

Molecular and ultrastructural detection of plastids in *Juniperus* (Cupressaceae) pollen

Rashmi Prava Mohanty, Mark Alan Buchheim, Richard Portman, Estelle Levetin

Department of Biological Sciences, The University of Tulsa, 800 South, Tucker Drive, Tulsa, Oklahoma 74104, USA

*For correspondence. E-mail estelle-levetin@utulsa.edu

ABSTRACT

Transmission electron microscopy (TEM), PCR and sequencing studies can provide important evidence about the presence of plastids in pollen grains. Their presence is critical if one proposes to develop plastid primers for the amplification of DNA from pollen for species identification. Differential interference contrast microscopy (DIC) and TEM were used to investigate the presence of plastids in pollen from *Juniperus* species, a major cause of airborne allergies in North America. Standard PCR techniques were used to amplify DNA from the pollen using universal primers targeting known plastid DNA genes including the *rbcL* and species-specific primers targeting the *matK* coding regions. Studies using TEM confirmed the presence of plastids in *Juniperus* pollen at three increasing stages of hydration. The *rbcL* and *matK* genes were successfully amplified using DNA extracted from pollen of *J. ashei*, *J. pinchotii* and *J. virginiana*. The results open a wide range of possibilities for using plastid primers in general pollen research especially when the interspecific variation among the standard set of nuclear genes (for e.g., ITS-1, ITS-2) is minimal. TEM results coupled with PCR results confirmed that plastid primers can be used to amplify DNA from pollen of *Juniperus*. This can be used to study the role of plastid DNA in the quantification of airborne, allergenic pollen from various *Juniperus* sources. Published on-line www.phytologia.org *Phytologia* 98(4): 298-310 (Oct 6, 2016). ISSN 030319430.

KEY WORDS: DNA, *Juniperus*, *matK*, PCR, plastids, pollen, *rbcL*, TEM.

Juniperus (Cupressaceae) is regarded as a major source of airborne allergens due to its wide distribution and heavy pollen production (Pettyjohn and Levetin, 1997; Bunderson et al., 2012). The allergenicity of pollen from *J. ashei*, *J. pinchotii* and *J. occidentalis* are the most significant, while other species such as *J. virginiana*, *J. communis*, and *J. horizontalis* are only occasionally reported as allergenic (Lewis et al., 1983).

Light microscopy (LM) has shown that *Juniperus* pollen grains are spherical when hydrated (Nepi et al., 2005). The exine is thin with granules on the surface; the intine is thick; and the protoplast appears pentagonal or star-like (Kurmann, 1994). The grains appear inaperturate in the light microscope; however, a small pore is visible when the pollen grains are viewed with a scanning electron microscope (SEM) (Kurmann, 1994). Although there are some differences in size, pollen grains from various *Juniperus* species cannot be distinguished by LM. In fact, pollen grains produced by members of the Cupressaceae are considered morphologically uniform (Lewis et al., 1983; Kurmann, 1994).

Cupressaceae pollen structure

Although there have been no specific transmission electron microscope (TEM) studies of *Juniperus* pollen, the ultrastructure of two members of the Cupressaceae, *Cupressus sempervirens* and *Cryptomeria japonica*, has been studied (Kurmann, 1994; Suárez-Cervera et al., 2003). These studies have primarily focused on the pollen wall layers. The sporoderm of cupressacean pollen consists of a thin exine and thick intine that surrounds the protoplast (Suárez-Cervera et al., 2003; Chichiriccò and Pacini, 2008). The exine ranges from 0.3-0.9 μm in thickness (Kurmann, 1994). The outer layer of the exine, the ectexine, is made up of granules while the inner layer, the endexine, is more electron dense and lamellate, ranging up to 0.5 μm in thickness (Kurmann, 1994; Suárez-Cervera et al., 2003). Chichiriccò and Pacini

(2008) described the detailed structure of the intine of *Cupressus arizonica*. The intine consists of three layers; the outermost layer is thin, homogeneous and rich in pectins. This outer intine layer is highly plastic and triples its diameter when hydrated. The middle layer is large, spongy and non-homogeneous. This layer is bordered by a mesh of large, branched fibrils and is rich in pectin. The innermost intine layer is considered to be the persistent wall of the sporoderm and consists of both cellulose and callose.

Pollen grains have storage reserves for use after pollen release and during pollen germination. These storage reserves can be oils, proteins and/or carbohydrates. Lipids are generally the main reserve for entomophilous pollen grains, while anemophilous pollen grains typically have starch as their main reserve (Stanley, 1971; Baker and Baker, 1979). Nonetheless, research has shown that the anemophilous pollen of the Cupressaceae contain large amounts of lipids and few starch grains (Owens, 1993).

