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A comparison of pollen counts: Light versus scanning electron microscopy

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Abstract

Palynologists use compound light microscopy (LM) for pollen identification and interpretation and scanning electron microscopy (SEM) for morphological comparisons and taxonomy. As we are unaware of any published reports comparing LM and SEM pollen counts and identifications of the same sample, we decided to examine a surface soil sample using both microscopes. Standard palynological extraction techniques were used. Two, 300 grain counts were made using LM, and two, 300 counts with SEM. Pollen grains viewed with SEM were also divided into three categories, “identifiable,” “obscured,” and “virtually impossible to identify”. Eighty-six (86) percent of the pollen grains counted with SEM were classified as “identifiable” or “obscured.” Pollen concentration values ranged from 385,714 (LM Count #2) to 900,000 (SEM Count #1) per gram of soil. Regardless of the microscope used, *Ligustrum* spp., Myrtaceae-type, and *Tilia* spp. had the greatest number of pollen grains. A total of 73 taxa were found. A scan of the unexamined portion of the stubs resulted in 20 additional taxa. There were no significant differences between the counts made with the two microscopes (ANOVA, $p > 0.05$, $F = 0.18$, $df = 76$). However, there were significantly more taxa found with SEM than with LM (t-test, $T = 0.05$). Sample preparation and the time needed to count, analyze, photograph and print the micrographs are the same regardless of the microscope used. The sample, information needed, and funding will determine which technique to use.

Keywords: Pollen analyses, scanning electron microscopy, light microscopy, soil analysis

Palynologists rely on light microscopy (LM) to identify and interpret the pollen spectrum of a particular sample. Scanning electron microscopy (SEM) is not normally used for counting and identifying pollen grains. Instead, SEM is mainly used for morphological comparisons and taxonomy where the increased resolution of SEM makes differentiation of pollen grains and taxa more obvious (for example The Northwest European Pollen Flora I–VIII (Punt, 1976; Punt & Clarke, 1980, 1981, 1984, 1988; Punt & Blackmore, 1991; Punt et al., 1995; Blackmore et al., 2003), Pollen et spores d’Europe et d’Afrique du Nord Series (Reille, 1992, 1995, 1998, 1999), and in Grayum’s contribution, 1992) and as an aid in taxonomy (Skvarla

& Larson, 1965; Ridgeway & Skvarla, 1969). Scanning electron microscopy of pollen is also used to create new terminology for describing pollen ornamentation (Rowley et al., 1988; Vezey & Skvarla, 1990; Vezey et al., 1991), developing a numerical approach to pollen sculpturing (Vezey et al., 1991), and even computer analysis of the exine (Vezey & Skvarla, 1990).

Some modern palynological textbooks include SEM micrographs of pollen (Ogden et al., 1974; Moore et al., 1991); however, the majority focuses only on LM (Herrera & Urrego, 1996; Beug, 2004). Likewise, some atlases include SEM micrographs (Nilsson et al., 1977; Bassett et al., 1978; Moar, 1993; Qiao, 2005) and only a few solely use SEM

micrographs for the pollen presentation (Adams & Morton, 1972, 1974, 1976, 1979; Bambara & Leidy, 1991; Jones et al., 1995; Wei et al., 2003).

Most entomopalynological (study of pollen on/in insects) studies use LM for pollen analyses (Jones & Coppedge, 1998). However, SEM is better for examining the pollen that adheres to insects, such as moths and butterflies (Lepidoptera). Since much of that pollen is on the proboscis, legs, thorax and head (Bryant et al., 1991), the samples can be mounted on SEM stubs and examined without processing. In these types of SEM studies, pollen identification is used to determine migration and dispersal routes, insect source zones and examine feeding resources (Hendrix et al., 1987; Benedict et al., 1991; Hendrix & Showers, 1992; Gregg, 1993; Lingren et al., 1993, 1994; Del Socorro & Gregg, 2001; Gregg et al., 2001).

Van Laere et al. (1969) saw the promise of SEM in such studies and even suggested that SEM might be an important technique for analyzing honey. Chen and Shen (1990) used SEM to examine the pollen of Formosan honey. However, they did not use SEM to make counts and pollen identifications.

Blackburn and Ford (1993) used SEM micrographs of filtered honey to determine the abundance of each pollen type in the honey. The abundance was calculated by placing a 1 cm² acetate grid over each micrograph. Unfortunately, there is no information about the actual pollen counts or pollen diversity.

Regardless, SEM is not routinely used in palynology and is seldom used to make pollen counts or pollen identification. In some situations the lack of availability and/or lack of funding may prevent the use of SEM. Nevertheless, if both LM and SEM were available, which should be chosen to conduct a pollen analysis of a single sample? We could not find any published comparisons between LM and SEM pollen counts and pollen identifications of the same sample, or any information about using SEM for sediment samples. The purpose of this research was to test and determine the advantages and disadvantages of each technique (LM and SEM), and to compare the pollen counts and pollen identifications from the same sample.

Material and methods

A surface sample (3.0 g) was collected following Adam and Mehringer (1975) in Tirana, Albania by mixing 30 individual "pinches" from the uppermost layer of soil at different locations within a 10 km² area. Unless otherwise stated, the sample was always centrifuged for 3 min at 1060 × g., the supernatant decanted, and resulting residue mixed (vortexed) on a vortex stirrer for 30 s after each step.

