trees. Based on the 5-min counts, we calculated the rate of flower visits for 1 h. A total of 17 h of direct observation of *A. mellifera* visits to flowers was made.

2.5. Statistical analysis

First, a comprehensive exploratory analysis considering both relative frequency (%) and frequency classes (Louveaux et al. 1978) of each pollen type among samples and between reference and evaluation groups was undertaken. A descriptive analysis of pollen spectra of the reference and evaluation groups was then performed. Next, a cluster analysis was conducted using the Bray-Curtis index to explore similarities among all 190 honey samples considering the relative frequencies of pollen types. The Mann-Whitney test was used to select significant variables from honey pollen spectra in the different reference and evaluation groups. Logistic regressions with the significant variables were then performed to explain the classification between groups (p value < 0.05).

2.6. Receiver operating characteristic curves

Receiver operating characteristic (ROC) curves consist of organizing classifiers and measuring the accuracy of their discrimination performance by calculating the area under the curve (AUC) (Bamber 1975; Swets 1988). This technique has been widely used in measuring the accuracy of diagnostic systems (Fawcett 2006) and to predict the distribution of species (Fielding and Bell 1997), as well as to measure the influence of climate change on the distribution of species (Giannini et al. 2012). In our study, a ROC curve was used to test the predictor variables previously selected by logistic regressions to classify honey, and to establish a cut-off value to discriminate aroeira honey samples. The experimental design with 10 reference honey samples and 180 evaluation honey samples to be classified is adequate for the performance of logistic models as well as for ROC curve analysis.

The maximum value of AUC is one, which indicates a perfect classification of samples. Therefore, we aimed to select the ROC curve with the highest AUC to establish the cut-off point for classifying the honey samples. To do this, it was necessary to analyze sensitivity and specificity values, which are parameters inherent to the ROC curve (Figure 3). Sensitivity is the true proportion of positive events in groups or, in other words, all the samples from the reference group. Specificity means the true negative proportion of samples.

All statistical analyses were done using R software (R Core Team 2017), with the ROC curve analysis being performed using the pROC package (Robin et al. 2011).

3. RESULTS

3.1. Pollen spectra of honeys

There were only six pollen types in the reference group of honey samples (Figure 4), with a mean percentage of *Myracrodruon* type of 99% ± 2.0 (mean ± 1 standard deviation is given throughout the text), ranging from 93 to 100% (Table I and Figure 5). Non-*Myracrodruon* pollen types occurred in frequencies lower than 1% ("Minor Pollen" Table I).

Forty-six pollen types were identified in the evaluation group, comprising 19 families (Table I), with a mean percentage of *Myracrodruon* pollen of 84% ± 17.7 (range 2–99%; Table I and Figure 5). Most non-*Myracrodruon* pollen grains were of *Eucalyptus*, *Mimosa*, and *Copaifera* (Table I and Figure 4). Not to family level identified

		Event (Experimental Design)		
		Reference group	Evaluation group	Total
Diagnosis (Result after ROC curve analysis)	Positive (Unifloral honeys)	a	b	a+b
	Negative (Heterofloral honeys)	c	d	c+d
	Total	a+c	b+d	a+b+c+d=N

Where:

a: Number of unifloral honey samples from the reference group (N= 10).

b: Number of samples from the evaluation group, classified as unifloral.

c: Number of samples from the reference group classified as heterofloral.

d: Number of samples from the evaluation group classified as heterofloral.

N: Total number of samples of the study (N=190).

Sensitivity (True positive) = a / a+c

Specificity (True negative) = d / b+d

Figure 3. Matrix of classification with the definition of ROC curve parameters considering the experimental design of this honey study. Adapted from Swets (1988).

pollen types occurred in 130 samples (Table I). The median of these pollen types in a honey sample was 2, ranging from 1 to 8 types, with median relative frequency of 2.8%, ranging from 1 to 24%. Pollen from *Eucalyptus* occurred in 50 samples, with a mean relative frequency of 4% ± 18.8 (Important Minor Pollen), while *Mimosa* occurred in 38 samples (3% ± 15.2); *Copaifera* had a mean frequency of 1% ± 28.4 (Minor Pollen) (Table I). Among the 46 pollen types identified, 41 occurred with frequencies ≤ 1%, and were considered as "present" (Table I).