Cupressaceae pollen has both aerodynamic and hydrodynamic properties. The aerodynamic properties are related to the small size of the pollen grains, which are readily dispersed by wind over long distances. The hydrodynamic properties are related to changes in the sporoderm that occur when pollen lands on the pollination drop produced by ovules (Tomlinson and Takaso, 1998; Fernando et al., 2005). Cupressaceae pollen swells at first contact with the pollination drop (Tomlinson and Takaso, 1998; Fernando et al., 2005). Experiments with *J. communis* indicated that there are various stages of hydration. At stage zero, the pollen was dehydrated, wrinkled and consisted of a star-like protoplast. In the next stage, hydration started in the intine, which increased its volume, and then continued to the protoplast. In the subsequent stage, the protoplast underwent complete hydration and became spherical. At this stage, the splitting and shedding of the exine was observed. During the final stage, the intine increased in volume, the protoplast increased in volume and the outer two layers of intine ruptured and were shed (Nepi et al., 2005). The mechanism of pollen rupturing—shedding the exine and parts of the intine when it comes in contact with the water, is thought to guarantee an easy entry of the pollen protoplast into the ovule (Ottley, 1909; Tomlinson and Takaso, 1998).

Airborne Cupressaceae pollen

Airborne pollen of the Cupressaceae is widely reported (qualitatively and quantitatively) by air sampling networks (Caiaffa et al., 1993; Pendell et al., 2008; Sabariego et al., 2012) and has been well documented in Oklahoma (Rogers and Levetin, 1998; Levetin and Van de Water, 2003). Several *Juniperus* species that occur in Oklahoma, Texas, and New Mexico have overlapping pollination periods. Pollen season start times show considerable year-to-year variability based on local meteorological conditions. The pollination season of *J. pinchotii* starts in late September and lasts through November (Levetin et al., 2012; Adams, 2014). *Juniperus ashei* can start releasing pollen as early as late November and continue through early February (Adams, 2014). *Juniperus virginiana* starts pollinating in early February in Tulsa and continues through April (Levetin, 1998; Adams, 2014). Even though Cupressaceae pollen is detected by air sampling during these overlapping periods, pollen from different *Juniperus* species cannot be distinguished microscopically (Lewis et al., 1983). In contrast to microscopic analysis, some molecular approaches offer opportunities to identify pollen at the species-level.

Molecular markers

Nuclear markers have been previously used in molecular studies of pollen grains (Zhou et al., 2007; Longhi et al., 2009). In contrast, the use of chloroplast markers for pollen studies has been limited. In the case of *Juniperus* species, numerous nuclear genes are available in the NCBI database, but interspecific variation is limited (unpublished observations). The low discrimination at the nucleotide level among the nuclear genes precludes them as potential sources for species-specific primers. Although the number of published chloroplast genes is limited, some variation at the interspecific level is present (unpublished observation).

Phylogenetic investigations of plastid genes have identified seven commonly used markers. Among these seven markers, four are parts of coding genes (*matK*, *rbcL*, *rpoB*, and *rpoC1*) and three are noncoding spacers (*atpF-atpH*, *trnH-psbA*, and *psbK-psbI*) (CBOLPlantWorkingGroup et al., 2009). The *matK* gene is approximately 1500 base pairs (bp) long and is situated within the intron of the chloroplast gene *trnK* (Hilu and Liang, 1997). The *matK* gene is the most rapidly evolving of all plastid coding regions (CBOLPlantWorkingGroup et al., 2009). The *matK* gene is the only chloroplast-encoded group II intron maturase whose function is to regulate plant development. Expression analysis of the *matK* gene reveals that “genetic buffers” are in operation, which compel its evolution and thus, low intraspecific variation is coupled with high interspecific differences (Lahaye et al., 2008). The *matK* gene has high discriminatory power when used in phylogenetic analyses (CBOLPlantWorkingGroup et al., 2009; Hollingsworth et al., 2011). The best characterized gene among the plastid regions is the *rbcL* gene (CBOLPlantWorkingGroup et al., 2009). The *rbcL* gene codes for the large subunit of ribulose-1, 5-biphosphate carboxylase (Chase et al., 1993). While *rbcL* has only modest discriminatory power in phylogenetic analyses when compared to *matK* (CBOLPlantWorkingGroup et al., 2009), it is convenient to amplify, sequence, and align in most of the land plants. The primers for *rbcL* are universal for virtually all land plants (CBOLPlantWorkingGroup et al., 2009; Hollingsworth et al., 2011).

