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Establishing Tobacco Origin from Pollen Identification: An Approach to Resolving the Debate

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Abstract

Previous research into pollen content of tobacco resulted in a debate. We address this debate and determine that pollen analysis may be able to assist with identifying geographical origin of tobacco. However, the value of any results should be assessed on a case-by-case regional basis until sufficient database information is available for an objective interpretation to be undertaken on a global basis. As a first step toward developing comparative data for South America, we analyzed a tobacco sample from Brazil in an effort to identify signature taxa from the state of Minas Gerais. We also assessed the role of honey additives to tobacco to assess this issue. Comparing the data to previously published data, we conclude that pollen signatures can distinguish broad geographic areas. We conclude that this forensic interpretation framework needs to be developed in context of the National Academy of Sciences recommendations for tightening methods in forensic science.

Keywords: forensic science, pollen, palynology, tobacco, sourcing, geographic origin, Brazil.

There have been two preliminary reviews of the palynological potential of tobacco analysis (1, 2). Both studies demonstrate that tobacco samples contain an abundance of regional pollen that is trapped on the surfaces of leaves and that the pollen from bulk tobacco is relatively easy to recover. However, these papers come to diametric conclusions regarding the value of pollen as regional markers of tobacco production. On one hand, Donaldson and Stephens conclude, "These differences in pollen types suggest that type distribution may vary with location, and the preliminary results are sufficiently encouraging to warrant efforts at characterizing pollen in tobacco from major tobacco-growing regions. On the larger scale, it is anticipated that pollen from different continents will provide very different signals in terms of flora species and their relative abundances. In this respect, it is noteworthy that the top 10 global producers of tobacco are located on five different continents. It is also likely that individual pollen types may be identified that are restricted to particular continents and even smaller geographic regions within those continents" (2:740). In contrast, Bryant and his colleagues (1:222) state, "Instead, we believe that because most pipe tobaccos are blends of tobacco grown in different geographical regions during different years, the notion that it is possible to identify a specific location of production or shipment based on pollen analysis is questionable at best." They do conclude that one of their samples, labeled "Turkish tobacco" contained pollen consistent with the area of Turkey.

The original paper on tobacco products by Donaldson and Stephens 2010 [2] examined one sample of tobacco obtained from The University of Kentucky consisting of a blend of local tobacco grown only on US farms in a limited region of the Eastern United States. The other sample they used as a comparison came from cigarettes purportedly made in China containing tobacco that was assumed to have been only of local Chinese origin.

Bryant and his colleagues [1] point out that because most commercial tobacco products are blends of tobacco, often from different geographical regions, it would be unwise to attempt to target the majority of tobacco products to one specific geographical location, especially if the argument was being used in a court of law. They ask if pollen data could be used to determine beyond a reasonable doubt that an analyzed pollen sample was consistent with a specific geographic area. Bryant and his colleagues answered in the negative. They applied this logic to their Turkish sample and argued that without additional comparison samples of tobacco from a variety of regions with similar flora it would be unwise to state in a court of law that such a "sample" is clear evidence that it is absolutely Turkish in origin. In essence, they ask if the combination of pollen in the Turkish tobacco could be found in the tobacco produced in any other region of the world, or even another region of the Middle East.

We believe that Bryant and his colleagues [1] missed the point of Donaldson and Stevens [2] paper. Donaldson and Stevens were presenting a case that pollen was useful in matching tobacco samples to broad geographical areas. In this context, the data collected would not necessarily be used in a court of law. Instead, such information would be useful in intelligence gathering regarding contraband transport of tobacco products. For example,

could tobacco from Cuba, after repackaging in Brazil, be identifiable as Brazilian versus Cuban upon import to the United States by pollen analysis? This is the broader type of issue raised by Donaldson and Stevens. They were interested in tracing trade in counterfeit tobacco products and presented a case that pollen analysis of tobacco products could provide information related to the geographical origin of tobacco production. They asserted that counterfeit tobacco products have elevated health risks, and infringe on trademark considerations that undermine the American economy. They presented an experimental comparison of USA and purported Chinese cigarette pollen counts and showed that there is a difference in pollen profiles between products that reflects geographical source of production. This difference was exhibited both by the types present in the samples and by variation between pollen abundance of the same types. They concluded that palynology holds promise in tracing counterfeit tobacco distribution. They highlighted that regionally specific pollen types should be sought after.

Bryant and his colleagues [1] point out that honey might be added as a sweetener to tobacco products. They cite a report that five companies add maple syrup, fructose, or honey to tobacco products. They argue that the Donaldson and Stephens [2] study is invalidated by the potential tobacco blending and the possible addition of honey to tobacco products.

Building on the goal of identifying regionally specific pollen markers, we designed an experiment using a Brazilian tobacco sample to assess unique pollen types to a tobacco-growing area. We devised this experiment to find an accurate and productive method in which tobacco products can be examined. With this method, authorities would be able to utilize forensic palynology as a means to trace the geographic origin of tobacco. We anticipate that the method can be applied to other smoked materials like marijuana. We are undertaking the analysis of a pollen sample from Brazilian pipe tobacco to assess whether pollen can be used to trace tobacco to a specific geographic region. We present ancillary studies with honey to determine whether commercial honey, added to tobacco, might alter the pollen spectrum. We also reviewed the Brazilian laws regarding tobacco production to determine whether adding honey to tobacco is permissible. We also examine the role of aeropalynology (studies of airborne pollen) as an independent check on the origin of tobacco samples.