3.2. Frequency of honeybee visits to pistillate and staminate flowers

Honeybees visited on average ten times more staminate flowers ($\overline{X} = 5.1 \pm 6.2$) than pistillate flowers ($\overline{X} = 0.5 \pm 3.5$) per hour (Mann-Whitney

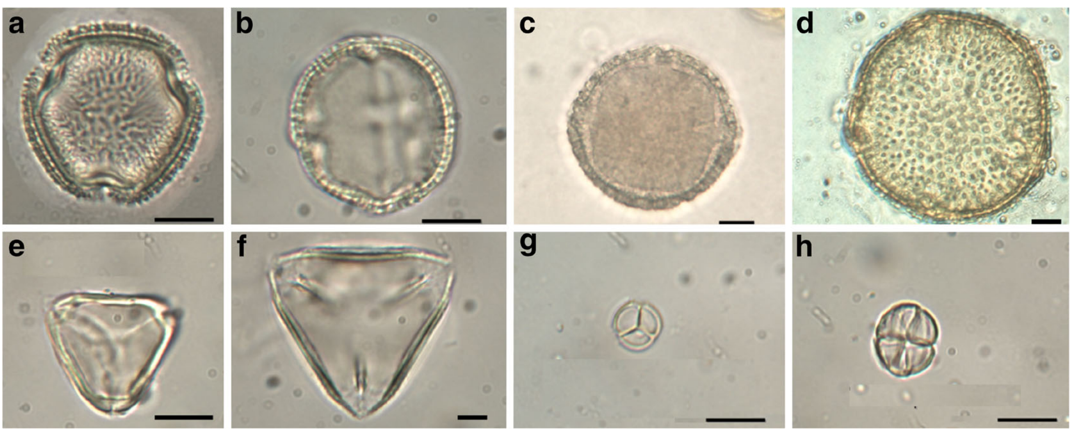

Figure 4. Main pollen types identified in honey samples analyzed in this study ($N = 190$). **a**, **b** *Myracrodruon* (Anacardiaceae). **c** *Cordia* (Boraginaceae). **d** *Waltheria* (Malvaceae). **e** *Eucalyptus* (Myrtaceae). **f** *Copaifera* (Fabaceae). **g** *Mimosa* sp1 (Fabaceae). **h** *Mimosa* sp2 (Fabaceae). Bars = 10 μm.

Table I. Mean percentage or presence (+) when relative frequency is less than 1%, in honeys from the reference group ($N = 10$) and from evaluation group ($N = 180$)

Pollen types	Reference group				Evaluation group			
	Mean %	sd	*N*	Frequency class	Mean %	sd	*N*	Frequency class
Amaranthaceae								
Althernanthera	+		1	MP	+		11	MP
Amaranthus					+		2	MP
Gomphrena					+		1	MP
Anacardiaceae								
Anacardiaceae non-identified	+		2	MP	+		22	MP
Myracrodruon	99%	1.97	10	PP	84%	17.69	174	PP
Tapirira					+		1	MP
Arecaceae					+		2	MP
Asteraceae								
Asteraceae non-identified					+		3	MP
Baccharis					+		6	MP
Chromolaema					+		1	MP
Elephantopus					+		1	MP
Vernonia					+		8	MP
Bignoniaceae								
Jacaranda					+		1	MP
Boraginaceae								
Cordia	+		1	MP	+			MP
Cecropiaceae								
Cecropia					1%	4.60	35	MP
Convolvulaceae								
Convolvulaceae non-identified					+		1	MP
Jacquemontia					+		1	MP
Merremia					+		17	MP
Euphorbiaceae								
Croton					+		1	MP
Euphorbiaceae non-identified					+		3	MP
Sebastiania					+		2	MP
Fabaceae								
Acacia					+		4	MP
Bauhinia					+		1	MP
Chaetocalyx					+		13	MP
Copaifera					1%	29.88	13	MP
Fabaceae non-identified					+		5	MP
Mimosa					3%	15.21	38	IMP
Senna					+		1	MP
Lamiaceae								
Hyptis					+		7	MP

Table I (continued)