The goals of the current investigation are to confirm the existence of plastids in *Juniperus* pollen through the use of TEM and to determine if plastid-specific primers can be used to amplify pollen DNA. A new toolbox for the differentiation of *Juniperus* plastid genes has the potential to provide a more effective means of identifying and quantifying pollen that causes widespread hay fever.

MATERIALS AND METHODS

LM and TEM of Juniperus pollen

Samples of *J. ashei* pollen were collected from Lampasas, Texas in January 2011, *J. pinchotii* pollen was collected from Sonora, Texas in October 2013 and *J. virginiana* pollen was collected in Tulsa, Oklahoma in March 2014.

Four samples for each pollen species were prepared for LM with differential interference contrast optics (DIC). The pollen from the first sample was not hydrated; whereas, the second and third samples were hydrated in water for one hour and 24 hours, respectively. Following hydration, the pollen was observed using DIC. The fourth pollen sample from each *Juniperus* species was stained with IKI (1%) solution and then observed using DIC for detection of starch grains in pollen.

For TEM studies, three small aliquots of pollen were prepared for each of the species. The first aliquot contained pollen without rehydration, the second aliquot was hydrated in deionized (DI) water for one hour to stimulate exine rupture, and the third aliquot was hydrated in DI water for 24 to 48 hours. The second and third aliquots were centrifuged at 16,004 g for 10 minutes and the supernatant was discarded. The pellet, containing pollen with a small amount of water, was pipetted and suspended in mini-petri dishes, containing 2% water agar (2g agar per 100 ml water) in the molten state. Similarly, the first aliquot of dry pollen was directly added to the liquid agar. After the agar solidified, a small block of agar containing pollen was cut from each petri dish. These blocks were fixed overnight in 5% glutaraldehyde. The following day, the blocks were washed with 0.1 M sodium phosphate buffer (pH 6.8) (a mixture of sodium phosphate monobasic (0.7 g) and sodium phosphate dibasic (1.31 g) added to 100 ml of water) three times at ten minutes per wash. The pollen blocks were post-fixed for one hour at room temperature with 2% osmium tetroxide dissolved in 0.1 M sodium phosphate buffer. The post-fixation was followed by the wash and dehydration steps with ascending concentrations of ethanol, starting from 25% to 100% for 10 min for each, followed by three steps with 100% acetone. The infiltration step was performed with Spurr's epoxy resin (Spurr, 1969). The following day, the blocks were embedded in 100% Spurr's epoxy resin in flexible silicone rubber molds. To cure the resin, polymerization was performed for 3 hours at 70° C. After curing, the blocks were trimmed with a razor blade and thin sections were cut

using an ultramicrotome and a diamond knife. Copper grids were used to collect the ultrathin sections, which were stained using lead citrate and uranyl acetate and observed using a Hitachi H7000 TEM.

Extraction and amplification of Juniperus pollen DNA

Samples of *J. ashei*, *J. pinchotii* and *J. virginiana* pollen weighing 0.1 mg were placed in 2 ml screw-capped tubes. One millimeter glass beads corresponding to approximately 700 μ l of volume were placed in each tube containing the pollen grains and 500 μ l of Fawley's extraction buffer (Fawley et al., 2004) (1 M NaCl, 70 mM Tris, 30 mM Na₂EDTA, pH 8.6), 15 μ l of 10 % CTAB extraction buffer and 10 μ l of β -mercaptoethanol was added to each tube. This was followed by bead-beating of the samples in a mini bead-beater (Biospec Products, Bartlesville, OK USA) for 3 min and incubation at 75° C for one hour. Incubation was followed by addition of 500 μ l of chloroform and isoamyl alcohol (49:1) and centrifugation at 16,004 g for 20 minutes. The aqueous phase was removed and placed in a separate microfuge tube. An additional 500 μ l of chloroform and isoamyl alcohol (49:1) was added to the tube with beads and centrifuged. The aqueous phase was added to the previous supernatant and an additional 500 μ l of chloroform and isoamyl alcohol (49:1) was added and centrifuged at full speed for 20 minutes. Forty five microliters of 3 M sodium acetate and 900 μ l of ice-cold, 100% ethanol were added to the aqueous phase. Samples were inverted and kept at -20° C for one hour. This was followed by centrifugation at 16,004 g for 15 min to pellet the nucleic acid which were washed with 70 % ethanol and dried by placing them in a 40° C water bath. Around 20-120 μ l of RNase free water was added to the dried pellet depending on the size of the DNA pellet.