This is a pilot study to define the methods of pollen recovery from tobacco and to assess the potential of such analysis to identify broad regional areas such as South America and also pinpoint a specific environment within Brazil. Our sample comes from tobacco grown in the Brazilian state of Minas Gerais. The vegetation of the Minas Gerais state is composed of a mosaic with tropical forests, cerrados, high-altitude grasslands, and rocky grasslands with seasonally dry tropical forests. In the cerrado region, rocky grassland species can be found with seasonally dry tropical forest. Common vascular plant families that have common distribution in these communities include Asteraceae, Poaceae, Fabaceae, Cyperaceae, Melastomataceae, Rubiaceae, Mimosaceae, Arecaceae, Euphorbiaceae, Sapindaceae, Caesalpiniaceae, Moraceae, and Myrtaceae [3, 4]. The common insect pollinated types have been documented through a review of melyssopalynology studies [5]. Barth reviewed honey, commercial pollen collections and propolis studies for the state of Minas Gerais. These samples were obtained from regions within the state including Barão dos Cocais, Bom Jesus, Caraça, São Gonçalo, and Serra da Piedade. The collections and analyses were made during both dry and humid climate periods covering several years. Melissopalynology from the area showed that *Eucalyptus* dominated the pollen spectra. Smaller quantities of pollen grains from *Alternanthera, Antigonon, Baccharis, Borreria, Croton, Eupatorium, Hyptis, Schinus, Serjania, Terminalia, Trichogonia*, and *Vernonia* were also detected. Interestingly, a monofloral honey with pollen of the *Mimosa scabrella* type was discovered in Minas Gerais. The honey data are relevant to the tobacco pollen debate because honey is sometimes used as an additive to certain tobacco products.

The basis for success in pollen recovery from tobacco is that the leaves are excellent "pollen traps" [6]. Hall's work [6], as reviewed by Bryant et al. [1] showed that native tobacco species in California collected between 4,207 pollen grains per gram of leaf to 114,999 pollen grains per gram of leaf. The tobacco leaf is covered with trichomes, which are fine hairs. The dense coverage of trichomes enables the leaf surface to accumulate particles from its environment [7]. The trichomes also secrete a sticky fluid, which increases the adhesive properties of the leaf surface [7]. Therefore, the pollen on the leaves represents the environment in which the tobacco is grown.

Materials and Methods

One brand of tobacco was used to determine an acceptable method for pollen identification. An extra mild Cavendish tobacco was purchased in Brazil for the use in a finamore pipe. The pipe tobacco is a product of the Tabacos Wilder Finamore Company of the city Juiz de Fora. This tobacco production center is located in the state of Minas Gerais, Brazil. The tobacco was packaged in a plastic bag and was extremely fibrous. A positive pressure, filtered air-ventilated laboratory was utilized to prevent contamination of other pollen grains. The tobacco was removed, and 15.04 grams was weighed electronically on a digital scale for the experiment. A solution of 3.0% potassium hydroxide (KOH) and distilled water (H2O) was used to rehydrate and loosen the tobacco. The solution also dissolved the plant resin that held the pollen to the leaves. The tobacco was placed in a 1000-mL plastic beaker to which the KOH solution was added. One dissolved tablet of 12,542 (batch # 124961) *Lycopodium clavatum* spores was added to the sample to allow for the determination of each type of pollen following the methods of Maher [8], Reinhard et al. [9], Donaldson and Stephens [2] and Bryant et al., [1]. The *Lycopodium clavatum* tablet is essential to palynology research to estimate the pollen content of the sample [10]. This basic solution was left at room temperature for 4 hours. Then, distilled water was added to dilute the KOH. The sample was filtered, using a 250-μm screen, over a 500-mL glass beaker to remove macroscopic debris. The solution was decanted into 50-mL centrifuge tubes, and the microfossils were concentrated by centrifugation. The plug of microfossils in the centrifuge tube was transferred to a fume hood for acetolysis to concentrate the pollen.

Acetolysis dissolves cellulose and chitin. A secondary effect of acetolysis is the darkening of the pollen grains, which emphasizes diagnostic features. Acetolysis is a process that can be dangerous if not executed properly. Long pants, laboratory coats, closed toed shoes, gloves, and goggles must be worn during the procedure. It is also necessary to execute all steps of this experiment in a fume hood. A series of water washes were necessary to remove the KOH. The sample was washed with distilled water three times. With each wash, distilled water was added to the centrifuge tube and the tobacco plug was broken up by agitation with an applicator stick and a vortex stirrer. After the water washes, glacial acetic acid was used to wash the sample to

replace the water in the sample. Water reacts violently to the acetolysis solution, but glacial acetic acid does not. It is important to mix the glacial acetic acid with the sample by using an applicator stick and vortex stirrer.

The acetolysis solution was prepared immediately before use. The solution was comprised of eight parts acetic anhydride and one part sulfuric acid. Normally, a mixture of nine parts acetic anhydride and one part sulfuric acid is recommended. However, for cellulose-rich samples, our laboratory has found that an 8-1 mixture is optimally effective [9, 10]. The acetic anhydride was measured in a graduated cylinder, and then, the sulfuric acid was added carefully to avoid a rapid reaction by slowly pouring the sulfuric acid down the side of the graduated cylinder. The solution was then added to the centrifuge tube and mixed with the plug of tobacco. The centrifuge tube was placed in a hot water bath at 99°C. After 20 minutes, the tube was centrifuged, and the supernatant was poured into a hazardous waste container.

