



Molecular approaches for the analysis of airborne pollen

A case study of *Juniperus* pollen

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ABSTRACT

Background: Pollen monitoring is a common and vital tool in the field of allergy, creating awareness in pollen sensitive individuals. Traditionally, pollen monitoring has been based on conventional microscopic counting techniques that are labor intensive and limited in the identification to the genus or family level. Molecular techniques provide an alternative approach that is less labor intensive and enable identification of any species by its genetic fingerprint.

Objective: To use quantitative polymerase chain reaction (qPCR) to evaluate pollen concentrations in air samples.

Methods: *Juniperus* pollen was selected as our model because of the importance of this pollen in the southcentral United States. We analyzed 105 air samples collected with a Burkard spore trap from 2013 to 2015 using species-specific primers and probes. To evaluate the feasibility of a molecular approach, we used duplicate air samples that allowed us to compare results from classical identification based on light microscopy with our qPCR results.

Results: Pollen concentrations from the qPCR data were significantly correlated with concentrations determined through light microscopy ($R = 0.902$, $P < .001$). We also confirmed an overlap in the pollination seasons between *Juniperus ashei* and *Juniperus pinchotii* and between *J ashei* and *Juniperus virginiana*.

Conclusion: We found that this method correctly identified different *Juniperus* species present in mixed air samples in the southcentral United States, an accomplishment that cannot be achieved using microscopic identification. We conclude that the qPCR method is more accurate and sensitive than current pollen monitoring techniques and, therefore, has the potential to be used in various pollen monitoring stations.

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Introduction

Current allergy warning systems provide daily information about the atmospheric pollen levels for pollen allergic patients. The American Academy of Allergy, Asthma, and Immunology established the National Allergy Bureau to disseminate information to the public about the concentrations of fungal spores and pollen from different monitoring stations in the United States, Canada, and Argentina.¹ Pollen and spores are collected at each National Allergy Bureau monitoring station using Burkard volumetric spore traps or Rotorod samplers.² Slides are prepared, and morphologic identification through microscopy is performed using pollen samples from the trap or sampler.^{3–5} However, many shortcomings have been documented with this approach. This method requires a trained

palynologist and is labor and time intensive.⁶ Apart from these issues, some combinations of pollen from different species within the same genus or family are difficult or impossible to differentiate on the basis of morphologic findings,⁷ which reduces the sensitivity of pollen monitoring with microscopy.

In addition, the pollen of some species, such as *Cupressus arizonica*, is more allergenic than other species (*Cupressus macrocarpa* and *Cupressus sempervirens*) in the same genus,⁸ and standard microscopy cannot distinguish the more allergenic from the less allergenic pollen. Barberini et al⁸ used aniline blue black staining to visualize protein and calcofluor staining for β -glucans to examine the pollen in these species. They found that the protein and β -glucan content were more abundant in the middle intine at the beginning of pollen hydration in *C arizonica* compared with *C macrocarpa* and *C sempervirens*.⁸ However, the intine composition and staining pattern of *C arizonica* was dynamic and after 60 minutes no longer differed from the other species. As a result, these methods would not prove useful for the routine analysis of air samples.

In the genus *Juniperus*, another member of the Cupressaceae family, pollen of *Juniperus ashei* is considered the most allergenic

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species in North America and is considered equivalent to ragweed in its allergenic potential.⁹ The allergenicity of pollen from *Juniperus pinchotii* is also significant, whereas *Juniperus virginiana* is not considered as allergenic as the other 2 species.⁷

Next-generation sequencing has become increasingly popular and has been used for identification and quantification of pollen in environmental samples.^{10,11} Kraaijeveld et al¹⁰ extracted DNA from 3 days of actual air samples and amplified the *trnL* gene. This method could not provide an absolute number of pollen grains; thus, only the relative abundance of pollen grains was mentioned. In addition, *trnL* is a chloroplast gene and the plastid number may vary from species to species.¹⁰ Apart from these limitations, air samples for only 3 days were compared in this study.¹⁰ Although Sickel et al¹¹ were able to identify different pollen samples by targeting the internal transcribed spacer 2 (ITS2), the technique does not provide a means to quantify the pollen. Keller et al¹² accomplished pollen identification by using DNA barcoding of the ITS2 region but a caveat associated with using ITS is the higher-than-normal substitution rates, which may cause variability in the ITS sequences among pollen derived from the same species.² In addition, primers used by Keller et al¹² could also amplify DNA from fungal species that may be present in pollen samples.^{10,13,14}

Longhi et al¹⁵ used a quantitative polymerase chain reaction (qPCR) approach to quantify different pollen grains. A simulated air sample tape was prepared in which different pollen grains were manually sprayed on the tape. The researchers found that it was possible to extract DNA from the pollen grains on the tape. No microscope slides of air samples were examined. Instead, simulated suspensions of pollen grains were prepared and quantified with qPCR and compared to pollen counts determined using a Fuchs-Rosenthal counting chamber.¹⁵ Although a qPCR approach is promising, the laboratory-based results of the study by Longhi et al¹⁵ may not extend to the field because actual air samples contain a mixture of spores, dust particles, and PCR inhibitory particles along with pollen grains.¹⁶