The *rbcL* region of *Juniperus* pollen DNA was amplified by the PCR using two universal primers: *rbcLFJuniperus* and *rbcLRJuniperus* (Wolf et al., 1994; Pryer et al., 2001). Similarly, the primers, *asheimatKF1* and *asheimatKR1* (Table 1.) were designed to amplify the *matK* region of *J. ashei* pollen DNA. Pollen DNA from *J. virginiana* was amplified using *JVmatKF1* and *JVmatKR1* (Table 1.). A total of 16 μ l of PCR mix (final concentration: 10 mM of forward and reverse primer, 5 units of *Taq* polymerase, 10 mM dNTP, 25 mM MgCl₂, PCR buffer and water; Promega, Madison, WI) was added to each PCR tube. For both *rbcL* and *matK* the following amplification protocols were implemented: an initial heating step of 5 min at 94° C, 36 repetitions of each of (1) a denaturation step of 1 min at 94° C, (2) an annealing step of 45s at 54° C and (3) an extension step of 1 min 10s at 72° C. All reactions were terminated with a final extension of 7 min at 72° C. Agarose gel electrophoresis (0.8% in TBE) was performed to verify the presence of suitable amplified products.

Sequence analysis

In order to verify the *matK* sequence for *J. ashei*, *J. virginiana* and *J. pinchotii*, *matKJF3*, *matKJR3*, *matKJF4*, *matKJR4* primers were designed (see Table 1.). Cycle-sequencing was performed with each of the primers and the amplicons were prepared for capillary sequencing using an ABI 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY, USA). All sequence fragments were assembled by using Sequencher v 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) followed by a nucleotide BLAST search with the query sequences.

RESULTS AND DISCUSSION

DIC and TEM of Pollen Grains

Three different stages of *Juniperus ashei* pollen were observed using DIC. Stage 1 characterized an intact pollen grain with exine, intine and a star-shaped protoplast (Figure 1A). In stage two, the exine was released leaving behind the protoplast with the intine. This transformation took place within an hour of hydration of the pollen. In the third stage, which occurred after 24 hours of hydration, the intine was also released. Stages two and three are evident in Figure 1B. Figure 1C depicts the pollen grain loaded with starch grains stained with IKI.

The DIC studies of *Juniperus* pollen were supplemented with the TEM studies, which provided more detailed images of the pollen. The wall of the intact pollen grain consisted of an outer exine, which is two layered including the ectexine and endexine, and a triple layer intine. The pollen grains contained amyloplasts bearing starch grains. Sections of intact pollen reveal the two-layered exine and three-layered intine (Figures 2A, B, C, D). A large nucleus, many lipid bodies without any membrane and a developing vacuole were also detected. The ectexine surface contains some orbicules (Figures 2A, B, C, D). The exine was shed from pollen that was hydrated for one or more hours. The middle layer of intine was swollen and the cytoplasm contained amyloplasts (Figures 3A, B, C, D). Cytoplasm from pollen which had lost both its exine and outer layers of intine after hydration for 24 hours showed several amyloplasts, a large nucleus and many lipid bodies (Figures 4A, B, C, D). Fig 5A and B reveals both amyloplasts and mitochondria in the *J. ashei* pollen after hydration for 24 hours.

PCR and DNA Sequencing

The *rbcL* gene was successfully amplified using DNA extracted from pollen of *J. ashei*, *J. pinchotii* and *J. virginiana* pollen DNA by using the *rbcL* primers (Figure 6A). Similarly, *matK* species-specific primers were used to successfully amplify pollen DNA from *J. ashei* and *J. virginiana* (Figures 6B and 6C). The *matK* primers for *J. pinchotii* were not species-specific and thus they amplified pollen DNA from all four species of *Juniperus*. Sequence analysis confirmed that the products of amplification for each set of primers corresponded to the targeted gene (KT698211, KT698212, KT698213, KT698214).

Though there are several reports in which LM and SEM were used to study Cupressaceae pollen (Duhoux, 1982; Kurmann, 1994; Chichiriccò and Pacini 2008; Danti et al., 2011), few of them used TEM. One previous TEM study focused on the morphology of *Cupressus sempervirens* pollen grains with emphasis on exine and intine changes during hydration (Kurmann 1994). Although no mention is made of plastids, one image appears to show starch grains (Figure 4, Kurmann, 1994). Similarly, Uehara and Sahashi (2000) present a TEM study of wall development in *Cryptomeria japonica* pollen. Again, starch grains are not mentioned but can be noted in several figures (Uehara and Sahashi, 2000). Suárez-Cervera et al (2003) used TEM of *Cupressus sempervirens* pollen grains for the localization of the pollen allergen. Although various organelles were labeled in the micrographs, no plastids or proplastids were either noted or visible in the published micrographs.