The plug of residue was washed three times in distilled water, just as was performed before the glacial acetic acid baths. A large amount of mineral (sand) was noted in the remaining sample. This mineral was eliminated by floating the remains in a zinc bromide solution of a specific gravity of 2.0. The floating sediment, about 3 mls in volume, was transferred to a 50-ml beaker and diluted with 40 mls of distilled water. The residue was concentrated by centrifugation and washed in a manner similar to the water wash proceeding acetolysis. The pollen was then transferred to a 1 dram pollen glass vial with ethanol. Glycerin was added to the vial, and the vial was placed on a low heat, hot plate to evaporate the ethanol leaving the pollen in the glycerol solution. The hot-plate temperature was approximately 35°C. The vial was sealed and labeled for archival purposes. Using an applicator stick, a drop of the sample was transferred to a microscope slide. A cover slip was placed over the preparation and sealed with commercial nail polish. The slide was examined under 400× and 1000× with a Jenaval compound microscope under DIC (differential interference contrast) conditions. Photography was done with a Ziess Optiphot system at 20× and 40× objectives with 10× oculars using DIC setting. A comparative collection of pollen from modern plants was used to identify the tobacco pollen. This collection is housed in the Laboratório de Ecologia Gustavo de Oliveira Castro, Escola Nacional de Saúde Pública, FIOCRUZ, Rio de Janeiro.

A minimum of 200 pollen grains was counted. For each type of pollen, the concentration was calculated per milliliter of sediment. This was calculated by:

$$\text{Pollen Concentration} = [(p/m)e]/w$$

p: pollen grains counted; *m*: *Lycopodium* marker grains counted; *e*: number of *Lycopodium* marker pollen grains added; *w*: weight or volume of sediment.

Table 1. Pollen counts for Cavendish pipe tobacco from Brazil. The Lycopodium spores were added during pollen processing for quantification [8-10].

Pollen Name	# of Grains	% of Sample	Pollen Concentration (Grains/Gram)
Lycopodium spore	215	–	–
Fern Adiantaceae spore	2	0.76	7.76
Celtis sp type (Ulmaceae)	42	16.0	162.87
Cheno Am	69	26.20	267.58
Fabaceae	11	4.20	42.66
Fern: Reticulate Trilete Spore	4	1.50	15.51
High Spine Asteraceae (*Aspilia foliacea*)	35	13.0	135.73
Lamiaceae	2	0.76	7.76
Fabaceae Papilionaceae	11	4.20	42.66
Matayba type (Sapindaceae)	22	8.40	85.30
Myrtaceae	9	3.40	34.90
Protium c.f.	15	5.70	58.17
Rutaceae	11	4.20	42.66
Salix	12	4.60	46.54
Zea Mays	18	6.80	69.80
Pollen Count	263	–	
Pollen Concentration			1019.92

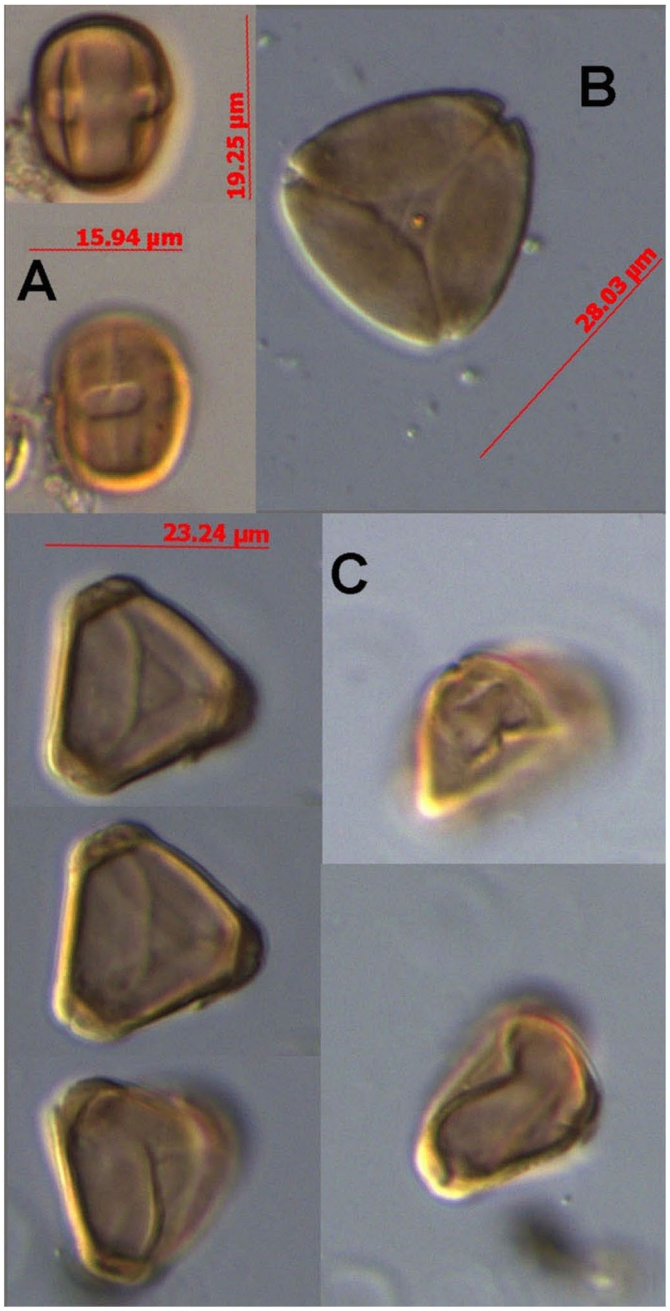

Figure 1. (A) Protium, Burseraceae, equatorial views. (B) Eugenia c.f., Myrtaceae, polar view. (C) Matayba, c.f., Sapindaceae, three polar views are to the left and two equatorial view are to the right. These pollen grains signal a tropical, New World environment and are found in Cerrado environments in Brazil.