Pollen extraction

One-half of the soil sample (1.5 g) was examined for pollen. One tablet of *Lycopodium clavatum* L. spores (Figure 1A) was dissolved in 5 drops of concentrated hydrochloric acid (HCl) for 5 min then added to the sample. The beaker containing the spores was rinsed twice with 95% ethyl alcohol (ETOH), and the rinse added to the sample.

The sample was then rinsed with glacial acetic acid.

Five ml of an acetolysis mixture (Erdtman, 1960) was added to the subsample. The subsample was heated for 6 min at 80°C in a pre-heated hot block. After which 5 ml of glacial acetic acid were added.

The subsample was rinsed with glacial acetic acid followed by two distilled water rinses. After each rinse the sample was centrifuged, decanted, and vortexed.

Four ml of zinc bromide (ZnBr₂) (specific gravity of 1.9) was added, and then thoroughly mixed into the subsample for one min. Afterwards, an additional 5 ml of ZnBr₂ was mixed into the subsample. The subsample was centrifuged for 5 min at 212 × g, and then for 5 min at 1060 × g. The dark band of material (pollen residue) that formed on the surface after centrifugation was removed. It consisted of about 2 ml of fluid.

To reduce the specific gravity of the removed pollen residue, 40 ml of 95% ETOH (specific gravity of 0.79) were added to the pollen residue. The pollen residue was concentrated using centrifugation.

The sample was rinsed twice with 95% ETOH.

One drop of the pollen residue was pipetted onto a SEM stub that was previously coated with TEMPFIX[®] adhesive. Three stubs were made, each with a single drop of the pollen residue.

Several drops of safranin-O stain and 8 ml of 95% ETOH were added to the remaining pollen residue. After centrifuging, decanting, and vortexing, the residue was transferred to a one-dram, glass vial. Five drops of glycerin were stirred into the vial and the vial was placed on a warm hot plate at 27°C overnight.

LM preparation

Before making each slide, the pollen residue was vortexed for 30 s (Jones & Bryant, 1998). One drop of residue was placed on a glass slide, allowed to spread, then covered with a cover slip, and the slide sealed.

SEM preparation

Stubs were coated with 400 Å of gold palladium in a Hummer II sputter coater. Marks for repositioning

Figure 1. (A) *Lycopodium clavatum* L. spore that was added as a tracer; (B) SEM after-coating mark made with nail polish; (C) SEM after-coating mark made with a pair of forceps; (D) SEM after-coating mark made with a diamond probe. Scale bars – 20 μm (A); 200 μm (B, D); 100 μm (C).

of the stub were added before and after coating. Marks made prior to coating included numbers or letters added to the sides, top, and bottom of the stub with a permanent marker. Marks made after coating included a drop of glitter nail polish (Figure 1B), an “i” made with a pair of forceps (Figure 1C), or a “+” made with a diamond scribe (Figure 1D). Stubs were examined in a JEOL JSM-6400 scanning electron microscope using an operating voltage of 15 kV and a working distance of 15 mm.

LM and SEM analyses

Four 300-grain pollen counts were made. One count was made from each of the two prepared glass slides, and one from each of two of the three prepared SEM stubs.

Pollen grains viewed with SEM were put into three categories. Grains where most of the diagnostic features could be seen were placed into Category 1 “identifiable” (Figure 1A). Grains in Category 2

were labeled “obscured”. Pollen in this category included grains that were sufficiently covered by debris so that many key ornamentation and/or aperturation details were partially obscured or were in a poor orientation (Figure 2A).

Category 3 grains were considered “virtually impossible to identify”. This category included grains that were badly degraded, severely broken, crumpled, badly folded, or so obscured by debris that sufficient morphological details were not visible (Figure 2B).

Pollen identification

Pollen identifications were made utilizing the USDA Areawide Pest Management Research Unit (APMRU) Modern Pollen Reference Collections, the Texas A & M University Palynology Laboratory Modern Pollen Reference Collection (TAMU), and comparisons with images in a number of published pollen atlases.

Figure 2. Two pollen grains from the SEM studies. (A) A Category 2 pollen grain “obscured” by a poor orientation for identification; (B) A Category 3 pollen grain “virtually impossible to identify” is so obscured by debris that identification is extremely difficult. Scale bar – 10 μ m.

For SEM analyses, when a pollen grain’s ornamentation looked different from other grains in the same family or genus, it was considered to represent a different taxon. Several different pollen types in the genera *Ligustrum*, *Tilia*, and *Plantago* were distinguishable during SEM analyses but were not initially apparent during LM analyses. During the SEM analyses, the pollen in each genus was separated into species or groups called “types.” To make the counts comparable, the number of pollen grains for the different types of *Ligustrum*, *Tilia*, and *Plantago* are lumped together in a genus ranking. For example, three different types of *Tilia* pollen were apparent during the SEM analyses but not during the LM. Therefore, there is a *Tilia* SEM total that is a summation of the number of pollen grains from each of the three *Tilia* types.

Asteraceae. The sunflower plant family is the largest vascular plant family with over 1,525 genera and over 22,750 species (Mabberley, 1997). Assigning specific genera to the pollen in this vast group is difficult even though each species has its own unique morphology. Nevertheless, some broad pollen groups were used. These groups included *Artemisia*, and *Cichorioideae*, both of which are distinguishable.