Our studies have specifically focused on different species of *Juniperus* pollen occurring in the southcentral United States. *Juniperus* pollen cannot be distinguished from pollen produced by other members of the Cupressaceae family.⁷ *Juniperus* species pollinate at different times during the year, and there are some periods when different *Juniperus* species release their pollen simultaneously. Pollen season start times have considerable year-to-year variability because of differences in local meteorologic conditions. The pollination season of *J. pinchotii* starts in late September and continues through November.^{17,18} *J. ashei* pollen release can start as early as late November and continue through early February.¹⁸ *J. virginiana* starts pollinating in early February in Tulsa and continues through April.^{18,19} *J. ashei* occurs in southcentral Oklahoma, the Edwards Plateau region of central Texas, and the Ozark Mountain region of Arkansas and Missouri. In Oklahoma, the species is found in the Arbuckle Mountains approximately 220 km southwest of Tulsa.⁹ *J. pinchotii* occurs naturally in west Texas, southwest Oklahoma, eastern New Mexico, and northern Mexico.¹⁸ *J. virginiana* occurs throughout the eastern United States.¹⁸ Of these 3 *Juniperus* species, only *J. virginiana* occurs naturally in Tulsa area. The plant distribution data, pollination time, and wind patterns indicate that *Juniperus* pollen documented in the Tulsa atmosphere from October through April come from 3 different sources. High levels of pollen are from local populations of *J. virginiana* during spring. Low levels of pollen are assumed to be carried to Tulsa by the winds from *J. pinchotii* in southwestern Oklahoma from October through November. Pollen of *J. ashei* are wind transported to Tulsa from populations in southern Oklahoma and Texas during December and January.⁹

Molecular techniques provide an alternative approach to microscope analysis that is less labor intensive and enables

identification of any species by its genetic fingerprint. The objective of this study was to ascertain whether qPCR could be used to determine airborne pollen concentrations for 3 *Juniperus* species from actual air samples and to define the pollination season overlap.

These species of *Juniperus* were chosen for our investigation because *Juniperus* is a significant source of allergy in Tulsa. Furthermore, the proposed molecular assay will markedly improve our understanding of the relative pollen contributions of different *Juniperus* species.

Methods

Collection of Airborne Pollen

Pollen was collected from the Tulsa atmosphere using a Burkard 7-day air sampler (Burkard Manufacturing, Rickmansworth, United Kingdom) placed on the roof of a building at University of Tulsa, Tulsa, Oklahoma, since December 1986. Every 7 days, the drum was changed and the Melinex tape was cut into 7 sections, each 48-mm long.⁴ Each tape section was transferred to a labeled slide and stained with glycerin jelly containing basic fuchsin; slides were then observed under the light microscope for pollen analysis. A second Burkard air sampler identical to the first was placed on the same site 2 m away from October 2013 until May 2015. The 48-mm tape sections from this air sampler were cut into small pieces (approximately 5 × 5 mm) and placed inside 2-mL screwcap tubes for molecular analysis. A total of 105 samples were selected for the molecular analysis.

DNA Extraction

Total genomic DNA was extracted using a previously described method, with some modifications.²⁰ One-millimeter glass beads were added on the top of the tape fragments in 2-mL screwcap tubes. A total of 500 μ L of Fawley's extraction buffer²⁰ (1 M sodium chloride, 70 mM Tris, 30 mM Na₂EDTA, pH 8.6) and 25 μ L of 10% cetrimonium bromide extraction buffer were added to the samples and subjected to bead-beating in a mini bead-beater (Biospec Products, Bartlesville, Oklahoma) for 3 minutes at full speed. Each tube was incubated at 75°C for 1 hour. After incubation, 500 μ L of chloroform and isoamyl alcohol (49:1) was added and the tubes centrifuged at 16,004 g for 20 minutes. The supernatant was removed and placed in a separate 1.5-mL microfuge tube. An additional 500 μ L of 49:1 chloroform and isoamyl alcohol was added to the tube with beads and then centrifuged at full speed for 20 minutes. The supernatant was added to the previous supernatant and an additional 500 μ L of 49:1 chloroform and isoamyl alcohol was added and centrifuged at full speed for 20 minutes. A total of 50 μ L of 3 M sodium acetate and 900 μ L of ice-cold 100% ethanol were added to the supernatant. Samples were gently inverted and kept at –20°C for 1 hour. The samples were centrifuged at 16,004 g for 15 minutes, and the nucleic acid was pelleted. The supernatant was removed and the pellet was washed with 70% ethanol. The washed pellet was dried by placing in a 40°C water bath. A total of 50 μ L of RNase free water was added to dissolve the dried pellet.

Standard Curve Sample Preparation

Standard curves were prepared by determining the number of pollen grains present in 1 mg of *J. ashei*, *J. pinchotii*, and *J. virginiana* pollen. Thus, 1 mg each of *J. ashei* pollen (collected from the Arbuckle Mountains in Oklahoma in December 2009 and Lampasas, Texas, in January 2011), *J. pinchotii* pollen (collected from Sonora, Texas, in October 2013), and *J. virginiana* pollen (collected in Tulsa, Oklahoma, in March 2014) were weighed and placed in 1.5-mL centrifuge tubes; 1 mL of 50% glycerol was added to each pollen

sample, and the suspensions were vortexed. A hemocytometer was used to determine the number of pollen grains in 1 mg of pollen. Approximately 10 μ L of the suspension was placed on each side of the hemocytometer. The number of pollen grains was determined by using hemocytometer conversion.²¹ For accuracy, the counts were repeated 10 times and averaged. Later, the DNA was extracted from pollen grains of *J ashei*, *J pinchotii*, and *J virginiana* by using the same protocol that was used for the Burkard air samples.