Here we report the presence of plastids and proplastids in all three stages of pollen hydration; these were visible in intact pollen with both exine and intine, pollen without the exine, and pollen without the exine and outer layers of the intine. Previous microscopical studies of *C. macrocarpa* pollen (Hidalgo et al., 2003) made no mention of starch grains. However, starch grains can be observed in the LM micrographs that recorded the early stages of microsporogenesis (see Figure 1f. in Hidalgo et al., 2003). Starch grains are no longer visible in mature *Cupressus* pollen (see Figure 1h in Hidalgo et al., 2003).

In most angiosperms chloroplasts are maternally inherited and plastid exclusion occurs at various stages including during first haploid mitosis, during sperm cell formation or development and during fertilization (Hagemann and Schröder, 1989). In gymnosperms like *Sequoia sempervirens*, chloroplast DNA was paternally inherited whereas, in *Cunninghamia konishii* (Cupressaceae), maternal inheritance of chloroplasts was seen and no paternal linkage was evident (Neale et al., 1989; Lu et al., 2001). Also, the F1 individuals from crosses between *Cunninghamia lanceolata* and *Cryptomeria fortunei* showed maternal inheritance of chloroplast DNA (Qi et al., 1998). Although it has been suggested that paternal inheritance of chloroplasts occurs in all gymnosperms (Neale and Sederoff, 1988; Neale and Sederoff, 1989; Reboud and Zeyl 1994), these studies indicate that maternal inheritance occurs in some members of the Cupressaceae. This emphasizes the importance of checking for the presence or absence of plastids in the pollen grains of other members of the Cupressaceae.

In our studies, we observed the contents of mature *J. ashei*, *J. pinchotii* and *J. virginiana* pollen grains when they came in contact with water. Expansion occurs mainly because of the intine swelling. The same expansion and exine rupture occurs when the mature pollen grain reaches a pollination drop and may facilitates rapid entry of pollen into the micropyle (Tomlinson and Takaso, 1998; Nepi et al., 2005). Our goal was to determine if hydration of the pollen grains was accompanied by changes to the microanatomy of the cell. About 10-16% of the pollen grains examined in this study showed at least one amyloplast as observed under TEM. We also determined that virtually all pollen grains had proplastids even when amyloplasts were not evident. This observation indicates that plastids are retained at least through the pollen hydration stage of gametophyte development. Further work is needed to determine subsequent stages of plastid inheritance in *Juniperus* species.

Primers from nuclear genes such as *needly*, *waxy*, *phantastica* and the internal transcribed spacer have been used as pollen markers (Zhou et al., 2007; Longhi et al., 2009) but the use of chloroplast primers is limited (Fumio et al., 2013). The preference for nuclear markers in pollen characterization may simply be a consequence of a large selection of nuclear genes that are available to the researcher. An alternative explanation for this limited use of plastid genes may be the uncertainty regarding the status of plastids in pollen grains. Regardless of why there is a dearth of pollen studies that use plastid genes, our initial results indicate that the use of chloroplast data from pollen for the study of plant dispersal, pollen dispersal (Mohanty et al., 2015) and diversity needs to be explored. Moreover, our results from preliminary experiments reveal that the plastid primers can be used in rapid quantification of *Juniperus* pollen as an alternative to the time-consuming and labor-intensive microscopy method (Mohanty et al., 2016).

Anemophilous pollen like that of *Juniperus* are light- weight and can travel long distances in large numbers if carried by wind (Levetin and Buck, 1986; Rogers and Levetin, 1998; Levetin and Van de Water, 2003; Bunderson et al., 2014). Population geneticists have exploited genetic evidence from pollen and seeds in the study of plant dispersal (Ennos, 1994; Jordano, 2010; Hampe et al., 2013). Confirmation of plastid gene diversity (Mohanty et al., 2015, 2016) in *Juniperus* pollen could be used to test hypotheses regarding introgression (Hall et al., 1961).

CONCLUSION

Our PCR results using chloroplast primers coupled with the TEM images of plastids in *Juniperus* pollen grains from three different species confirmed that chloroplast primers can be used for the amplification of DNA from pollen of *Juniperus*. The evidence of chloroplast DNA in pollen of *Juniperus* opens up new avenues for the study of *Juniperus* population dynamics (e.g., the invasive *J. virginiana*). Of more immediate impact is the role that plastid DNA will play in the quantification of airborne, allergenic pollen from various *Juniperus* sources.

ACKNOWLEDGEMENTS

The authors thank the University of Tulsa Research Office for providing grants to conduct this research.