Pollen types	Reference group				Evaluation group			
	Mean %	sd	*N*	Frequency class	Mean %	sd	*N*	Frequency class
Malvaceae								
Bombacopsis					+		1	MP
Bombax					+		2	MP
Malvaceae non-identified					+		1	MP
Waltheria	+		2	MP	+		1	MP
Melastomataceae					+		1	MP
Myrtaceae								
Eucalyptus					4%	18.85	50	IMP
Myrcia					+		3	MP
Myrtaceae non-identified					+		3	MP
Non-identified pollen types	+		1	MP	4%	4.84	129	IMP
Nyctaginaceae					+		1	MP
Poaceae					+		16	MP
Polygalaceae					+		1	MP
Rubiaceae								
Borreria					+		4	MP
Diodia					+		3	MP
Richardia					+		3	MP
Rubiaceae non-identified					+		1	MP
Sapindaceae								
Serjania					+		2	MP

sd standard deviation, *PP* Predominant Pollen (> 45%), *SP* Secondary Pollen (16–45%), *IMP* Important Minor Pollen (3–15%), *MP* Minor Pollen (< 3%)

test, $W = 1497.5$, p value < 0.001) (Figure 6). Both staminate and pistillate flowers produced small volumes of nectar (< 0.8 µL, $\overline{X} = 0.4 \pm 0.12$, $N = 42$) during the entire flower lifespan. Besides nectar, honeybees intensively collected pollen in the staminate flowers. The rate of honey bee visits to staminate flowers was high throughout the day while visits to pistillate flowers occurred primarily in the afternoon.

3.3. Statistical analysis

3.3.1. Similarity among samples

Cluster analysis of all 190 honey samples (Figure 7) revealed a well-delimited group with a low number of pollen types and a high percentage of *Myracrodruon* pollen. Furthermore, as the percentage of *Myracrodruon* pollen decreased, the number of pollen types in the honey samples increased, forming other groups of samples, such as one characterized by the presence of *Mimosa* and another group characterized by the presence of *Eucalyptus* pollen.

3.3.2. Selection of variables and logistic regressions

The selection of variables for logistic regression by Mann-Whitney test revealed that only five differed between the reference and the evaluation groups: *Cordia*, *Eucalyptus*, *Mimosa*, and *Myracrodruon* pollen types, and the number of pollen types (Table II).

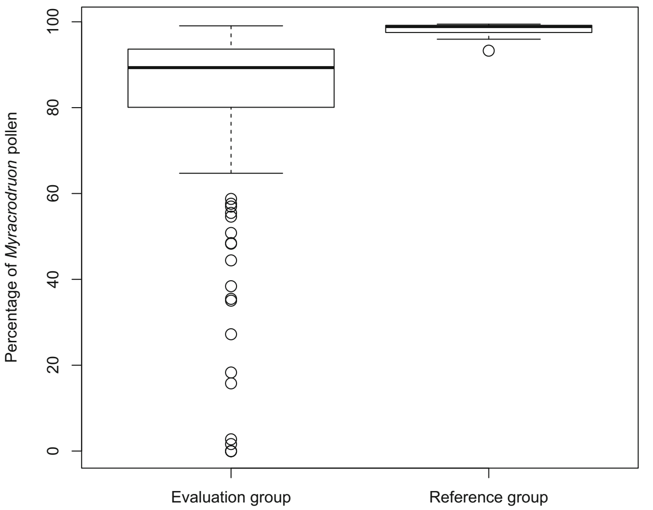

Figure 5. Relative frequency (%) of *Myracrodruon* pollen in honey samples of the evaluation group (N=180) and the reference group (N=10).

We tested several logistic regressions with all possible combinations of the significant variables, including interactions, and only these two significant regressions were obtained: (1) percentage of *Myracrodruon* pollen and (2) number of pollen types in the sample (Table III).

Interpreting the odds ratio of each regression found that for every percent of increase in the relative frequency of *Myracrodruon* pollen in a sample, there was a twofold increase in the chance of it belonging to the reference group. In contrast, for each new pollen type identified in a honey