Primers and Probes Design for qPCR

All primers and probe pairs were designed by performing multiple sequence alignment of target sequences using the CLC Genomics Workbench (Qiagen, Waltham, Massachusetts) and Mesquite version 3.02.²² Alignments from sets of target sequences were searched manually for potential probe sites. All potential probes were designed, prepared (Eurofins MWG Operon LLC, Huntsville, Alabama), and tested for feasibility. Comparative analysis of published *matK* sequence data obtained from numerous Cupressacean taxa (including *J ashei*, *J pinchotii*, and *J virginiana*) revealed a potential primer and probe site that could be used to uniquely target DNA from *J ashei*. However, none of the other standard markers (*trnL*, *rbcL*, ITS2) had variation that could be exploited for use in developing probes for qPCR. To identify additional probe sites, a transcriptome analysis was performed using leaf tissue of *J ashei* (collected from Devil's Knob, Arkansas), *J pinchotii* (collected from Palo Duro, Texas), and *J virginiana* (collected from Tulsa, Oklahoma).²³ Two homolog sets of sequences for all 3 taxa were identified and selected for the design of species-specific primers and probes for *J pinchotii* and *J virginiana*. Downstream bioinformatics analyses confirmed the sequences to be single-copy nuclear genes.²³ The species-specific primer and probe set for *J ashei*, JUNASHprobe01, JUNASHf01, and JUNASHr01 (Table 1) was designed (see above), tested, and selected for use in qPCR. The TaqMan probes were dual labeled at the 5' and 3' ends with 6-carboxy-fluorescein group and Black Hole Quencher (Eurofins MWG Operon LLC, Huntsville, Alabama). Sequences with variability from the transcriptome data for *J pinchotii* and *J virginiana* were selected, and primers and probe for these sequences were designed for *J pinchotii* (PINCH1615probe, PINCH1615Forward, PINCH1615-Reverse; this sequence is available as part of the Transcriptome Shotgun Assembly project, which has been deposited at the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank under the accession No. GEWX000000000; the version described in this article is the first version [GEWX010000000]) and *J. virginiana* (JV980probe, JV980Forward, JV980Reverse; this sequence is available as part of the Transcriptome Shotgun Assembly project, which has been deposited at DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank under the accession No. GEWV000000000; the version described in this article is the first version [GEWV010000000]) (Table 1).

Table 1
List of Primers for Polymerase Chain Reaction and Primers and Probes for Quantitative Polymerase Chain Reaction Analysis

Primer name	Primer sequence (5'-3')
JUNASHprobe01	ACTCTTTACAAAATCATTAGAATCCA
JUNASHf01	ATGTGCTAAACCTTTGGC
JUNASHr01	CTCTGTGAACGAGTTTT
PINCH1615probe	AGACCCCTTGAGGTGTTCTCTGTTGT
PINCH1615For	AGCCGTCCATCTTTAATTA
PINCH1615Rev	AAGCCAATAGCACCTTA
JV980probe	TTCCTCCACTGCCCGGCCA
JV980Forward	ATTCTGACTTCATTGCAGCCT
JV980Reverse	AGACAACCTGAGACTGTAGT

Both in silico (comparisons of various sequence reads and/or published Cupressacean data) and experimental analyses were undertaken to assess primer and probe specificity for the 3 target species (*J ashei*, *J pinchotii*, and *J virginiana*),²⁴ for other *Juniperus* species (*Juniperus chinensis*, *Juniperus communis*, *Juniperus horizontalis*, and *Juniperus scopulorum*), and for other Cupressaceae genera (*Cryptomeria japonica*, *Thuja* species, and *Taxodium distichum*).²³

qPCR Protocols

Quantitative PCR reactions were performed with a Step One Plus Real-time PCR system (Applied Biosystems Inc, Foster City, California). An assay solution was prepared for each species by adding 18 μ L of forward primer, 18 μ L of reverse primer, 5 μ L of probe, and 59 μ L of water. A final volume of 20 μ L, composed of 1 μ L of assay solution, 10 μ L of Gene Expression Mastermix (Applied Biosystems Inc), 6 μ L of water, and 3 μ L of DNA, was prepared for each qPCR sample. The following protocol was implemented for qPCR reactions: an initial holding stage of 2 minutes at 50°C, followed by the cycling stage of 40 repetitions of each of (1) 15 minutes at 95°C and (2) 1 minute at 60°C.

Standard Curves From qPCR Assays

To determine the standard curves to be used in the qPCR assays, DNA was extracted from 1 mg of pollen grains of *J ashei*, *J pinchotii*, and *J virginiana*. Standard curves were obtained by using 10-fold serial dilution of each of the species pollen DNA. Three technical replicates were performed for each serial dilution, and each standard curve was repeated 3 times. The limit of quantification (LOQ) was calculated by the minimum number of pollen grains in the standard curve. The specificity of each assay was checked with the DNA of other *Juniperus* species.

Two standard curves were generated for *J ashei* (*matK* sequences) (one set collected from the Arbuckle Mountains in Oklahoma December 2009 and one set collected from Lampasas, Texas, in January 2011), to check the reproducibility because plastid numbers may vary between the pollen grains. An analysis of covariance (ANCOVA) was performed to determine whether the number of pollen grains and cycle threshold value relationship have different slopes and intercepts for the 2 different collection sources. This analysis was completed using R software.²⁴

Comparison With Microscopic Counts

All qPCR counts were converted to pollen concentrations and expressed as pollen grains per cubic meter. The Burkard air sampler draws 14.4 cm³ of air each day. Consequently, we converted our qPCR pollen counts to pollen concentrations by dividing the counts by 14.4. Pollen concentrations from microscopy for each day were determined from a mean of the counts from 3 longitudinal traverses. Similarly, the mean of 3 qPCR replicates was computed for each day. The pollen concentrations obtained from standard microscopy were compared with pollen concentrations obtained from qPCR using a Spearman correlation coefficient as implemented in R.²⁴

Results

qPCR Assay and Standard Curve

Standard curves were obtained by analyzing 10-fold serial dilutions of pollen DNA starting from 257,000 pollen grains to 3 pollen grains for *J ashei*, 153,300 to 2 pollen grains for *J pinchotii*, and 292,800 to 3 pollen grains for *J virginiana*. The coefficient of determination (R^2) was 0.9938 and 0.9873 for *J pinchotii* (Fig 1A) and *J virginiana* (Fig 1B), respectively. The standard curves obtained with *J ashei* pollen DNA samples from 2 different sources