Martin (1963) split the pollen of the subfamily *Asteroideae* into two major groups based on the length of the surface “spines.” *Asteroideae* pollen

with surface “spines” longer than 2.5 μ m were categorized as a high “spine” (HS) group, and those with shorter “spines” were put into a low “spine” (LS) group.

Myrtaceae. This large family of tropical and subtropical plants contains over 130 genera and more than 4,600 species (Mabberley, 1997). Since the 1800s, members of this family have been exported as ornamental or timber trees to tropical and subtropical regions of the world. Most of the pollen types in the *Myrtaceae* share similar morphological characteristics. However, with SEM, different *Myrtaceae* pollen grains were initially separated into distinctive types. Similar to *Ligustrum*, *Tilia*, and *Plantago* for comparison between LM and SEM counts, there is a *Myrtaceae* SEM total that is a summation of the number of pollen grains from each *Myrtaceae* type.

Pollen statistics

The number of taxa was totalled for each technique (LM and SEM), a grand total was summed, and pollen concentration values were calculated using the following formula:

$$\frac{(\text{number pollen grains counted}) \times (\text{number of tracer spores added})}{(\text{number of grams of sample processed}) \times (\text{number tracer spores counted})}$$

ANOVA and t-tests were used to compare the

number of taxa among the counts and between the two techniques (Jones & Bryant, 2001, 2004).

Results and discussion

SEM analysis

Prior to examining the first stub, a palynologist using SEM for pollen analyses must decide two main things. First, the palynologist must decide which type of photomicroscopy medium to use. Secondly, the palynologist must determine how to reposition a stub if the count is interrupted and the stub must be removed and replaced at a later time.

Micrographs can be made with film and/or digitally. Two types of film are used for SEM: negative and positive. The advantage of using negative film is the cost, generally about \$0.50 to 0.70 (U.S.A) per sheet (Table I). The resolution is much better than positive film. It is also easier to

make enlargements and multiple copies from negative film than from positive film. The disadvantages of negative film include the developing time and uncertainty of the quality of the image at the time the micrograph is taken (Table I).

The main advantage of positive film is time (about 45 s) and the “instant” micrograph that is generated (Table I). However, positive film is expensive costing about \$2.00 to 3.00 (U.S.A.) per micrograph. In addition, numerous copies and/or enlargements are difficult to make because there is no negative.

Newer SEM models can use digital imagery for generating micrographs. One advantage of digital imagery is that the image is already digitized so that image manipulation (i.e., removal of debris) and enhancement (i.e., color, contrast, and brightness) are easy (Table I). Some computer software programs even allow the viewing of numerous micrographs at one time making the comparison of two or more pollen grains easy.

Table I. Advantages and disadvantages of negative and positive film and digital imagery for pollen microscopy.

NEGATIVE FILM	
Advantages	Disadvantages
1. Good resolution and clear micrographs	Do not get an instant micrograph for comparisons
2. Can correct contrast/brightness when printing	More time consuming, (i.e. development of film, prints).
3. Can remove some debris from micrograph when printing	Can be sensitive to light, fungi, etc
4. Can make as many prints as desired	Problems when developing can cost time and money
5. Can enlarge the negative or small areas to whatever size needed without false magnification	Not sure what the negative will look like until it is developed
6. Have multiple copies (negative and print) so if one gets lost, the data are not	If the negative isn't usable, it is difficult to find the exact pollen grain again
7. Will last for 50–100 years or longer	
POSITIVE FILM	
Advantages	Disadvantages
1. Instant micrograph	Expensive
2. Can quickly be used for pollen comparisons, counts, etc.	Micrograph must be digitized to make multiple copies
3. Faster than negative film for obtaining prints	Resolution is not as good as negative film
4. Good to show clients	It is the only copy so if lost so are the data
5. Chemical exposure is much less	If past expiration date, may not produce a good micrograph
6. Do not have to stand in a darkroom for long periods of time to develop negatives and prints	Brightness/contrast levels may be incorrect so may need to take multiple micrographs
7. Once digitized can remove debris, enhance contrast, etc.	
DIGITAL IMAGERY	
Advantages	Disadvantages
1. Micrograph is already digitized	Amount of storage need for each high quality micrograph
2. Easy to email, add to manuscripts, etc.	Cost of computer hardware, storage devices, software, etc.
3. Various software can be used to remove debris, adjust contrast/brightness, etc.	Technology advances may reduce the life of storage devices (CD, DVD, etc.)
4. Can put multiple micrographs on a screen for comparison	Resolution is not as good as negative film
5. No exposure to chemicals for making micrographs	It is the only copy so if lost so are the data
6. Do not have to stand in a darkroom for long periods of time to develop negatives and prints	Time needed to learn image analysis software packages

Some disadvantages of digital imagery include the large amount of memory needed for each micrograph, disk storage space needed (hard disk, CDs or DVDs), and the initial cost for computer, software, etc. Obtaining a high quality resolution micrograph took about 4 min. The resolution of the printed digital image was not as good as the same images taken with either negative or positive film. The JEOL SEM used for this manuscript is over 15 year old. No doubt there have been many improvements in the digital imagery system of newer SEM scopes.