To address the issue of pollen in honey that might be blended into tobacco, we processed several samples from large- and small-scale commercial honey producers using standard methods [11]. These included *Eucalyptus* honey from Brazil, *Citrus* from Portugal, commercial honey from large retailers, spun honey, and three *Trifolium* samples from small-scale family farms in Indiana, Nebraska, and Tennessee.

Results

The pollen concentration reveals that each gram of the tobacco contained over 1,000 pollen grains. The pollen counts for this experiment are presented in Table 1. Along with these counts, photographs of the pollen grains are presented in Figures 1-4. Four of the 13 common plant families listed above for the environments of Minas Gerais were found in the pollen counts. The only trees positively identified include *Celtis* (elm), *Salix* (willow), and Rutaceae (*Citrus*). We also found Myrtaceae pollen. Myrtaceae is a family of trees, shrubs, and creepers, which includes *Eucalyptus* and is found mainly in the Pacific region, Australasia, and Tropical America [7]. The remaining identified pollen grains are flowering plants, grasses, and weeds, such as cheno-am (goosefoot family and pigweed genus), Asteraceae (sunflower family), Fabaceae (bean family), *Matayba* in the Sapindaceae, Lamiaceae (mint family), *Protium* cf in the Burseraceae, and *Zea mays* (corn). Two more plant taxa were identified by spores: trilete Pteridophyte (fern) and Adiantaceae

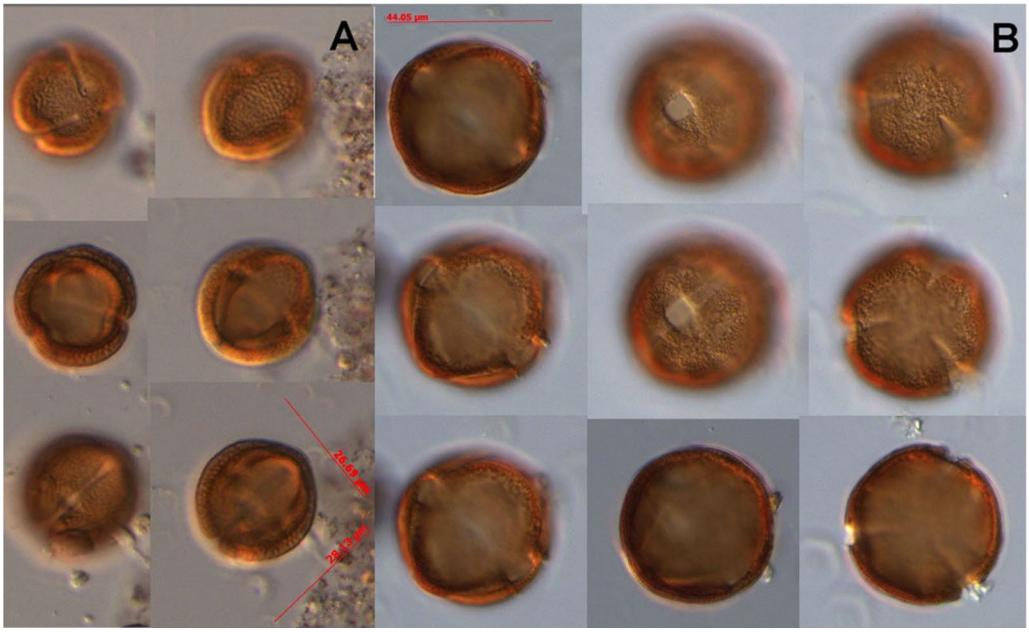

Figure 2. (A) Rutaceae, the upper left images show the polar view and the remainder show the equatorial morphology. (B) unknown genus in the Fabaceae, the three right images show the polar view, the remainder show the equatorial view. The Rutaceae, citrus family, signals a tropical or subtropical environment. We cannot identify the Fabaceae, bean family, to a specific genus at this point. With more research, we may be able to link this pollen type to a specific ecological regime.