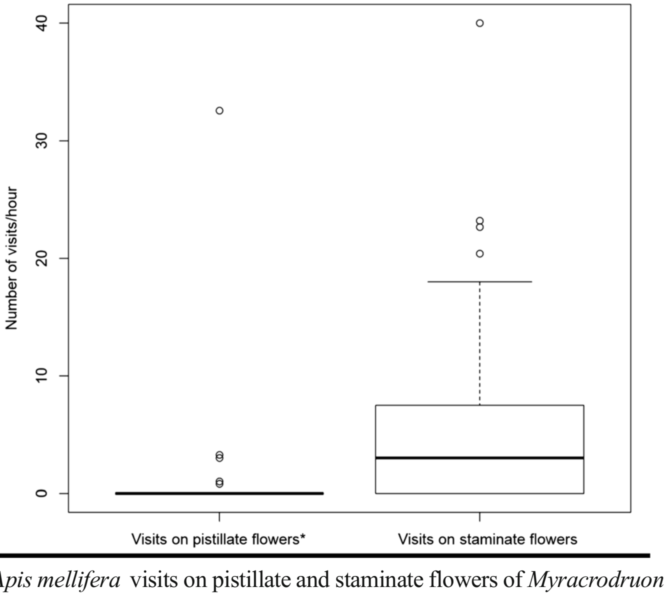

Figure 6. Number of *Apis mellifera* visits on pistillate and staminate flowers of *Myracrodruon urundeuva* per hour (N = 16 trees, being seven with pistillate flowers and nine with staminate flowers). * Significantly different from staminate flowers (Mann-Whitney test, p value < 0.001).

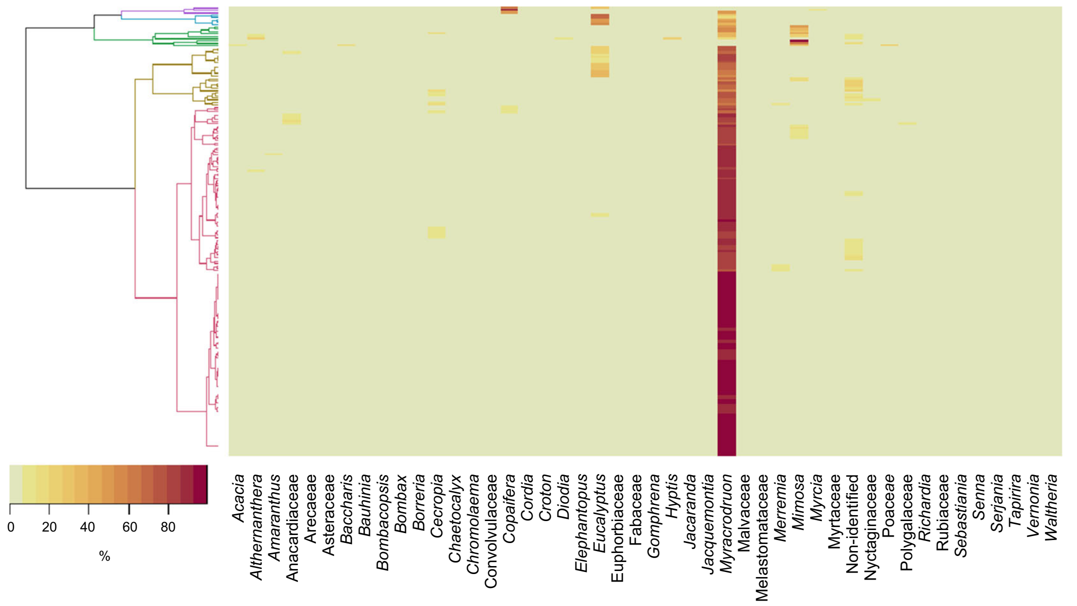

Figure 7. Dendrogram representing hierarchical clustering analysis (Bray-Curtis index) of honey samples ($N = 190$) using the relative frequency of pollen types. The heat map representation shows the percentage of each pollen type (columns) on each honey sample (rows).

sample with a relative frequency ≥ 1%, the chance of the sample belonging to the reference group decreased by a multiple of approximately five (Table III and Figure 8a, b).

3.3.3. Receiver operating characteristic curves

A ROC curve was constructed for each of the two significative regressions to compare their area under the curve (AUC) (Figure 8c). A higher AUC (0.945) was obtained for the first regression (% of *Myracrodruon* pollen). Thus, the percentage of *Myracrodruon* pollen was considered the best predictor for classifying honey samples as of the reference or evaluation group.