Printing compound photographic plates from digital images was difficult. Trying to match the contrast and brightness for each print was time consuming and used a lot of photographic printer paper and ink. Putting four micrographs together to be printed on one photographic sheet was not an option in the software used to generate micrographs for this manuscript. Therefore no matter how large or small the final micrograph, a whole sheet of paper had to be used. Finally, the heat press used to attach a print and adhesive to a mounting board scratched, melted, and scarred the micrograph made from the computer regardless of how low the temperature was. This is most likely due to the heat sensitivity of the ink used for most computer printers. There are other types of techniques such as spray adhesives that can be used to put micrographs made from computer printers onto plates for publication and exhibition.

Because the objective of this study was to compare LM and SEM pollen counts and identifications, it was important to have instant access to the different pollen types. Therefore, positive film was used for the SEM analyses. The positive micrographs were put into plastic photo holders, placed into a 3-ring binder, and arranged by ornamentation then aperature. This binder was kept next to the SEM so that new grains could be compared to previously photographed ones. Over 200 LM and over 150 SEM pollen micrographs were taken during the counts.

Pollen counting. Re-positioning the SEM stub in the exact location to continue a count is next to impossible. To allow exact repositioning, marks were made prior and after coating the stub. Marking prior to coating did not work because the coating covered the marks that were made on the top and sides of the stub. Marks on the bottom of the stub were not covered by the coating but were hidden when the stub was put in the specimen holder making re-positioning impossible.

The after-coating mark made with the nail polish (Fig. 1 B) was the easiest to apply because a drop of nail polish could be added without picking up and

holding the stub. Once applied, the nail polish had to dry for several days before the stub could be examined. Unfortunately, the nail polish mark was the most difficult mark to use to consistently reposition the stub in the same location. In addition, there was concern that the drop may obscure important pollen types.

Marking with the forceps worked well (Figure 1C). However, several forceps were bent during the marking process. If forceps are used, they should be a coarse type that does not easily bend. The “i” made with the forceps was easily seen but could be mistaken as an accidental scratch on the stub by an inexperienced technician.

Marking with a diamond scribe worked best (Figure 1D). Diamond scribes are common in palynological laboratories and are used for marking numbers or codes on vials and glassware. Virtually any type of mark can be made with a diamond scribe. The “t” made with the diamond probe was more obvious when examining the stub (Figure 1D) than the “i” (Figure 1C) made with the forceps. Also, the cross bars of the “t” helped in re-positioning the stub correctly each time.

Pollen data interpretation and LM/SEM comparison.

Debris in a pollen sample is common and something palynologists work around. When using glycerine and LM, a pollen grain usually can be separated from the debris by gently rolling it around. Debris on the stub can partially or entirely cover a pollen grain. When the diagnostic features are hidden, identification of the pollen grain can be impossible. To calculate the difficulty in pollen identification during SEM analyses, pollen grains seen with the SEM were put into three categories: Category 1 “identifiable” (Figure 1A), Category 2 “obscured” (Figure 2A) and Category 3, “virtually impossible to identify” (Figure 2B).

Overall, the majority of the pollen grains counted with the SEM (86%) were classified as either Category 1 or 2 (calculated from Table II). Only 14% of the grains were considered to be in Category 3.

In the SEM Pollen Counts #1 and #2, over 50% of the grains were classified as Category 1 (54 and 70% respectfully, Table II). Category 3 contained the fewest number of pollen grains in both SEM counts (16 and 12%, respectfully, Table II).

All of the pollen in Category 1 was identified or in a few cases some were listed as unknown types. The unknown category was used for pollen grains that were identifiable provided there were sufficient SEM images to match the unknown type.

Table II. Comparison of the identification quality of pollen grains when using SEM.

Categories	Count 1 Actual #	Percent of count	Count 2 Actual #	Percent of count	Overall total	Percent of overall total
Category 1	162	54.0	211	70.3	373	62.2
Category 2	91	30.3	54	18.0	142	23.7
Category 3	47	15.6	35	11.7	82	13.7
Total	300	100.0	300	100.0	600	100

Pollen grains in Category 2 were not easy to identify. In most cases, only an expert familiar with the pollen taxa in the sample and with what pollen grains look like with SEM could identify these grains with certainty. Eventually, all but a very few of the Category 2 pollen grains were identified and assigned to a specific taxon.

Category 3 pollen were grains that were degraded, severely broken, crumpled, badly folded, or so obscured by debris that insufficient morphological detail was visible. Nevertheless, many Category 3 grains were identified. However, it took days and sometimes weeks of examining the micrographs, many at high magnification, and comparing them with hundreds of other micrographs. The main factors that influence identification of Category 3 pollen are time, funding, and the condition of the pollen grain (how many of the diagnostic feature are hidden).

Since Category 3 grains made up only 16% and 12% of the total, they normally will not affect the final pollen interpretation. In cases involving forensics, the time and money involved may be worth the effort to identify Category 3 pollen grains.

Tracer spores and pollen concentration values. A comparison of the LM and SEM pollen counts and identifications would not be complete without comparing concentration values. We selected *Lycopodium clavatum* spores because there was no record of this plant growing in or near the sampled area.

Maher (1981) indicated that the ratio of pollen grains in a sample should not exceed twice the number of tracer spores added before processing. The most efficient pollen/marker ratio that gives the greatest precision for the least amount of counting is when the ratio of pollen to tracer spores is at or close to 2:1. Furthermore, the greater the ratio of pollen to tracer spores beyond 2:1, the larger number of pollen and tracer spores must be counted to obtain the same level of accuracy provided by smaller counts with ratios of 2:1 or less. When a sample is pollen rich and only a minimal number of tracer spores are added, encountering even one additional tracer spore during a count drastically changed the pollen concentration value (Maher, 1981).