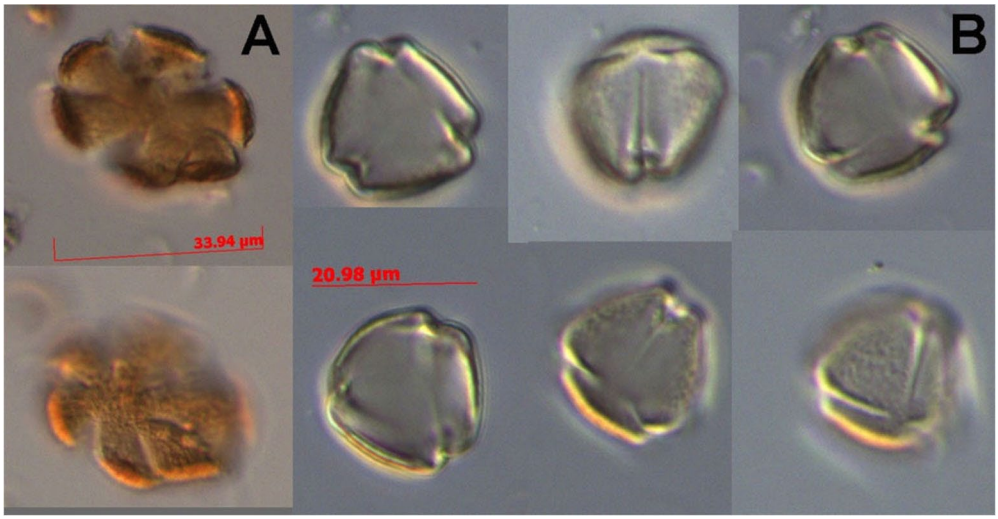

Figure 3. (A) Lamiaceae, mint family, polar view. (B) Fabaceae, Papilionaceae, various polar views. These pollen grains are well preserved but can be found in a great variety of regions and therefore A subfamily of the bean family that has hundreds of species in many environments and therefore do not help identify the origin of tobacco production.

(fern subfamily). Cheno-am is a large portion of the pollen signal due to the abundance of goosefoot weeds that grow in cultivated fields. The lack of *Nicotiana* (tobacco) is most likely due to the cultivation processes discussed by Bryant and his colleagues, which involves removing the flowers [1].

Pollen grains of importance for this experiment will include those which can be found in the tropics of Central and South America, and especially the cerrado environment of Minas Gerais. *Matayba, Protium* cf, Myrtaceae pollen, and the Adiantaceae spores are common to this general area. The presence of these pollen grains indicates this tobacco was grown in the tropical region of Central and South America. Additionally, the *Zea mays* and Fabaceae grains indicate this tobacco was grown and cultivated among other agriculture products in an area containing few trees. The complete spectrum of pollen grains represents the specific cerrado environment of the Brazilian state of Minas Gerais. The pollen of the Asteraceae compares favorably with *Aspilia foliacea*, which is a cerrado plant.

The amount of pollen in the honey samples varied. The highest concentration is the Nebraska farmer's market honey at a concentration of 164 pollen grains per milliliter of honey. The family farm honey from Indiana had a concentration of 54. The Portugal Citrus honey had a concentration of 12 grains per milliliter. The Brazilian Eucalyptus honey contained 50 grains per milliliter. The commercial spun honey had a concentration of 10. Pollen was not detected in the remaining samples.

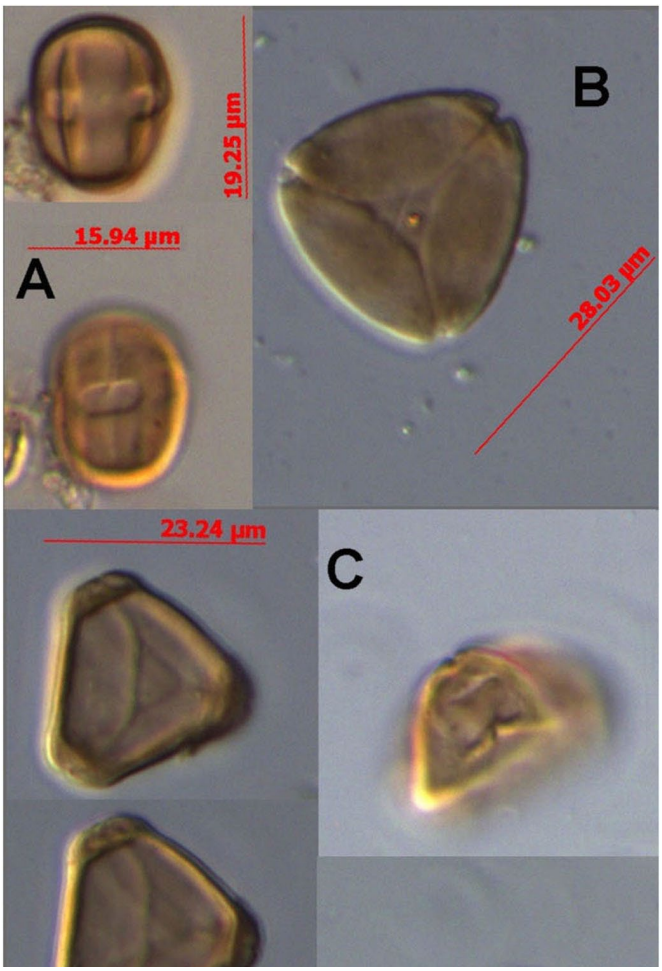

Figure 4. (A) Celtis, equatorial views, showing one of three pores. (B) Asteraceae, Aspilia foliacea c.f. Celtis, hackberry, is found in many environments and therefore is of little use in tracing specific geographic origin of tobacco production. Normally, Asteraceae, sunflower pollen, is of little use. However, in this case, the pollen is morphologically consistent with Aspilia foliacea, which is a Brazilian flower.

Discussion

Donaldson and Stephens [2] concluded that it is important to link tobacco to broad regions of production. Their experiment measured the pollen concentration from two different brands of tobacco grown on different continents. The samples have large differences in pollen concentrations and the types of pollen grains observed, as seen in Table 2. Additionally, the pollen concentrations for Donaldson and Stephens differ greatly from the tobacco cultivated in the tropics of Brazil. This research indicates that pollen extracted from tobacco can signal the location of geographic cultivation.