When using the ROC curve to establish the cut-off point for *Myracrodruon* pollen between the reference and the evaluation group, it was necessary to be certain that the classification of the reference group samples was fully correct. In other words, none of the reference honey samples could be classified as negative (i.e., non-aroeira honey), and that the sensitivity value must equal one. In the

Table II. Results of Mann-Whitney tests between reference ($N = 10$) and evaluation groups ($N = 180$) for significant variables of the pollen spectra of honey samples

Variables	Reference group		Evaluation group		*p* value
	Mean (%)	se[a]	Mean (%)	se	
Cordia	0.22	0.22	0.00	0.00	< 0.001
Eucalyptus	0.00	0.00	4.07	0.88	0.06
Mimosa	0.00	0.00	3.03	0.82	0.09
Myracrodruon type	98.00	0.62	82.00	1.53	< 0.001
Number of pollen types	1.60	0.27	3.37	0.12	< 0.001

se standard error

Table III. Results of the significative logistic regressions for classifying honey samples between reference ($N = 10$) and evaluation ($N = 180$) groups

Regressions	Coefficients	se	Z value	Odds ratio	p value
% of *Myracrodruon* pollen	0.74	0.22	− 3.37	2.09	< 0.001
Number of pollen types	− 1.71	0.49	− 3.52	0.18	< 0.001

se standard error

present study, when the sensitivity was one, the specificity value was 0.71 and the corresponding percentage of *Myracrodruon* pollen was equal to 93.2% (Figure 8d). Applying this cut-off value to the evaluation group, 52 (30%) of the 180 honey samples were considered pure aroeira honey (Table IV).

4. DISCUSSION

Results of the present study show that the pollen spectra of honey collected in the seasonally dry tropical forest belt of Brazil during the flowering period of *Myracrodruon urundeuva* can be used for indicating its botanical origin. According to

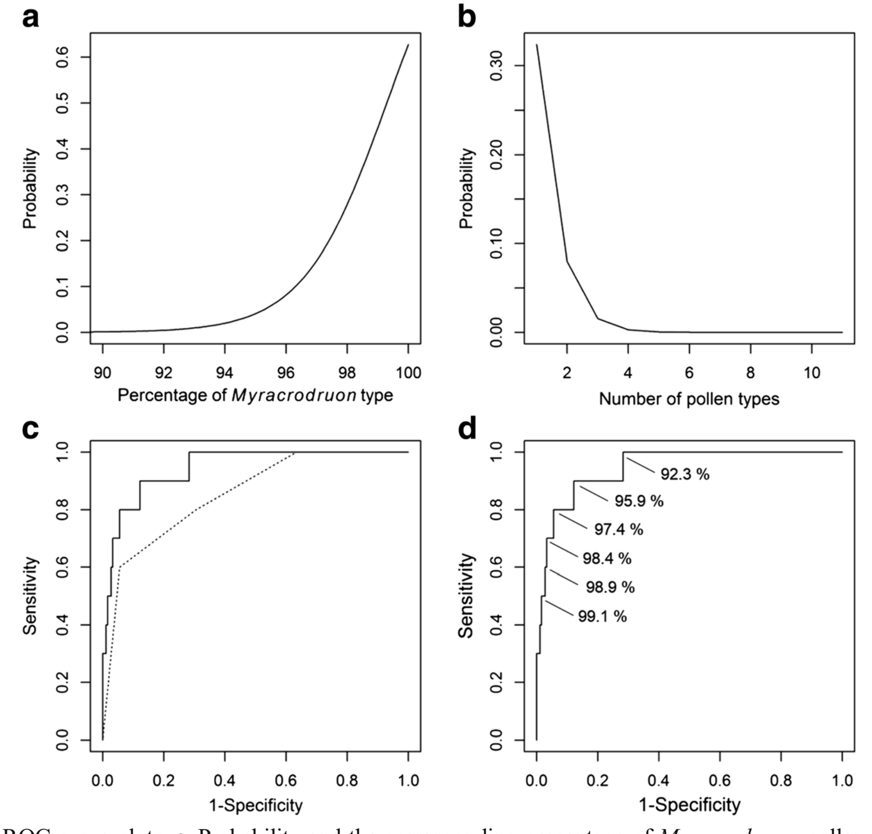

Figure 8. ROC curve plots. **a** Probability and the corresponding percentage of *Myracrodruon* pollen in a honey sample. **b** Probability and the corresponding number of pollen types in a honey sample. **c** Receiver operating characteristic (ROC) curves of the percentage of *Myracrodruon* pollen (bold line, AUC = 0.945) and for the number of pollen types in a honey sample (dotted line, AUC = 0.853). **d** ROC curve of the percentage of *Myracrodruon* pollen in a honey sample and the corresponding cut-off levels for several values of sensitivity and specificity.