Previous studies of surface samples (Bryant et al., 1994; Mack & Bryant, 1974) contained no more than 20,000–25,000 pollen grains per gram. We did not want to “overwhelm” the potential pollen in the soil sample with too many tracer spores; therefore, one tablet containing $13,500 \pm 500$ tracer spores was added.

Pollen concentration values ranged from a low of 385,714 (LM Count #2) to a high of 900 000 (SEM Count #1) per gram of soil (Table III). Because this sample contained such a prolific amount of pollen and the number of tracer spores added was underestimated, the pollen concentration values varied greatly among the four counts (Table III).

The abundance of pollen in this soil sample was a surprise and represents the highest pollen concentration value either of us has found in similar surface samples. This high pollen concentration value is expected in samples collected from sediments where preservation conditions are ideal, such as acidic peat bogs or samples collected in the middle of a pine forest shortly after the pines shed their pollen.

Taxa diversity. Regardless which microscope was used or which count examined, the same three taxa (*Ligustrum* spp., Myrtaceae-type, and *Tilia* spp.) had the greatest number of pollen grains. The top three taxa in the LM analyses were *Tilia* spp. (28 and 23%), *Ligustrum* spp. (27 and 32%), and Myrtaceae-type (20 and 17%) (Table III), while the top three found in the SEM analyses were Myrtaceae-type (27 and 13%), *Tilia* spp. (18 and 22%), and *Ligustrum* spp. (15 and 36%) (Table III).

The variations in the pollen grain numbers among the counts may have resulted from the size of Myrtaceae pollen grains. Myrtaceae-type pollen is small and may not have been drawn up very far into the pipette when preparing the SEM stub. Thus, those grains would have been expelled first when the stub was made. This would also affect the LM counts because the slides were made from the remaining residue after the SEM stubs were prepared. Removal of the smaller Myrtaceae grains during the stub preparation would lessen their numbers in the remaining sample for the LM analyses.

The number of taxa found in each count ranged from 22 (LM Count #1) to 52 in (SEM count #2,

Table III. Family, taxon, actual grain counts (NOG), and the percentage of each taxon in the count (POG) made with light (LM) and scanning electron (SEM) microscopy. LM/SEM total represents the actual pollen count from LM and the summed total for all the types in that category made from SEM.

Family	Taxon or type	LM				SEM			
		Count 1		Count 2		Count 1		Count 2	
		NOG	POG	NOG	POG	NOG	POG	NOG	POG
Aceraceae	<i>Acer</i>							1	0.3
Arecaceae						1	0.3		
Asteraceae	Cichorioideae type					2	0.7		
Asteraceae	Asteroideae LS type	2	0.7	3	1.0				
Asteraceae	Asteroideae HS type	1	0.3	1	0.3				
Berberidaceae								1	0.3
Betulaceae	<i>Carpinus</i>	5	1.7	3	1.0	5	1.7	3	1.0
Betulaceae	<i>Alnus</i>	1	0.3	1	0.3	2	0.7	3	1.0
Betulaceae	<i>Betula</i>					3	1.0	2	0.8
Brassicaceae				1	0.3	1	0.3	1	0.3
Caprifoliaceae	<i>Lonicera</i>			2	0.7				
Caryophyllaceae						1	0.3		
Chenopod./Amar.	Cheno-Ams			3	1.0	3	1.0	2	0.6
Cupressaceae		2	0.7	2	0.7	11	3.7	4	1.4
Cupressaceae	<i>Juniperus</i>							1	0.3
Ericaceae		1	0.3	1	0.3				
Ericaceae	SEM type 1							1	0.3
Ericaceae	SEM type 2							1	0.3
Fabaceae	<i>Acacia</i>	1	0.3					1	0.3
Fabaceae	<i>Melilotus</i>			1	0.3	1	0.3	1	0.3
Fabaceae	SEM type 2					1	0.3	1	0.3
Fagaceae	<i>Castanea</i>	5	1.7	3	1.0	4	1.3	2	0.6
Fagaceae	<i>Fagus</i>					1	0.3	3	1.0
Fagaceae	<i>Quercus</i>	1	0.3	1	0.3	5	1.7	4	1.4
Juglandaceae	<i>Juglans</i>			1	0.3				
Liliaceae	<i>Erythronium</i>					1	0.3		
Liliaceae								1	0.3
Malvaceae		1	0.3						
Myrtaceae	LM/SEM total	60	20.1	50	16.7	82	27.3	40	13.3
Myrtaceae	SEM type 2					52	17.3	33	11.0
Myrtaceae	SEM type 3					24	8.0	6	1.9
Myrtaceae	SEM type 1					3	1.0	1	0.3
Myrtaceae	SEM type 4					1	0.3		
Myrtaceae	SEM type 5					2	0.7		
Oleaceae	Ligustrum LM/SEM total	80	26.8	96	32.0	46	15.3	108	36.0
Oleaceae	<i>Ligustrum</i> SEM type 1					10	3.3	5	1.7
Oleaceae	<i>Ligustrum</i> SEM type 2					31	10.3	97	32.3
Oleaceae	<i>Ligustrum</i> SEM type 3					5	1.7	6	2.0
Oleaceae	<i>Olea</i>	12	4.0	20	6.7	20	6.7	19	6.4
Oleaceae	<i>Syringa vulgaris</i>					3	1.0	6	2.0
Pinaceae	<i>Pinus</i> - Diploxylon type	1	0.3	1	0.3				
Pinaceae	<i>Pinus</i> - Haploxylon type	1	0.3	1	0.3				
Pinaceae	Undetermined type	19	6.4	14	4.7	14	4.7	11	3.6
Plantaginaceae	<i>Plantago</i> LM/SEM total	1	0.3	1	0.3	12	4.0	7	2.5
Plantaginaceae	<i>Plantago</i> SEM type 1					2	0.7		
Plantaginaceae	<i>Plantago</i> SEM type 2					10	3.3	5	1.7
Plantaginaceae	<i>Plantago</i> SEM type 3							2	0.8
Poaceae		13	4.4	12	4.0	19	6.3		
Poaceae	<i>Zea</i>					1	0.3		
Polygonaceae	<i>Rumex</i>					2	0.7		
Ranunculaceae	<i>Caltha palustris</i>					1	0.3	2	0.6