Bryant and colleagues [1], call into question the use of one or two discerning pollen grains. They state that while these "unique" grains may contribute to locality, they cannot be used arbitrarily as confirmation of a distinct geographical location. Additionally, they indicate pipe tobacco is most often a mixture of more than one type of tobacco. The tobacco is blended together with tobacco from previous or subsequent cultivation years, in which the pollen rain will have differed. Therefore, it is unlikely to obtain a pollen signature for forensic use to indicate a location for cultivation and manufacturing. We agree with these researchers regarding "unique" grains. However, the entire spectra of pollen taxa encountered reveal differences that have geographic relevance.

We plotted our data with those of previous research (1 & 2) in Table 2. These data highlight the slight to major differences in pollen variation between widely separated geographic origins. We will first address Donaldson and Stephens [2] data. The purported Chinese sample has only four taxa unique to it: Apiaceae (carrot family), Cyperaceae (sedge family), small Poaceae (wild grass), and *Dodonaea* (a genus of 70 tropical and subtropical species). These four taxa make up 16.9% of the count. The USA sample also has four taxa unique to the count: Brassicaceae (mustard family), Lactuceae (chicory, dandelion, and related species), Plantaginaceae (plantain family), and *Quercus* (oak). These taxa make up just 1.6% of the pollen counted. All of the taxa unique to the USA tobacco count are cosmopolitan. Their absence in the Chinese count could be an issue of sampling error. Thus, nothing in the USA sample is specific to North America.

The real difference between the Chinese and USA samples lies in the abundance of pollen in a limited number of shared categories [2]. There are 20 (8%) Myrtaceae pollen grains in the Chinese sample for each one in the USA sample (0.4%). There are 11 sunflower family pollen grains in the USA sample (71.3%) for each one in the Chinese sample (6.3%). Cultivated cereal pollen is 9.1% of the Chinese sample compared to 2.4% of the USA sample. Wild grass pollen makes up 32.9% of the Chinese sample compared to 6.7% of the USA sample. Thus, the samples present total spectra that are distinct from each other.

Our Brazilian sample bears out the assertion by Donaldson and Stephens that pollen reflects broad geographic origin [2]. The data presented in Table 2 show that 13 taxa are unique to the Minas Gerais sample compared with the USA and Chinese samples. These include seven taxa that can be found in Southeast Brazil: ferns (trilete and Adiantaceae), Rutaceae (citrus family), *Protium* (a dominant tree in Minas Gerais), *Matayba* (a common species in Minas Gerais), and *Aspilia foliacea* c.f. (a flower endemic to Minas Gerais). The latter pollen type, *A. foliacea*, is a provisional

Table 2. Comparison to the Brazilian sample to data from Bryant et al. [1] and Donaldson and Stephens [2].

Pollen Name	Williams et al. Brazil % Sample	Donaldson & Stephens (U.S.) % Sample	Donaldson & Stephens (China) % Sample	Bryant et al. (Turkish)	Bryant et al. (Virginia)	Bryant et al. (Blend)
Acer				0.4		
Fern Adiantaceae spore	0.76	–	–			
Alnus				0.4		
Apiaceae	–	–	1.1	0.8		
Asteraceae *Ambrosia*				2.5	39.4	14.1
Asteraceae *Artemisia*	–	1.6	1.1	7.5		
Asteraceae *Centaurea*				0.8		2.9
Asteraceae *Cirsium*				0.4		
Asteraceae High Spine		71.3	6.3	2.5	4.1	5.4
Asteraceae High Spine (*Aspilia foliacea*)	13.0					
Asteraceae Lactuceae	–	0.4	–	0.4		1.0
Betula						0.5
Brassicaceae	–	0.4	–	2.5	0.4	
Caryophyllaceae	–	2.0	0.6			
Castanea						0.5
Casuarina					3.3	
Cedrus				0.4		
Celtis sp (Hackberry)	16.0	–	–		0.4	
Cheno am	26.20	6.3	4.5	12.1	13.7	10.2
Cheno/Caryoph	–	2.0	2.8			
Cyperaceae	–	–	2.8	0.8	0.4	
Dodonaea	–	–	10.2			
Ericaceae				0.4		0.5
Fabaceae	4.20	–	–	0.4		
Fabaceae Papilionaceae	4.20	–	–			
Fern: Reticulate Trilete Spore	1.50	–	–			
Lamiaceae	0.76	–	–			
Ligustrum				0.4		
Magnolia					0.8	
Matayba type (Sapindaceae)	8.40	–	–			
Myrtaceae	3.40	0.4	8.0			
Oleaceae *Olea*				0.8		0.5
Picea				0.4		
Pinus	–	2.0	2.3	10.4	0.8	2.4
Plantaginaceae	–	0.4	–			
Plantaginaceae *Plantago*				2.1		
Poaceae	–	2.4	9.1			
Poaceae (<40 microns)	–	6.7	30.1	20.8	19.5	25.9
Poaceae *Triticum*				1.7		1.0
Poaceae *Zea Mays*	6.80	–	–		0.8	1.5
Poaceae (>40 microns)	–	–	2.8			
Protium c.f.	5.70	–	–			
Quercus	–	0.4	–	2.1	0.4	3.4
Rosaceae					3.3	23.9
Rutaceae	4.20					
Rutaceae *Citrus*					1.2	
Salix	4.60					
Typha latifolia type					1.2	
Typha/Sparganium				0.4		
Ulmus/Zelkova					0.4	
Unidentifiable		3.9	18.2	0.8	3.3	
Unknown				0.8	5.4	0.5
Viola	–			26.7	0.8	5.9