Table IV. Matrix of classification showing the result of honey samples classification based on a cut-off value of 93.2% of *Myracrodruon* pollen in honey samples, as established by receiver operating characteristic (ROC) curve analysis

Classification result/initial groups	Reference group	Evaluation group	Total
Unifloral honeys	10	52	62
Heterofloral honeys	0	128	128
Total	10	180	190

our data, unifloral aroeira honey should contain the following: (i) at least 93% of *Myracrodruon* pollen and (ii) just up to three pollen types in the frequency class "Minor Pollen" (1–3%), using the categories of Louveaux et al. (1978).

Pollen grains of some species are over-represented in honey showing, thus, extraordinary high percentages in pollen spectra (Louveaux et al. 1978). When compared to other established unifloral over-represented honeys, like *Brassica napus* (> 86%) (Von Der Ohe et al. 2004), *Eucalyptus* spp. (> 90%) (Barth 1989; Bastos et al. 2003), *Castanea sativa* Mill. (> 90%) (Oddo et al. 2004), and *Mimosa scabrella* Benth. (92%) (Azevedo et al. 2017), the percentage of *Myracrodruon* pollen in aroeira honey is also very high.

The uniform pollen spectra, with the extraordinary high percentage of aroeira pollen, for this unifloral honey can be explained by the striking seasonality of the synchronized blooming period of this species, when few other melliferous plants set flower in the Brazilian SDTF belt. Moreover, it reflects the importance of the species as source of pollen besides nectar for honeybees. The mass flowering trees of this dioecious species produce five times more staminate flowers than pistillate flowers (Kiill et al. 2010), and our study showed that honeybees have a strong preference for the staminate flowers (visitation rates about tenfold higher) when compared to foraging on pistillate nectar flowers. A similar strong preference of honeybees (fivefold more visits) for staminate flowers of *M. urundeuva* was also reported in the Caatinga (Kiill et al. 2010). Moreover, trees with staminate flowers are more frequent than those with pistillate flowers in *M. urundeuva* populations. In the present study, 60% of the trees had staminate flowers while 40% of trees had pistillate flowers, which is similar to the findings of Kiill et al. (2010), with 61 and 39%, respectively. Furthermore, trees of *M. urundeuva* are highly abundant in the Brazilian SDTF (Santos et al. 2007; Caetano et al. 2008; Santos et al. 2012), which might be a consequence of an allelopathic effect caused by secondary compounds produced by their leaves (Moreira et al. 2007). All these factors together favor massive entry of the small pollen grains into the honey.

Originating from a dioecious tree species in near-natural environments, aroeira honey is exceptional among honeys. Dioecy is found in 5 to 6% of angiosperm species (Renner 2014) and it is expected that flowers of dioecious plants attract primarily nectar feeders to guarantee pollination (Richards 1997). Highly eusocial bees demonstrate the ability to discriminate between flowers with the more rewarding morph in tropical dioecious species (Renner and Feil 1993). This may have the result that plants with staminate flowers can temporarily be the main pollen source, for example to honeybees in flowers of *Spondias mombin* L. (Anacardiaceae), *Pouteria stipitata* Cronquist (Sapotaceae), and species of *Zanthoxyllum* (Rutaceae) (Roubik 1989), but that pistillate flowers are barely visited and, thus, pollinated by these bees (Renner and Feil 1993). The strong honeybee preference for staminate flowers of *M. urundeuva* found in our study corroborates with the above mentioned examples.

The floral display of male plants in *M. urundeuva* is increased in number of flowers and inflorescences in comparison to female plants, similar to other dioecious trees. In general, these species invest more in pollen production when compared to hermaphroditic species (Bawa 1980). This is also true for other dioecious neotropical Anacardiaceae such as *Astronium fraxinifolium* Schott (Cornacini et al. 2017), *Schinopsis brasiliensis* Engl. (Kiill et al.

2010), *Schinus terebinthifolia* Raddi (Lenzi and Orth 2004), and *Tapirira guianensis* Aubl. (Lenza and Oliveira 2005), which are strongly attractive to highly eusocial bees. Pollen of these plants is most likely over-represented in honeys like those of *Myracrodruon* in aroeira honey. Pollen of most dioecious species, however, is under-represented in honey samples because pistillate and staminate flowers attract pollinators mainly by nectar (Louveaux et al. 1978).

The presence of *Eucalyptus* pollen in the honey samples was negatively correlated with the presence of aroeira pollen, indicating that, when present, honeybees also use the widely known highly melliferous *Eucalyptus* flowers (Simeão et al. 2015), when its flowering overlaps with that of *M. urundeuva*. *Eucalyptus* plantations in the semi-arid region of northeast Brazil are usually in irrigated areas due to limited rainfall concentrated over short periods of the year, which causes lower yields (Azar and Larson 2000).