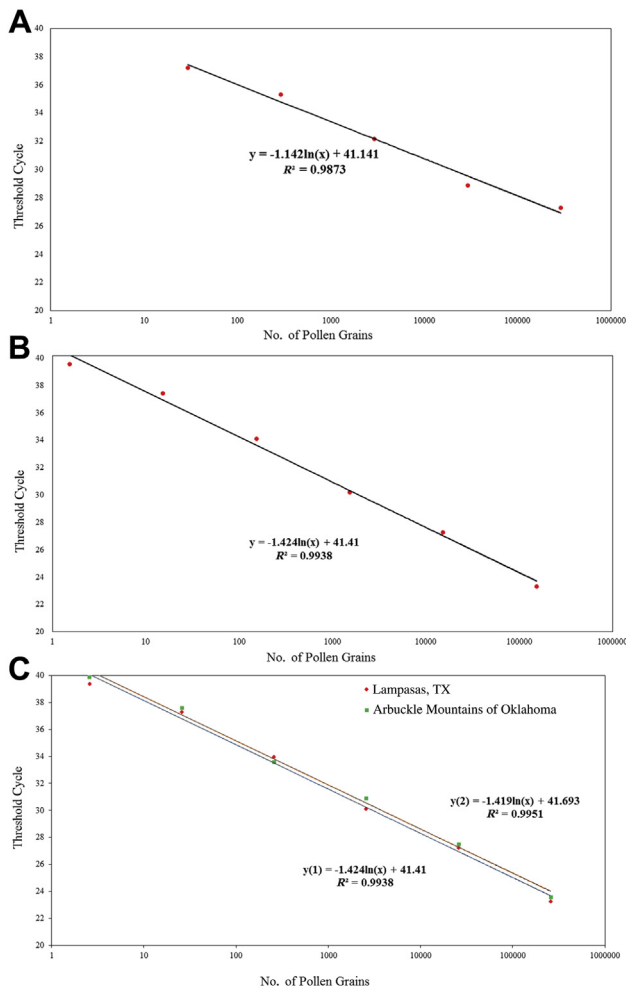


Figure 1. Standard curve for *Juniperus pinchotii*, *Juniperus virginiana*, and *Juniperus ashei* pollen with species-specific primers and probes. Slope equations and R^2 values for the linear regression are shown. A, Association between cycle threshold value and number of pollen grains for *J. pinchotii*. B, Association between cycle threshold value and number of pollen grains for *J. virginiana*. C, Comparison of the standard curves for *J. ashei* pollen with species specific primers and probes obtained from pollen collected from 2 different sources (Lampasas, Texas, and the Arbuckle Mountains of Oklahoma).

(Lampasas, Texas, and Arbuckle Mountains in Oklahoma) produced R^2 values of 0.9938 and 0.9951, respectively (Fig 1C). The ANCOVA confirmed that the slope values and the intercepts for both the sources were not significantly different at $P = .09$ and $P = .03$, respectively. The LOQ values of 3, 2, and 29 pollen grains were achieved for *J. ashei*, *J. pinchotii*, and *J. virginiana*, respectively.

Each assay was checked for the amplification of DNA by primers and probe designed for qPCR. Each of the primer and probe sets were species specific when applied to the primary target species (*J. ashei*, *J. pinchotii*, *J. virginiana*) when applied to other *Juniperus* (*J. chinensis*, *J. communis*, *J. horizontalis*, *J. scopulorum*) and when applied to several ornamental Cupressaceae (data not shown). That is, none of the assays produced an amplification product when DNA of nontarget species was evaluated. Thus, we concluded that our assays were species specific.

Pollen Counting by qPCR and Comparison With Microscopy

Pollen counts from both qPCR assays and microscopy are presented in eTable 1. The qPCR pollen concentration for total *Juniperus* pollen revealed a significant correlation with the pollen

concentration determined by using standard microscope counts ($R = 0.902$, $P < .001$) (Fig 2).

Airborne Pollen Quantification

Results from qPCR analysis revealed that in the year 2013 *J. pinchotii* pollen was reported to occur in the Tulsa atmosphere on 5 days between October and November; the peak was documented on October 27 (Fig 3A) (eTable 1). In 2014, *J. pinchotii* pollen was registered in Tulsa on 11 days between October and December, with a peak observed on October 25 (Fig 3B) (eTable 1). In 2013, *J. ashei* pollen was documented in the Tulsa atmosphere on 14 days between November 2013 and February 2014. The peak was observed on January 12, 2014 (Fig 4A) (eTable 1). The 2014–2015 pollen season for *J. ashei* was documented from November through February on 16 days, with the peak on January 19, 2015 (Fig 4B) (eTable 1). Pollen from *J. virginiana* during 2014 and 2015 was recorded from January until April (Fig 5A and B) (eTable 1). Five days of pollination season overlap were recorded between *J. ashei* and *J. pinchotii* in November and December during the years 2013 to 2014 (Fig 6A) (eTable 1). Similarly, 8 days of pollination season overlap were detected for *J. ashei* and *J. virginiana* during January and February of the years 2014 to 2015 (Fig 6B) (eTable 1).

Discussion

Our results suggest that the DNA extraction protocol we used can be applied to a wide range of airborne pollen types found on Burkard air sample tapes. This method depends on laboratory techniques that do not require a trained palynologist. Even for closely related plant taxa, species-level assignment is possible by this method. Our method provides the absolute number of pollen grains for each of the *Juniperus* species. In addition, the minimum number of pollen grains detectable (LOQ) by our qPCR assays reached a value as small as 3 and 2 for *J. ashei* and *J. pinchotii*, respectively. These values are even lower than the LOQ of 47 pollen grains recorded by Longhi et al.¹⁵ On some of the days on which the microscopic counts were 0 (eg, November 12, 2014, December 8, 2014, and December 18, 2014), our qPCR assays indicated the presence of *Juniperus* pollen in the atmosphere (Table 2).