The data show that speciose and ubiquitous taxa are found in every, or nearly sample. However, speciose types such as cheno-am exhibit percentage variation. Importantly, 26 taxa are unique to single samples. Nine types are unique to the Brazilian sample and eight are unique to the Turkish sample. These unique types have value in signaling the geographic origin of tobacco samples in combination to variation in ubiquitous types.

identification. Potentially, these subregional signature pollen types for Minas Gerais make up 33.8% of the Brazilian tobacco sample count. Other cosmopolitan taxa include Lamiaceae (mint family), Fabaceae (bean family), Fabaceae Papilionaceae (large bean subfamily including many cultivated species), *Salix* (willow), *Zea mays* (maize), and *Celtis* (hackberry). With regard to abundance, Cheno-am pollen marks the Brazilian sample as distinct from the USA and Chinese samples. Over 26% of the pollen is cheno-am compared with 6.3 for USA and 4.5 for the Chinese samples. High cheno-am counts are typical of disturbed environments. At face value, the data from the Brazilian sample suggests a disturbed area near established tropical forest in which tobacco and maize are cultivated. Therefore, the Minas Gerais sample presents a distinct profile, which signals a geographic region.

The Bryant et al. [1] data are very interesting, and we believe that the samples do show a regional Turkish signature (Table 3).

Table 3. The pollen spectrum from the Brazilian analysis presented in this article is compared to that of a Turkish sample presented by Bryant et al. [1].

Pollen Name	Brazil	Turkey
Asteraceae High Spine (*Aspilia foliacea*)	13.0*	
Celtis sp (Hackberry)	16.0	
Fabaceae Papilionaceae	4.20	
Fern Adiantaceae spore	0.76*	
Fern: Reticulate Trilete Spore	1.50*	
Lamiaceae	0.76	
Matayba type (Sapindaceae)	8.40*	
Myrtaceae	3.40*	
Poaceae *Zea Mays*	6.80	
Protium c.f.	5.70*	
Rutaceae	4.20*	
Salix	4.60	
Cheno am	26.20	12.1
Fabaceae	4.20	0.4
Acer		0.4*
Alnus		0.4*
Apiaceae	–	0.8
Asteraceae *Ambrosia*		2.5
Asteraceae *Artemisia*	–	7.5*
Asteraceae *Centaurea*		0.8*
Asteraceae *Cirsium*		0.4
Asteraceae High Spine		2.5
Asteraceae Lactuceae	–	0.4
Brassicaceae	–	2.5*
Cedrus		0.4*
Cyperaceae	–	0.8
Ericaceae		0.4*
Ligustrum		0.4*
Oleaceae *Olea*		0.8*
Picea		0.4*
Pinus	–	10.4*
Plantaginaceae *Plantago*		2.1
Poaceae (<40 microns)	–	20.8
Poaceae *Triticum*		1.7
Quercus	–	2.1*
Typha/Sparganium		0.4
Unidentifiable		0.8
Unknown		0.8
Viola	–	26.7

The two samples have very little overlap, which signals different ecological origins. Asterisks mark types that are unique to the Brazilan Cerrado environment and types that typify pollen counts in Turkey today. The number of types unique to each environment underscores the value of pollen as regional markers of tobacco production.

In their three samples, Bryant and colleagues identified 35 pollen types. We compared the counts with published aeropalynology data from the Mediterranean region [12-16]. Ten of the pollen types were unique to the Turkish sample and, indeed, five of them are typical of Turkish flora and pollen rain: *Artemisia, Cedrus, Cirsium,* and *Ligustrum*. Higher counts of *Pinus* and *Olea* in the tobacco are typical of Turkey and other Mediterranean countries. Of the 26 taxa found in the Turkish sample, 16 are noted in the pollen rain of Turkey and three, (*Artemisia*, *Olea*, and *Pinus*) can be considered key types in Turkey's aeropalynology today. It is apparent to us that Bryant and his colleagues [1] collected data that revealed a Turkish pollen spectrum [12-16]. Therefore, their data support the notion that pollen can trace the geographic origin of tobacco. Their data do not refute the utility of pollen in tracing tobacco commerce as they assert.

To highlight the value of pollen in tracing tobacco production area of cultivation, we are presenting our data compared with the Turkish [1] data in Table 3. As can be seen, there is very little overlap between the pollen spectra of these samples. Only two pollen types were found in both samples. These are the ubiquitous cheno-am types which is shared by 1,300 species [17]. The second is the Fabaceae family, which includes numerous genera and species. Twelve types are unique to the Minas Gerais sample and 25 are unique to the Turkish sample. As discussed previously, the Minas Gerais sample is dominated by tropical vascular plants with a small showing of ferns. These are typical of Brazilian Cerrado vegetation. The Turkish types represent an arid Mediterranean environment that is almost precisely what would be expected from Turkey. The contrast between these two samples clearly supports Donaldson's and Stephens's [2] prediction that pollen data from tobacco could mark the continental and subcontinental origin of tobacco.