Table III. Continued

Family	Taxon or type	LM				SEM			
		Count 1		Count 2		Count 1		Count 2	
		NOG	POG	NOG	POG	NOG	POG	NOG	POG
Ranunculaceae				1	0.3	1	0.3		
Rhamnaceae								2	0.6
Rutaceae	<i>Citrus</i>			1	0.3				
Salicaceae	<i>Populus</i>			1	0.3				
Saxifragaceae	<i>Saxifraga</i>			1	0.3				
Sparganiaceae	<i>Sparganium</i>							1	0.3
Tiliaceae	<i>Tilia</i> LM/SEM total	83	27.9	70	23.3	52	17.7	67	22.3
Tiliaceae	<i>Tilia</i> SEM type 1					32	10.7	40	13.3
Tiliaceae	<i>Tilia</i> SEM type 2					4	1.3	4	1.4
Tiliaceae	<i>Tilia</i> SEM type 3					16	5.3	23	7.7
Ulmaceae	<i>Ulmus</i>			2	0.7				
Vitaceae	<i>Parthenocissus</i>					1	0.3	1	0.3
Vitaceae	<i>Vitis</i>			1	0.3				
SEM Unknown 1						1	0.3		
SEM Unknown 2								3	1.0
SEM Unknown 3						1	0.3		
SEM Unknown 4						1	0.3		
SEM Unknown 5						1	0.3		
LM Unknown A		3	1.0						
LM Unknown B				2	0.7				
LM Unknown C		3	1.0						
Degraded (ID impossible)		2	0.7	3	1.0				
<i>Lycopodium</i> spores		3		7		3		5	
Total number of taxa		22		30		41		52	
Total number of grains		298		300		300		300	
Pollen concentration values		894,000		385,714		900,000		540,000	

SEM taxa separated) (Table III). Some taxa were found only in the SEM analyses and other only in the LM analyses (Table III).

Likewise, when all taxa from both counts for each technique (LM vs. SEM) are examined, more taxa were found in the SEM counts (52) than in the LM counts (33) (Table III). This is primarily due to the increased resolution of the SEM, which enabled similar looking taxa to be separated.

A total of 73 pollen taxa were found in the four counts (Table IV). Nine taxa were found in all the LM and SEM counts (Table IV). These included not only the three most dominant types: *Tilia*, *Ligustrum*, and Myrtaceae-type, but also minor types such as: *Carpinus*, *Alnus*, *Olea*, *Castanea*, *Pinus*, *Plantago*, and pollen in the Cupressaceae. Some taxa such as *Juglans* sp. were found only in the LM counts, and others, such as Fabaceae SEM type 2, were only found in the SEM counts. Seventeen taxa (17) were found in only the LM analyses while 28 taxa were found only in the SEM analyses (Table IV).

After the end of the SEM counts, the unexamined portion of both stubs (approximately ½ of the surface) and the unexamined third stub were scanned for new taxa. Twenty (20) new taxa were found that

were not previously encountered during the SEM counting process (Table V) bringing the grand total of taxa to 93. Some of these taxa were airborne types such as *Typha angustifolia* L. and *Platanus* sp., while others were entomophilous types such as *Trifolium* sp. and *Aesculus* sp.

Of the 93 total taxa, 22% were not encountered in the 1,198 pollen grains counted (calculated from Tables III and V). Light microscopy accounted for 56% of the total number of taxa, while SEM accounted for 79%. Undoubtedly, the additional 20 taxa missed during one of the four counts represent minor pollen taxa in the soil sample. Nevertheless, some of those minor pollen taxa might be critical data in identifying minor vegetation components, in identifying a unique, geographical location; or being an important factor in forensic investigations.

It is not surprising that SEM accounted for more taxa, nor that some of the pollen taxa appeared only in the LM counts while other taxa appeared only in the SEM counts. Finding pollen in any mixed surface soil, fossil sediments, or honey sample is essentially a "hit or miss" attempt. After processing a sample for analysis, and then preparing a slide or SEM stub for counting, the

Table IV. The total number of pollen grains (NOG) and percentage (POG) of grains per taxon for the LM and SEM counts and an over all total number of each taxon pollen grains and its percentage. LM totals do not include degraded grains.