Microscopy does not allow investigators to detect any pollination season overlap for different intrageneric species that possess identical pollen morphologic findings.^{2,6,10–12,15,25–28} Our study of 105 air samples allowed us to identify pollen from different species of *Juniperus* and to detect overlapping days of pollination season for different sets of *Juniperus* species. All these analyses are based on local sampling data, and the presence of *J. pinchotii* and *J. ashei* pollen in the Tulsa atmosphere is a consequence of the long-distance transport. Even though *J. virginiana* populations are local to Tulsa, the pollen grains recorded in January may have their source well to the south of Tulsa. Field observations of *J. virginiana* trees in Tulsa that coincided with atmospheric pollen records revealed that the pollen cones were not mature at that time (unpublished observations). Our data also confirm that the pollination season duration of different *Juniperus* species has year to year variability.

Although the qPCR data revealed a significant statistical correlation with the microscopic counts, differences in the actual pollen count numbers were recorded by the 2 methods (eTable 1). A number of factors may account for the differences. Two Burkard samplers were used in the investigation: the first for the microscopic analyses and a second for qPCR studies. Thus, minor differences in the functioning of the 2 samplers could yield different results.^{29,30} For the microscopic analysis, the slides were analyzed by counting 3 longitudinal traverses, which represents 10.3% of the slide. Pollen is not distributed uniformly on air sample slides, and thus counting a small portion of a slide significantly underestimates the total amount of pollen on the slide.^{31,32} By contrast, the DNA

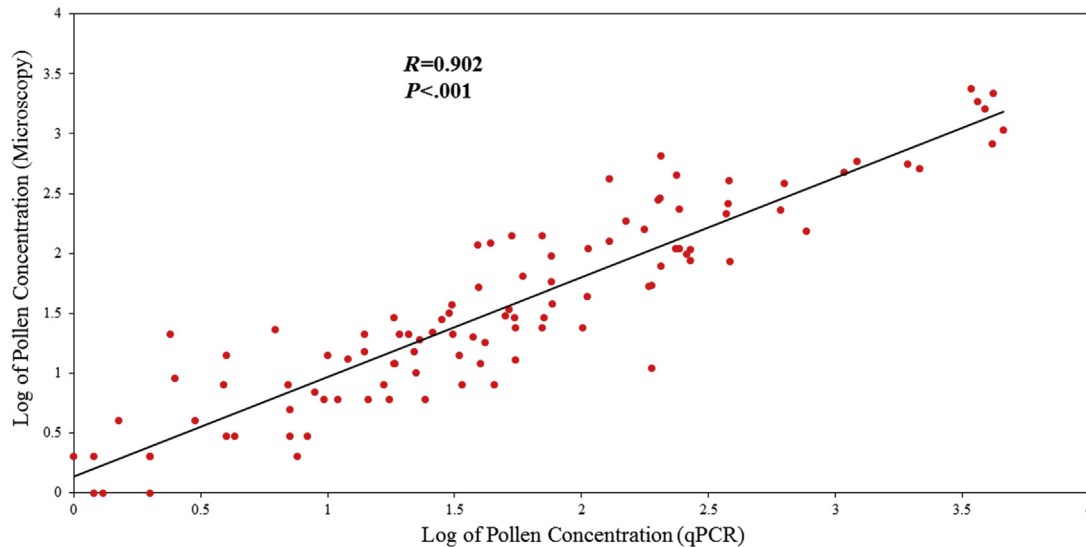


Figure 2. Comparison between *Juniperus* pollen concentrations (pollen grains per cubic meter) estimated by microscopy and pollen concentrations obtained by quantitative polymerase chain reaction (qPCR).

was extracted from the entire tape for the qPCR analysis. In addition to bias attributable to sampler or technique, examiner bias (ie, pollen count variation attributable to differences in the abilities of the researcher) is also a possible source of the differences between qPCR and microscopy.

Our method for detecting *J ashei* pollen involved the use of data from *matK*, a plastid-encoded gene. The problem with using plastid data is that plastid number may vary in the pollen.³³ No data exist for plastid numbers in pollen grains of *J ashei*, and such an investigation is beyond the scope of this project. Instead, we tested for reproducibility in qPCR-based counts by quantifying standardized *J ashei* pollen in a dilution series starting from 257,000 down to

3 pollen grains from 2 sources of pollen with different geographic origins. If variability in plastid number exists, it should manifest itself in nonreproducibility when geographically distinct, but standardized sources are tested. The counts from different sources had high levels of reproducibility, which suggests that the plastid number is not a confounding problem for the use of plastid primers in qPCR analysis of *J ashei* pollen.

The qPCR method described here can be applied to other taxa, such as species of grass (Poaceae), which produce pollen that is morphologically indistinguishable.¹⁰ Grass pollen exposure is one of the major causes of allergic rhinitis in many parts of the world, and the grass family comprises more than 10,000 species across the