Mimosa-type pollen (minor pollen) was present only in the pollen spectra of the evaluation group honeys, although numerous species from this and other genera of Mimosoideae are native and a common source of nectar and pollen for bees in SDTF (Simon and Proença 2000; Santos et al. 2012, 2007, 2006). Most of these species, however, flower in the rainy season and are important constituents of heterofloral honeys collected outside the dry season in this region (Bastos et al. 2003; Novais et al. 2009; Silva and Santos 2014). Samples with any percentage of *Mimosa*-type pollen had decreased percentages of *Myracrodruon* pollen (Spearman's correlation $r = -0.37$, p value $= 0.0$). We suppose that mixtures of dry and rainy season honeys contain more *Mimosa*-type pollen. To obtain rather pure aroeira honey, beekeepers, therefore, should collect honey immediately after the blooming period of *M. urundeuva*.

The establishment of the cut-off value or threshold on pollen relative frequency in unifloral honeys can be assessed by diagnostic system techniques. They provide a discrimination of two groups or alternatives (e.g., being a unifloral honey) by using a specific threshold or cut-off value (Swets 1988). The success of these applications relies on the accuracy of the models given by the ROC curve analysis (Liu et al. 2011). The ROC curve analysis proved to be a suitable tool for determining a cut-off value for classifying unifloral honey. To our knowledge, this is the first use of this approach in melissopalynology, and we recommend its application for the evaluation of other kinds of unifloral honeys. However, to do this, it is necessary to define a standard reference group of monitored honey samples. In addition, other honey predictors, not only related to the pollen spectra of honey, may also be used in the ROC curve analysis, such as variables from physicochemical honey analysis.

Unifloral honeys produced from natural or near-natural plant cover are rare in the world and include manuka honey derived from *Leptospermum scoparium* J.R. Forst. & G. Forst. and *L. ericoides* A. Rich. (Myrtaceae) in New Zealand (Moar 1985), *Euphorbia* honey (Euphorbiaceae) in Marroco (Terrab et al. 2014), Humboldt's willow honey (*Salix humboldtiana* Willd., Salicaceae) in Argentina (Fagúndez 2016), and bracatinga honey (*Mimosa scabrella*, Fabaceae) in southern Brazil (Azevedo et al. 2017), among which aroeira honey soon may be added.

We conclude that aroeira honey shows a uniform pollen spectra with an extraordinary high percentage of pollen from this plant that allows the palynological definition of this honey as unifloral. This high relative amount of aroeira pollen in the honey is the result of synchronized mass flowering of this dioecious species in the dry season, when blooming of other bee plants is scarce in the Brazilian SDTF belt, as well as its high abundance in natural and semi-natural SDTF vegetation, and the strong preference of honeybees for trees with pollen- and nectar-producing staminate flowers over those with only nectar-producing pistillate flowers. The ROC curve analysis, applied to melissopalynology for the first time, proved to be an efficient tool for defining this unifloral honey. The presented results are a basic contribution for the control of origin and quality standard, which, together with the physicochemical characteristics, are important in establishing the protected denomination of origin (PDO) for aroeira honey. Considering pollination biology, it would be interesting to evaluate the pollination effectiveness of honeybees in comparison to native stingless bees, which are also common native flower visitors of *M. urundeuva*.

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AUTHOR CONTRIBUTIONS

EMAFB conceived the research. PC performed the analysis. CS and EMAFB contributed to the experimental design. EMAFB contributed to the pollen identification. PC, CS, and EMAFB interpreted results. PC and CS wrote the paper. All authors read and approved the final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Discrimination du miel unifloral à partir d'un arbre dioïque à floraison massive de forêt sèche tropicale du Brésil à travers les specters de pollen: conséquences de la préférence des abeilles pour les fleurs mâles

Myracrodruon urundeuva* / courbe-ROC / mélissopalynologie / *Apis mellifera

Unterscheidung von unifloralen Honigen eines diözischen massenblütigen Baumes aus dem brasilianischen wechselfeuchten Tropenwald anhand des Pollenspektrums: Konsequenzen auf die Honigbienenpräferenz für männliche Blütenstände

Myracrodruon urundeuva* / ROC-Kurve / melissopalynologie / *Apis mellifera

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