The issue of potential pollen alteration of tobacco by addition of honey must be addressed [1]. We have shown that honey from Portugal, Brazil, and the USA contains honey. So, if honey really is a tobacco additive in some countries, pollen spectra of honey and tobacco could be blended. Importantly, it is impossible for Brazilian tobacco producers to legally add honey to tobacco products. With regard to Brazil, the Agência Nacional de Vigilância Sanitária (National Sanitation Surveillance Agency) specifically prohibits the addition of honey to tobacco products. For the sake of argument, what if this law did not exist and honey could be added to tobacco? In our laboratory, the analysis of pollen from a Brazilian honey sample revealed the presence of 50 *Eucalyptus* pollen grains per milliliter of sample. No other taxa were present. Conceivably, the addition of pollen honey such as this could have altered the spectrum of the tobacco sample by spiking the Myrtaceae count. However, the spectrum would not have been otherwise altered, and we believe that we would be able to trace the pollen to Minas Gerais, Brazil even if honey had been added. In a recent review of melissopalynology of Brazil, it was shown that honeys from all parts of Brazil are dominated by insect pollinated plants [5]. In Minas Gerais, honey pollen is dominated by *Eucalyptus* with lesser amounts of *Alternanthera, Antigonon, Baccharis, Borreria, Croton, Eupatorium, Hyptis, Schinus, Serjania, Terminalia, Trichogonia*, and *Vernonia* as noted above. Importantly, the lesser taxa did not appear in the tobacco counts at all. This shows that pollinators are targeting a specific cluster of plant species that are largely independent of the taxa that are trapped by tobacco plants.

With regard to the Turkey sample, the only data from Turkish honey are published online [18]. The pollen concentrations in terms of number of grains per milliliter are not presented for these data. However, the main pollen types are relevant to answering the question regarding alteration of tobacco pollen spectra by addition of honey. The main plant taxa represented in Turkish honey are *Castanea sativa, Centaurea cyanus, Ceratonia siliqua, Citrus, Erica manipuliflora*, honeydews, *Leopoldia, Lotus, Melilotus alba, Punica granatum*, and *Sophora japonica*. Of these, only *Centaurea* and Ericaceae are common to both honey and tobacco. We think that the honey pollen spectra are especially different from both Brazil and Turkey with regard to wind-pollinated taxa. Honey samples are almost exclusively dominated by the pollen of insect-pollinated (entomophilous) taxa. In contrast, the tobacco pollen samples are dominated by wind-pollinated (anemophilous) plants. Although we show that some honey samples contain noteworthy amounts of pollen, those honey pollen spectra are very different and distinct from tobacco samples. Therefore, even if honey was added to tobacco, a trained palynologist will be able to identify this type of alteration by the increased presence of a restricted number of entomophilous types.

Conclusion

Based upon the results, tobacco is efficient at trapping and retaining pollen grains from the environment. Additionally, it is likely that most pollen grains survive tobacco processing to be examined. The distribution of pollen located within a tobacco sample has the ability to offer a "robust fingerprint" of its path from cultivation to distribution. The presence of a limited number of South American pollen grains indicates that it is possible to link the cultivation and manufacturing of this pipe tobacco to Minas Gerais, Brazil. Importantly, we are able to show that the pollen spectrum from a purported Turkish sample previously reported by Bryant and his colleagues [1] also presents an environmental signature consistent with Turkey. We show this by documenting the similarity of the pollen spectrum sample with aeropalynological studies of Turkey [12-16]. We suggest that aeropalynology be used as a independent check on the regional origin of tobacco samples. If tobacco pollen from a suspect locality matches the pollen rain of the locality, then the tobacco is more likely to come from that region. If there is discrepancy, then alteration of the count may have occurred through the processes mentioned in the literature [1]. Importantly, we demonstrate the validity of the Donaldson and Stevens [2] approach in finding continental regional and subregional pollen fingerprints of tobacco cultivation by using aeropalynology and tobacco pollen counts.

We acknowledge that the mixing of tobaccos from different regions could be confusing to palynologists tasked with tracing a tobacco sample to its region of origin [1]. The first stage in addressing this problem is collecting tobacco pollen data from known regions. In the case of this sample, it was collected from Minas Gerais and we feel confident that it is not blended. Therefore, we are presenting here a geographic marker pollen spectrum for this state of Brazil. In the coming years, we will expand our study to other Brazilian plantations. We hope that other researchers collect data from other tobacco producing regions.

We also acknowledge that producers may mix honey with tobacco [1]. Our processing of pollen from honey shows that pollen is common in honey. However, when comparing honey pollen and tobacco pollen from Minas Gerais and Turkey, there is very little overlap between what plants insects select for foraging and the plants whose pollen is inadvertently trapped in tobacco. We suggest that if honey and tobacco from the same geographic area are mixed, this would result in a very strong signal of geographic origin of tobacco. However, if honey and tobacco from two very different geographic areas are mixed, the palynologists would detect this by discovering entomophilous pollen from one geographic area and anemophilous pollen from the other geographic area. A well-trained palynologist would realize that two areas are represented in the same sample.

The 2009 NAS report on the status of forensic science identified deficiencies in investigation science [19]. Specific to palynology, recent workers have confronted problems in quantification and interpretation in forensic work [10]. This parallels criticism leveed at newer and unscientific developments in archaeopalynology [17]. We are presenting our analysis as a first step in developing regional pollen signatures from known tobacco producers. As can be inferred in this review, pollen data are complex and commercial processes have the potential to confuse palynologists working on tobacco samples. We hope that our methods and approach are useful to other palynologists interested in tracing sources of contraband tobacco products based on sound methodology and with the incorporation of the established fields of aeropalynology and melissopalynology. We anticipate that such a methodology will lead to a scientific approach to the challenges of pollen analysis.

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