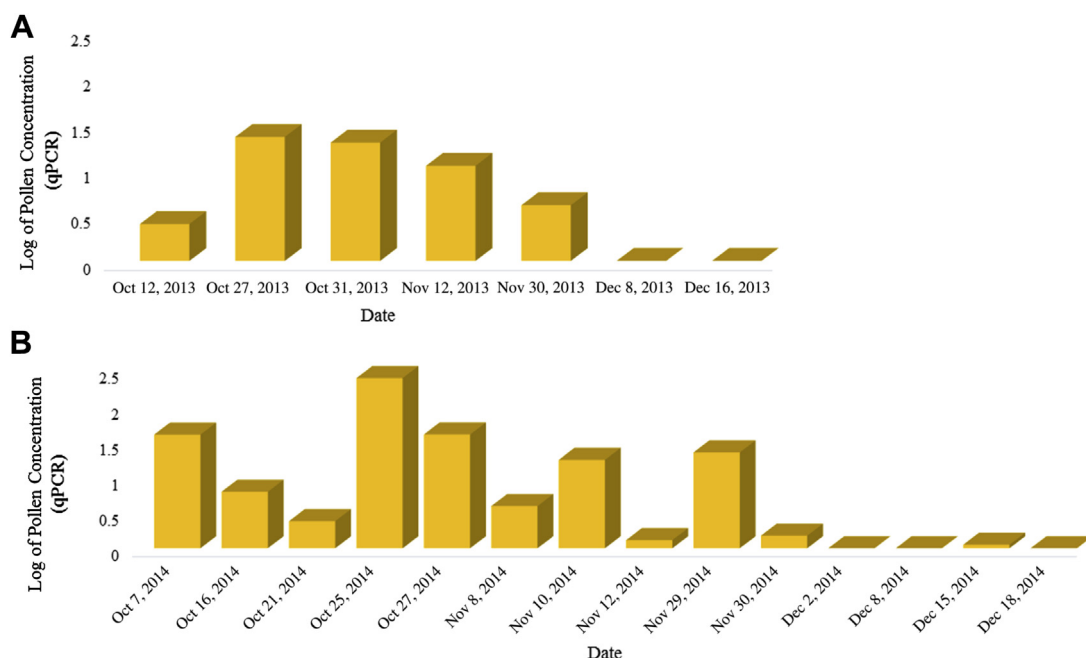


Figure 3. *Juniperus pinchotii* pollen documented in Tulsa based on long distance transport events. A, First occurrence in 2013 was recorded on October 12, with the peak on October 27, and the last occurrence was recorded on November 30. B, First occurrence in 2014 was recorded on October 7, with the peak on October 25, and the last occurrence was recorded on December 15. All concentrations are in pollen grains per cubic meter. qPCR indicates quantitative polymerase chain reaction.

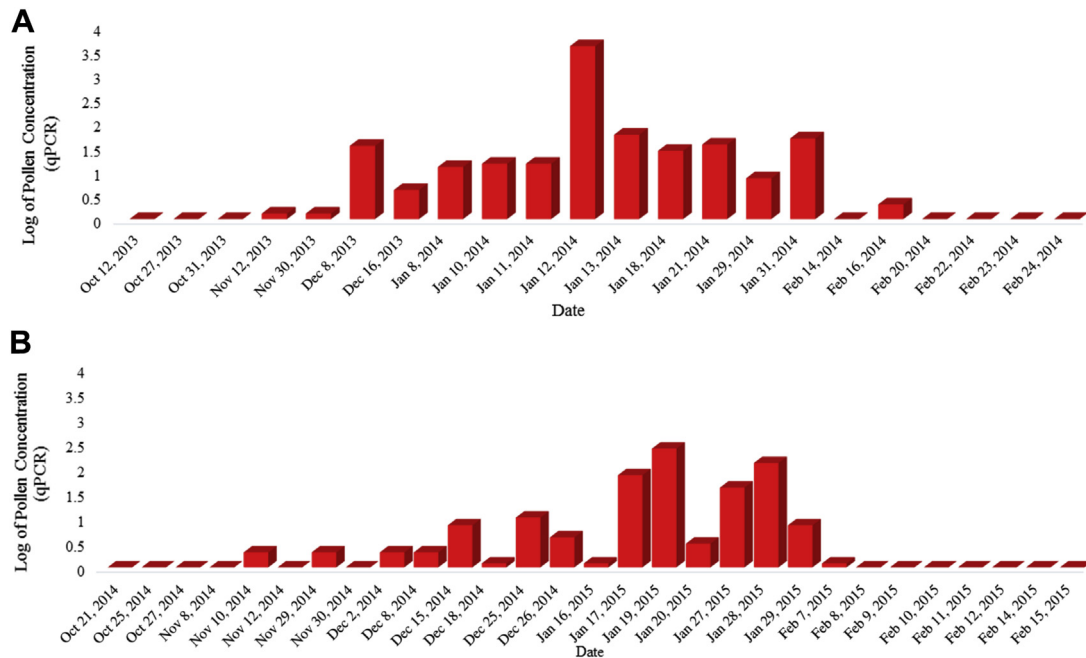


Figure 4. *Juniperus ashei* pollen documented in Tulsa based on long distance transport events; A, First occurrence for 2013 to 2014 was documented on November 12, 2013, with the peak on January 12, 2014, and the last occurrence was recorded on February 16, 2014. B, First occurrence for 2014 to 2015 was recorded on November 10, 2014, with the peak on January 19, 2015, and the last occurrence was recorded on February 7, 2015. All concentrations are in pollen grains/per cubic meter. qPCR indicates quantitative polymerase chain reaction.

globe.³⁴ Knowing the identity of grass pollen prevalent in the atmosphere during different periods would be beneficial to the practicing allergist.

Limitations to the qPCR method, in general, are most likely going to manifest in the absence of suitable sequence data for a species of interest. We were able to identify suitable targets for developing primers and probe sets by first generating transcriptomics data from *J. pinchotii* and *J. virginiana*.²³ In addition, analysis of entirely new

and unknown sequences can be time intensive. With the expansion of sequencing methods, this problem can be solved. Another limitation for a qPCR approach would be a time lag in reporting pollen concentrations to allergy patients because currently this technique is unlikely to be used daily because of expense. A more cost-effective approach to the qPCR method might involve weekly pollen monitoring that could serve as a viable alternative to daily monitoring. The numerous pipetting steps may also serve as a