Family	Taxon or type	LM Totals		SEM Totals		Overall Total	
		NOG	POG	NOG	POG	NOG	POG
Aceraceae	<i>Acer</i>			1	0.1	1	0.1
Arecaceae				1	0.2	1	0.1
Asteraceae	Cichorioideae type			2	0.3	2	0.2
Asteraceae	Asteroideae LS type	5	0.8			5	0.4
Asteraceae	Asteroideae HS type	2	0.3			2	0.2
Berberidaceae				1	0.2	1	0.1
Betulaceae	<i>Carpinus</i>	8	1.3	8	1.3	16	1.4
Betulaceae	<i>Alnus</i>	2	0.3	5	0.9	7	0.6
Betulaceae	<i>Betula</i>			5	0.9	5	0.5
Brassicaceae		1	0.2	2	0.3	3	0.2
Caprifoliaceae	<i>Lonicera</i>	2	0.3			2	0.2
Caryophyllaceae				1	0.2	1	0.1
Chenopod./Amar.	Cheno-Ams	3	0.5	5	0.8	8	0.6
Cupressaceae		4	0.7	15	2.5	19	1.6
Cupressaceae	<i>Juniperus</i>			1	0.2	1	0.1
Ericaceae	Ericaceae	2	0.3			2	0.2
Ericaceae	SEM type 1			1	0.1	1	0.1
Ericaceae	SEM type 2			1	0.1	1	0.1
Fabaceae	<i>Acacia</i>	1	0.2	1	0.1	2	0.2
Fabaceae	<i>Melilotus</i>	1	0.2	2	0.3	3	0.2
Fabaceae	SEM type 2			2	0.3	2	0.2
Fagaceae	<i>Castanea</i>	8	1.3	6	0.9	14	1.1
Fagaceae	<i>Fagus</i>			4	0.7	4	0.4
Fagaceae	<i>Quercus</i>	2	0.3	9	1.5	11	0.9
Juglandaceae	<i>Juglans</i>	1	0.2			1	0.1
Liliaceae	<i>Erythronium</i>			1	0.2	1	0.1
Liliaceae				1	0.1	1	0.1
Malvaceae		1	0.2			1	0.1
Myrtaceae	LM/SEM total	110	18.4	122	20.3	232	19.4
Myrtaceae	SEM type 1			4	0.6	4	0.3
Myrtaceae	SEM type 2			85	14.2	85	7.1
Myrtaceae	SEM type 3			30	5.0	30	2.5
Myrtaceae	SEM type 4			1	0.2	1	0.1
Myrtaceae	SEM type 5			2	0.3	2	0.2
Oleaceae	<i>Ligustrum</i> LM/SEM total	176	29.4	155	25.7	331	27.6
Oleaceae	<i>Ligustrum</i> SEM type 1			15	2.5	15	1.3
Oleaceae	<i>Ligustrum</i> SEM type 2			128	21.3	129	10.8
Oleaceae	<i>Ligustrum</i> SEM type 3			11	1.8	11	0.9
Oleaceae	<i>Olea</i>	32	5.4	39	6.5	71	5.9
Oleaceae	<i>Syringa vulgaris</i>			9	1.5	9	0.7
Pinaceae	<i>Pinus</i> - Diploxylon	2	0.3			2	0.2
Pinaceae	<i>Pinus</i> - Haploxylon	2	0.3			2	0.2
Pinaceae	Pinaceae - Undetermined	33	5.5	25	4.1	58	4.8
Plantaginaceae	<i>Plantago</i> LM/SEM total	2	0.3	19	3.2	22	1.8
Plantaginaceae	<i>Plantago</i> SEM type 1			2	0.3	2	0.2
Plantaginaceae	<i>Plantago</i> SEM type 2			15	2.5	15	1.2
Plantaginaceae	<i>Plantago</i> SEM type 3			2	0.4	2	0.2
Poaceae	Poaceae	25	4.2	19	3.2	44	3.7
Poaceae	<i>Zea mays</i>			1	0.2	1	0.1
Polygonaceae	<i>Rumex</i>			2	0.3	2	0.2
Ranunculaceae	<i>Caltha palustris</i>			3	0.4	3	0.2
Ranunculaceae		1	0.2	1	0.2	2	0.2
Rhamnaceae				2	0.3	2	0.1
Rutaceae	<i>Citrus</i>	1	0.2			1	0.1
Salicaceae	<i>Populus</i>	1	0.2			1	0.1
Saxifragaceae	<i>Saxifraga</i>	1	0.2			1	0.1
Sparganiaceae	<i>Sparganium</i>			1	0.1	1	0.1
Tiliaceae	<i>Tilia</i> LM/SEM total	153	25.6	119	19.8	272	22.7
Tiliaceae	<i>Tilia</i> SEM type 1			72	12.0	72	6.0
Tiliaceae	<i>Tilia</i> SEM type 2			8	1.4	8	0.7
Tiliaceae	<i>Tilia</i> SEM type 3			39	6.5	39	3.3

Table IV. Continued.