LITERATURE CITED

- Adams, R. P. 2014. *Juniperus of the world : the genus Juniperus*, 4th ed. Trafford Publishing, Liberty Drive, Bloomington, Indiana.
- Baker, H. G., and I. Baker. 1979. Starch in angiosperm pollen grains and its evolutionary significance. *Am. J. Bot.* 66: 591-600.

- Bunderson, L., P. Van De Water, J. Luvall, and E. Levetin. 2014. Influence Of Meteorological Conditions On Mountain Cedar Pollen. *J. Allergy. Clin. Immunol.* 133: AB17.
- Bunderson, L. B., P. Van de Water, H. Wells, and E. Levetin. 2012. Predicting and quantifying pollen production in *Juniperus ashei* forests. *Phytologia* 94: 417-438.
- Caiaffa, M. F., L. Macchia, S. Strada, G. Bariletto, F. Scarpelli, and A. Tursi. 1993. Airborne Cupressaceae pollen in southern Italy. *Ann. Allergy.* 71: 45-50.
- CBOLPlantWorkingGroup, P. M. Hollingsworth, L. L. Forrest, J. L. Spouge, M. Hajibabaei, S. Ratnasingham, M. van der Bank, M. W. Chase, R. S. Cowan, D. L. Erickson, A. J. Fazekas, S. W. Graham, K. E. James, K.-J. Kim, W. J. Kress, H. Schneider, J. van AlphenStahl, S. C. H. Barrett, C. van den Berg, D. Bogarin, K. S. Burgess, K. M. Cameron, M. Carine, J. Chacón, A. Clark, J. J. Clarkson, F. Conrad, D. S. Devey, C. S. Ford, T. A. J. Hedderson, M. L. Hollingsworth, B. C. Husband, L. J. Kelly, P. R. Kesanakurti, J. S. Kim, Y.-D. Kim, R. Lahaye, H.-L. Lee, D. G. Long, S. Madriñán, O. Maurin, I. Meusnier, S. G. Newmaster, C.-W. Park, D. M. Percy, G. Petersen, J. E. Richardson, G. A. Salazar, V. Savolainen, O. Seberg, M. J. Wilkinson, D.-K. Yi, and D. P. Little. 2009. A DNA barcode for land plants. *Proc. Natl. Acad. Sci.* 106: 12794-12797.
- Chase, M. W., D. E. Soltis, R. G. Olmstead, D. Morgan, D. H. Les, B. D. Mishler, M. R. Duvall, R. A. Price, H. G. Hills, Y.-L. Qiu, K. A. Kron, J. H. Rettig, E. Conti, J. D. Palmer, J. R. Manhart, K. J. Sytsma, H. J. Michaels, W. J. Kress, K. G. Karol, W. D. Clark, M. Hedren, S. G. Brandon, R. K. Jansen, K.-J. Kim, C. F. Wimpee, J. F. Smith, G. R. Furnier, S. H. Strauss, Q.-Y. Xiang, G. M. Plunkett, P. S. Soltis, S. M. Swensen, S. E. Williams, P. A. Gadek, C. J. Quinn, L. E. Eguiarte, E. Golenberg, G. H. Learn, Jr., S. W. Graham, S. C. H. Barrett, S. Dayanandan, and V. A. Albert. 1993. Phylogenetics of Seed Plants: An Analysis of Nucleotide Sequences from the Plastid Gene *trnD*. *Ann. Missouri Bot.* 80: 528-580.
- Chichiriccò, G., and E. Pacini. 2008. *Cupressus arizonica* pollen wall zonation and in vitro hydration. *Plant Syst. Evol.* 270: 231-242.
- Danti, R., G. Della Rocca, R. Calamassi, B. Mori, and M. Mariotti Lippi. 2011. Insights into a hydration regulating system in *Cupressus* pollen grains. *Ann. Bot.* 108: 299-306.
- Duhoux, E. 1982. Mechanism of exine rupture in hydrated taxoid type of pollen. *Grana* 21: 1-7.
- Ennos, R. 1994. Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* 72: 250-259.
- Fawley, M. W., K. P. Fawley, and M. A. Buchheim. 2004. Molecular diversity among communities of freshwater microchlorophytes. *Microb. Ecol.* 48: 489-499.
- Fernando, D., M. Lazzaro, and J. Owens. 2005. Growth and development of conifer pollen tubes. *Sex. Plant. Reprod.* 18: 149-162.
- Fumio, N., U. Jun, S. Yoshihisa, K. Ryo, T. Nozomu, F. Koji, M. Hideaki, I. Satoshi, and K. Hiroshi. 2013. DNA analysis for section identification of individual *Pinus* pollen grains from Belukha glacier, Altai Mountains, Russia. *Env. Res. Lett.* 8: 014032.
- Hagemann, R., and M.-B. Schröder. 1989. The cytological basis of the plastid inheritance in angiosperms. *Protoplasma* 152: 57-64.
- Hall, M. T., J. McCormick, and G. G. Fogg. 1961. Hybridization between *Juniperus ashei* Buchholz and *Juniperus pinchotii* Sudworth in southwestern Texas. *Butler University Botanical Studies* 14: 9-28.
- Hampe, A., M.-H. Pemonge, and R. J. Petit. 2013. Efficient mitigation of founder effects during the establishment of a leading-edge oak population. *Proceedings of the Royal Society of London B: Biological Sciences* 280:20131070.
- Hidalgo, P. J., C. Galán, and E. Domínguez. 2003. Male phenology of three species of *Cupressus*: correlation with airborne pollen. *Trees* 17: 336-344.
- Hilu, K., and H. Liang. 1997. The *matK* gene: sequence variation and application in plant systematics. *Am. J. Bot.* 84: 830-830.