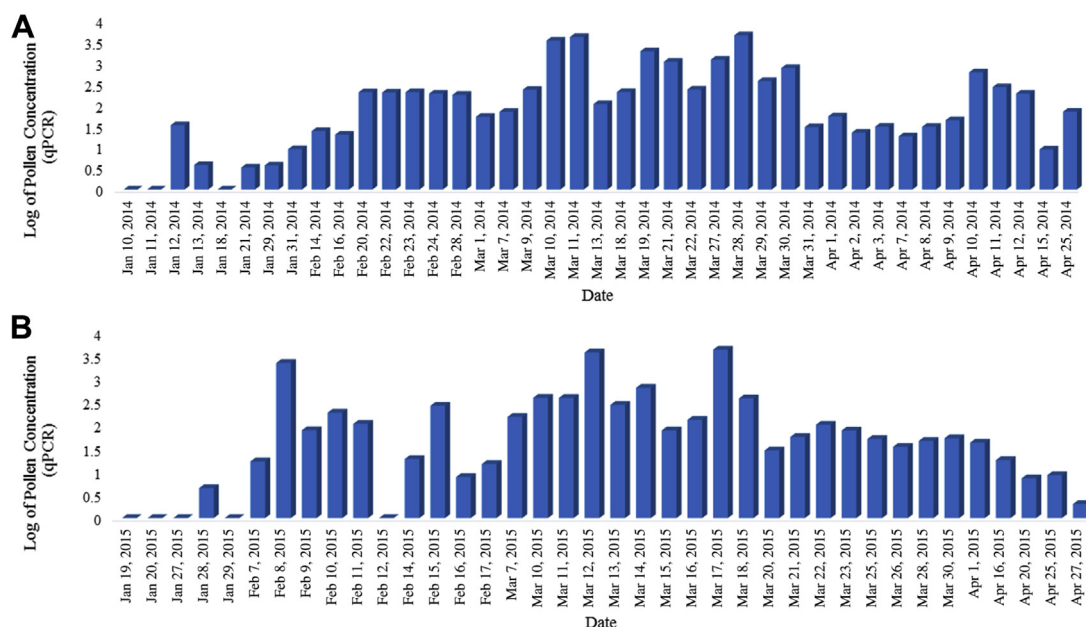


Figure 5. *Juniperus virginiana* pollination season. A, First occurrence for 2014 was recorded on January 12 in Tulsa, with the peak on March 28, and the last occurrence was recorded on April 25. B, In 2015, the first occurrence was recorded on January 28, with the peak on March 17, and the last occurrence was recorded on April 27. All concentrations are in pollen grains per cubic meters. qPCR indicates quantitative polymerase chain reaction.

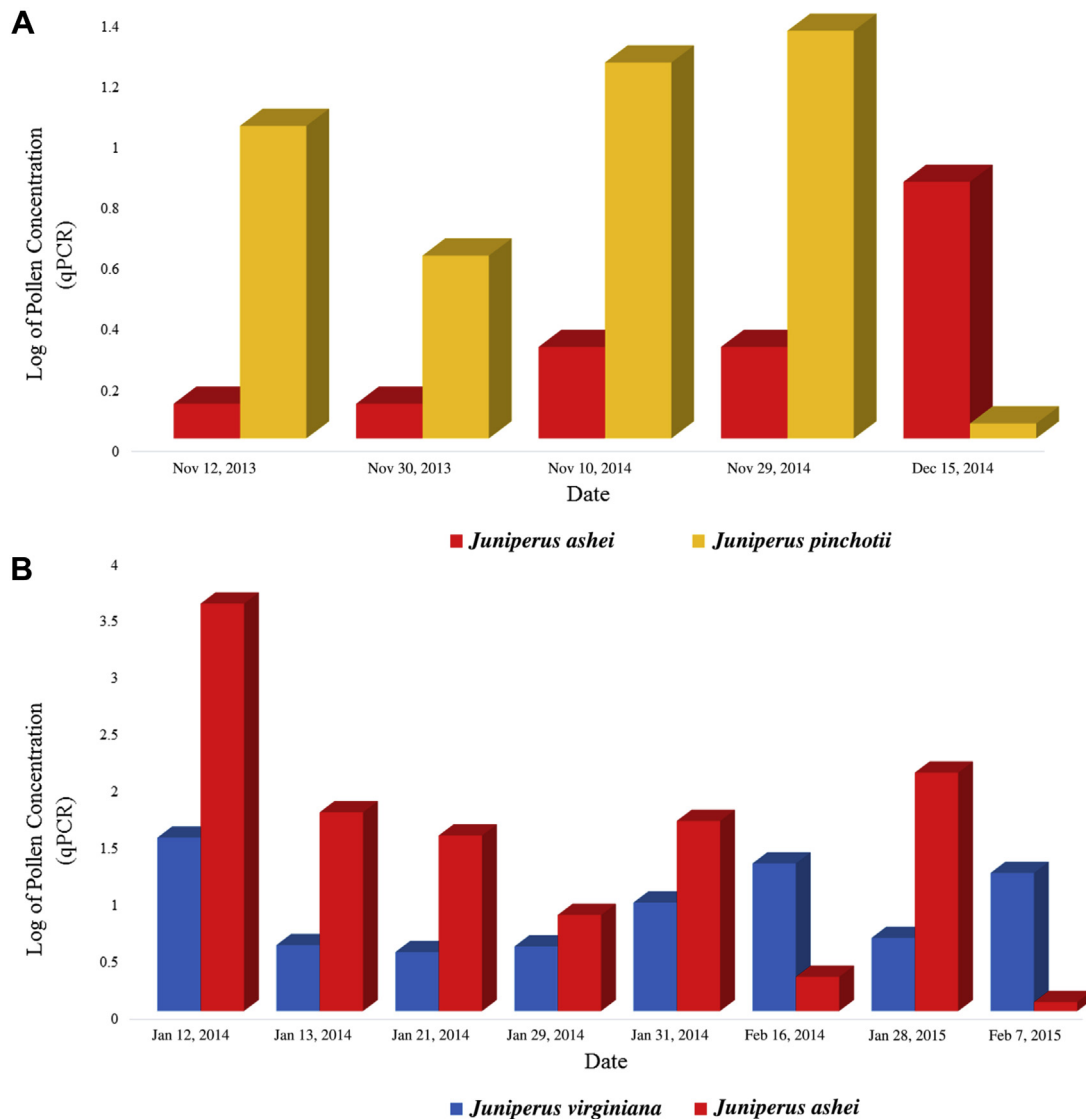


Figure 6. Overlapping pollination season and concentrations (in pollen grains per cubic meters) of the pollen. A, Five days of overlapping pollination season between *Juniperus ashei* and *Juniperus pinchotii*. B, Eight days of overlapping pollination season between *J ashei* and *Juniperus virginiana*. qPCR indicates quantitative polymerase chain reaction.

source of error. Using a minimum of 3 replicates for each sample would resolve this source of error but would also add to the time and expense of the qPCR approach. Any routine use of qPCR for pollen reporting is likely to require some elements of automation.