Family	Taxon or type	LM Totals		SEM Totals		Overall Total	
		NOG	POG	NOG	POG	NOG	POG
Ulmaceae	<i>Ulmus</i>	2	0.3			2	0.2
Vitaceae	<i>Parthenocissus</i>			2	0.3	2	0.2
Vitaceae	<i>Vitis</i>	1	0.2			1	0.1
SEM Unknown 1				1	0.2	1	0.1
SEM Unknown 2				3	0.6	3	0.3
SEM Unknown 3				1	0.2	1	0.1
SEM Unknown 4				1	0.2	1	0.1
SEM Unknown 5				1	0.2	1	0.1
LM Unknown A		3	0.5			3	0.3
LM Unknown B		2	0.3			2	0.2
LM Unknown C		3	0.5			3	0.3
Degraded (ID impossible)		5	0.8			5	0.4
Number of taxa		33		52		73	

potential for finding or missing a specific pollen type will depend on the amount of the sample examined and the number of pollen grains actually counted (Traverse, 1988).

The results of the present study confirm similar results from an earlier study (Jones and Bryant, 1998) where five drops were extracted from a single processed honey sample. For each drop, a total of 500 pollen grains were counted. Once the counts were finished, a cursory examination of the slide was made to find taxa that were not previously recorded. The results of that study showed that none of the five, 500-grain counts accounted for more than 60% of the total 130 taxa present in the sample.

Like the results in this study, Jones and Bryant (2004) found that some taxa only occur in one count of a sample while others occur in all the counts.

Furthermore, the number of newly discovered taxa increased as the size of the total pollen count increases.

How long one spends looking for new taxa depends on the amount of funding and the information needed from the study. Obviously, if only the majority of taxa are needed and the top 2 or 3 taxa are the most important, then doing a 1200+ grain count is not needed. However, if looking for a particular pollen taxon or determining the complete pollen taxa diversity in the sample is warranted, then a 1200 grain count is not sufficient.

Are all pollen counts equal: A question of SEM vs. LM

When the SEM types of *Ligustrum*, Myrtaceae, and *Tilia* are combined into their generic and family classification, there was no significant differences in the number of taxa among the counts ($p > 0.05$, $F = 0.18$, $df = 113$), between the two LM counts ($p > 0.05$, $F = 0.28$, $df = 51$), nor between the two SEM counts ($p > 0.05$, $F = 0.01$, $df = 61$). Even when the SEM taxa were separated, there were no significant differences between the SEM counts ($p > 0.05$, $F = 0.06$, $df = 77$).

However, a t-test indicated that there was a significant difference in the number of taxa between the SEM and LM ($T = 0.05$) counts. Even so, with only two counts for each technique, more research is needed to determine significant differences between the two techniques.

Conclusion: What to use, LM or SEM?

Time wise there was little difference between counting a pollen sample using LM or SEM. In

Table V. Additional pollen types found in the SEM examination.

Family	Taxon or type
Aceraceae	<i>Acer</i> - 2 additional types
Apocynaceae	<i>Apocynum</i>
Asteraceae	Medium process - 2 types
Asteraceae	Cichorioideae - 2 types
Caryophyllaceae	Type 2
Fabaceae	<i>Trifolium</i>
Hippocastanaceae	<i>Aesculus</i>
Lamiaceae	
Oleaceae	<i>Fraxinus</i>
Parnassiaceae	<i>Parnassia</i>
Platanaceae	<i>Platanus</i>
Ranunculaceae	<i>Clematis</i>
Rhamnaceae	<i>Rhamnus</i>
Rutaceae	<i>Zanthoxylum</i>
Salviniaceae	<i>Salvinia</i>
Scrophulariaceae	<i>Lindernia</i>
Typhaceae	<i>Typha angustifolia</i>

addition, the sample extraction, processing time, and micrograph production were the same regardless of which microscope was used. One major advantage of LM is convenience and the major advantages of SEM are increased resolution and number of taxa.

When using LM, several things should be remembered. First, there is a possibility of lumping two or more different taxa into one category. How much this effects the overall interpretation of the data depends on the sample and what information is needed from the sample. Second, LM analyses accounted for the least diversity of pollen taxa. Again, the importance of the pollen taxa diversity depends on the type of information needed.

When using SEM, several things should be considered. First, at least two micrographs should be taken of each pollen taxon, one of the whole grain and the second of a close up of the ornamentation of the grain.

Second, the micrographs should be taken at similar magnifications. For example, all pollen grains classified as *Tilia* should be taken at 1,500–1,800X and the high magnification of the ornamentation at 5,000X. This standard magnification will change according to the taxon and the family. For example, at 1,500X, a whole Malvaceae pollen grain will not fit on the negative. On the other hand, at 1,500X, *Mimosa strigillosa* Torr. & A. Gray is still very small and the whole grain needs to be photographed at 5,000X.

Third, micrographs generated should be housed in a dry, cool area where they can be kept for future use. Digital images also must be cared for and kept in a secure and dry location. When cared for properly, micrographs and negatives should last for at least 50+ years. How long digital imagery will last depends on technology. At the current rate of new computer technology, common types of storage (CD, DVD) may be obsolete and unreadable in 10–20 years. By keeping the micrographs for years, regardless of their format, there is the chance that an unknown pollen type in the sample will be identified at a later date.

Whenever confronted with the need to decide whether or not to examine a pollen sample using LM, SEM, or both, the sample, the information needed from the sample, and the cost will most likely dictate whether to use LM, SEM, or both. Additional factors for deciding which type of microscope to use are the availability of a SEM, the deadline for the project, and whether precise (genera, species, or sub species levels) or general (genera or family levels) pollen data are needed. For the moment, we suggest that those who are comfortable using LM should continue using LM and those who like to use SEM and have the

availability and funding needed for SEM analyses should consider using SEM for statistical pollen counts and identifications.

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