- Hollingsworth, P. M., S. W. Graham, and D. P. Little. 2011. Choosing and Using a Plant DNA Barcode. PLoS ONE 6: e19254.
- Jordano, P. 2010. Pollen, seeds and genes: the movement ecology of plants. Heredity 105: 329-330.
- Kurmann, M. H. 1994. Pollen morphology and ultrastructure in the Cupressaceae. Acta. Botanica. Gallica. 141:141-147.
- Lahaye, R., M. van der Bank, D. Bogarin, J. Warner, F. Pupulin, G. Gigot, O. Maurin, S. Duthoit, T. G. Barraclough, and V. Savolainen. 2008. DNA barcoding the floras of biodiversity hotspots. Proc. Natl. Acad. Sci. 105: 923-2928.
- Levetin, E. 1998. A long-term study of winter and early spring tree pollen in the Tulsa, Oklahoma atmosphere. Aerobiologia 14: 21-28.
- Levetin, E., and P. Buck. 1986. Evidence of mountain cedar pollen in Tulsa. . Ann. Allergy. 54:5.
- Levetin, E., L. Bunderson, P. Van de Water, and J. Luvall. 2012. Is Red-Berry Juniper an Overlooked Fall Allergen in the Southwest? J. Allergy. Clin. Immunol. 129:AB91.
- Levetin, E., and P. K. Van de Water, 423-442. 2003. Pollen count forecasting. . Immunol. Allergy. Clin. North. Am. 23:19.
- Lewis, W. H., P. Vinay, and V. E. Zenger. 1983. Airborne & allergic pollen of north america. The john hopkins University Press, Baltimore, Maryland.
- Longhi, S., A. Cristofori, P. Gatto, F. Cristofolini, M. S. Grando, and E. Gottardini. 2009. Biomolecular identification of allergenic pollen: a new perspective for aerobiological monitoring? Ann. Allergy Asthma Immunol. 103: 508-514.
- Lu, S.-Y., C.-I. Peng, Y.-P. Cheng, K.-H. Hong, and T.-Y. Chiang. 2001. Chloroplast DNA phylogeography of *Cunninghamia konishii* (Cupressaceae), an endemic conifer of Taiwan. Genome 44: 797-807.
- Mohanty, R. P., M. A. Buchheim, J. Anderson, and E. Levetin. 2015. Detection of Airborne *Juniperus* Pollen By Conventional and Real-Time PCR from Burkard Air Samples. J. Allergy. Clin. Immunol. 135: AB231.
- Mohanty, R. P., M. A. Buchheim, and E. Levetin. 2016. Rapid Quantification of *Juniperus* Pollen Proves Overlapping Pollen Seasons. J. Allergy. Clin. Immunol. 137: AB279.
- Neale, D., and R. R. Sederoff. 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor. Appl. Genet. 77: 212-216.
- Neale, D. B., K. A. Marshall, and R. R. Sederoff. 1989. Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. Proc. Natl. Acad. Sci. 86: 9347-9349.
- Neale, D. B., and R. R. Sederoff. 1988. Inheritance and evolution of conifer organelle genomes. Genetic manipulation of woody plants. 44: 251-264.
- Nepi, M., M. Guarnieri, S. Mugnaini, L. Cresti, E. Pacini, and B. Piotta. 2005. A modified FCR test to evaluate pollen viability in *Juniperus communis* L. Grana 44: 148-151.
- Ottley, A. M. 1909. The development of the gametophytes and fertilization in *Juniperus communis* and *Juniperus virginiana*. Botanical Gazette. 48: 31-46.
- Owens, J. N. 1993. Pollination Biology. In: D.L. Bramlett, G.R. Askew, T.D. Blush, F.E. Bridgwater and J.B. Jett. eds. Pollen management Handbook, 91-13. USDA Forest Service, Washington DC.
- Pendell, G. G., F. Hu, F. Pacheco, J. Portnoy, and C. Barnes. 2008. Seasonal and Daily Patterns of Cupressaceae Pollen in Kansas City. J. Allergy. Clin. Immunol. 121: S21.
- Pettyjohn, M., and E. Levetin. 1997. A comparative biochemical study of conifer pollen allergens. Aerobiologia 13: 259-267.
- Pryer, K. M., A. R. Smith., J. S. Hunt., and J.-Y. Dubuisson. 2001. rbcL data reveal two monophyletic groups of filmy ferns (Filicopsida: Hymenophyllaceae). Am. J. Bot. 88: 1118-1130.
- Qi, W., H. Yang, Y. Xue, and S. Hu. 1998. Inheritance of chloroplast and mitochondrial DNA in Chinese fir (*Cunninghamia lanceolata*). Acta Botanica Sinica 41: 695-699.
- Reboud, X., and C. Zeyl. 1994. Organelle inheritance in plants. Heredity 72: 132-140.
- Rogers, C. A., and E. Levetin. 1998. Evidence of long-distance transport of mountain cedar pollen into Tulsa, Oklahoma. Int. J. Biometeorol. 42: 65-72.