The results of this investigation indicate that a qPCR approach to identifying and quantifying pollen can provide data with greater resolution than is possible with microscopy. As the qPCR approach becomes economically viable, this method has the potential to be adopted by monitoring networks, thus providing a standardized approach to the rapid quantification and identification of pollen.

Table 2
Comparison of Sensitivity of the qPCR Method and Microscopy Method

Date	No. of pollens (Microscopy)	No. of <i>Juniperus ashei</i> pollens (qPCR)	No. of <i>Juniperus pinchotii</i> pollens (qPCR)
December 8, 2014	0	8	0
November 12, 2014	0	0	5
December 18, 2014	0	3	0

Abbreviation: qPCR, quantitative polymerase chain reaction.

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2016.11.015>.

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Supplementary Data

eTable 1

Complete Overview of the Pollen Concentration Determined by Microscopy and qPCR for 105 Samples^a

Date	Pollen concentration (Microscopy) (Mean of 3 counts), pollen grains/m ³	Pollen concentration (qPCR) (Mean of 3 replicates), pollen grains/m ³			Total pollen concentration (qPCR), pollen grains/m ³
		<i>Juniperus virginiana</i>	<i>Juniperus ashei</i>	<i>Juniperus pinchotii</i>	
10/12/2013	8		0	1.5	1.5
10/27/2013	14		0	21	21
10/31/2013	20		0	18.1	18.1
11/12/2013	5		0.3	9.7	10
11/30/2013	2		0.3	3	3.3
12/8/2013	13		32	0	32
12/16/2013	2		3	0	3
1/8/2014	12		11		11
1/10/2014	20	0	13		13
1/11/2014	14	0	13		13
1/12/2014	1,617	33	3,839		3,872
1/13/2014	63	3	55		58
1/18/2014	21	0	25		25
1/21/2014	19	2	34		36
1/29/2014	5	3	6		9
1/31/2014	23	8	46		54
2/14/2014	5	23.3	0		23.3
2/16/2014	20	19	1		20
2/20/2014	289	203	0		203
2/22/2014	280	199	0		199
2/23/2014	78	204	0		204
2/24/2014	53	188	0		188
2/28/2014	157	176			176
3/1/2014	139	52			52
3/7/2014	139	69			69
3/9/2014	108	233			233
3/10/2014	2,391	3,431			3,431
3/11/2014	2,187	4,189			4,189
3/13/2014	109	105			105
3/18/2014	650	205			205
3/19/2014	558	1,924			1,924
3/21/2014	474	1,079			1,079
3/22/2014	453	235			235
3/27/2014	582	1,213			1,213
3/28/2014	1,074	4,608			4,608
3/29/2014	259	376			376
3/30/2014	153	765			765
3/31/2014	31	29			29
4/1/2014	28	53			53
4/2/2014	9	21			21
4/3/2014	20	30			30
4/7/2014	11	17			17
4/8/2014	36	30			30
4/9/2014	121	43			43
4/10/2014	230	606			606
4/11/2014	86	268			268
4/12/2014	10	188			188
4/15/2014	6	8			8
4/25/2014	23	69			69
10/7/2014	116		38		38
10/16/2014	22		5.2		5.2
10/21/2014	20		1		1
10/25/2014	232		241		241
10/27/2014	51		38.2		38.2
11/8/2014	7		2.9		2.9
11/10/2014	28	1	16		17
11/12/2014	0	0	<1		<1
11/29/2014	18	1	21		22
11/30/2014	3	0	1		1
12/2/2014	1	1	0		1
12/8/2014	0	1	0		1
12/15/2014	4	6	<1		<7
12/18/2014	0	<1	0		<1
12/25/2014	13	9			9
12/26/2014	13	3			3
1/16/2015	1	<1			<1
1/17/2015	28	70			70

(continued on next page)

eTable 1 (continued)

Date	Pollen concentration (Microscopy) (Mean of 3 counts), pollen grains/m ³	Pollen concentration (qPCR) (Mean of 3 replicates), pollen grains/m ³			Total pollen concentration (qPCR), pollen grains/m ³
		<i>Juniperus virginiana</i>	<i>Juniperus ashei</i>	<i>Juniperus pinchotii</i>	
1/19/2015	109	0	242		242
1/20/2015	3	0	2		2
1/27/2015	11	0	39		39
1/28/2015	125	3	124		127
1/29/2015	7	0	6		6
2/7/2015	7	15	<1		16
2/8/2015	512	2,153	0		2,153
2/9/2015	37	76	0		76
2/10/2015	52	183	0		183
2/11/2015	43	105	0		105
2/12/2015	1	0	0		0
2/14/2015	11	17	0		17
2/15/2015	98	258	0		258
2/16/2015	1	7			7
2/17/2015	5	13			13
3/7/2015	186	148			148
3/10/2015	84	383			383
3/11/2015	408	380			380
3/12/2015	1,869	3,626			3,626
3/13/2015	107	268			268
3/14/2015	387	628			628
3/15/2015	95	75			75
3/16/2015	417	127			127
3/17/2015	820	4,156			4,156
3/18/2015	212	370			370
3/20/2015	27	27			27
3/21/2015	12	54			54
3/22/2015	23	100			100
3/23/2015	57	75			75
3/25/2015	29	49			49
3/26/2015	7	33			33
3/28/2015	7	44			44
3/30/2015	33	51			51
4/1/2015	17	41			41
4/16/2015	5	17			17
4/20/2015	2	6			6
4/25/2015	2	7			7
4/27/2015	1	1			1

Abbreviation: qPCR, quantitative polymerase chain reaction.

^aEach sample reading is the mean of 3 counts or 3 replicates. The data in bold correspond to an overlapping pollination season.