- Sabariego, S., P. Cuesta, F. Fernandez-Gonzalez, and R. Perez-Badia. 2012. Models for forecasting airborne Cupressaceae pollen levels in central Spain. *Int. J. Biometeorol.* 56: 253-258.
- Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of ultrastructure research* 26: 31-43.
- Stanley, R. G. 1971. Pollen chemistry and tube growth. *In: Heslop-Harrison J, ed. Pollen: development and physiology*, 131-155. Butterworths Publishers, London, United Kingdom.
- Suárez-Cervera, M., Y. Takahashi, A. Vega-Maray, and J. Seoane-Camba. 2003. Immunocytochemical localization of Cry j 1, the major allergen of *Cryptomeria japonica* (Taxodiaceae) in *Cupressus arizonica* and *Cupressus sempervirens* (Cupressaceae) pollen grains. *Sex Plant Reprod.* 16: 9-15.
- Tomlinson, P., and T. Takaso. 1998. Hydrodynamics of pollen capture in conifers. *In: Owens, SJ, Rudall, PJ. eds. Reproductive biology in systematics, Conservation and economic botany.* Royal Botanic Gardens, Kew, Richmond, United Kingdom.
- Wolf, P. G., P. S. Soltis, and D. E. Soltis. 1994. Phylogenetic Relationships of Dennstaedtioid Ferns: Evidence from *rbcL* Sequences. *Mol. Phylogenet. Evol.* 3: 383-392.
- Zhou, L. J., K. Q. Pei, B. Zhou, and K. P. Ma. 2007. A molecular approach to species identification of Chenopodiaceae pollen grains in surface soil. *Am. J. Bot.* 94: 477-481.

Table 1. List of *rbcL* and *matK* primers used in PCR and sequencing.

Primer name	Primer sequences (5'-3')
<i>asheimatKF1</i>	ATCCAACAGGTTATTCTTG
<i>asheimatKR1</i>	TGGATTCTAATGATTTTGT
<i>JVmatKF1</i>	CGTAAACAGAATCAGAAT
<i>JVmatKR1</i>	GATTCTCTTTCTTTTGAAA
<i>matKJF3</i>	GTTCTCCCTGTTCCCTT
<i>matKJR3</i>	TCAAGACTGCATATCCT
<i>matKJF4</i>	TCATCTGTTTCATTTTGGC
<i>matKJR4</i>	CTCTGTGAACGAGTTTTT
<i>rbcLFjuniper</i>	ATGTCACCACAAACAGAACTAAAGCAAGT
<i>rbcLRjuniper</i>	TCACAAGCAGCAGCTAGTTCAGGACTC
<i>rbcLBFjuniper</i>	GCAAATACTTCGTTGGCTCA
<i>rbcLARjuniper</i>	TGAGCCAACGAAGTATTTGC



Figure 1. Differential interference contrast micrographs of *Juniperus* pollen (A-C); (A) Intact pollen grain of *Juniperus ashei* with exine, intine and the star-shaped protoplast. (B) Pollen grains of *Juniperus ashei* in which the exine was released leaving behind the intine with protoplast and one pollen grain in which exine and intine both were released leaving behind only protoplast. (C) Pollen grain of *Juniperus ashei* with numerous starch grains visible when stained with IKI solution.

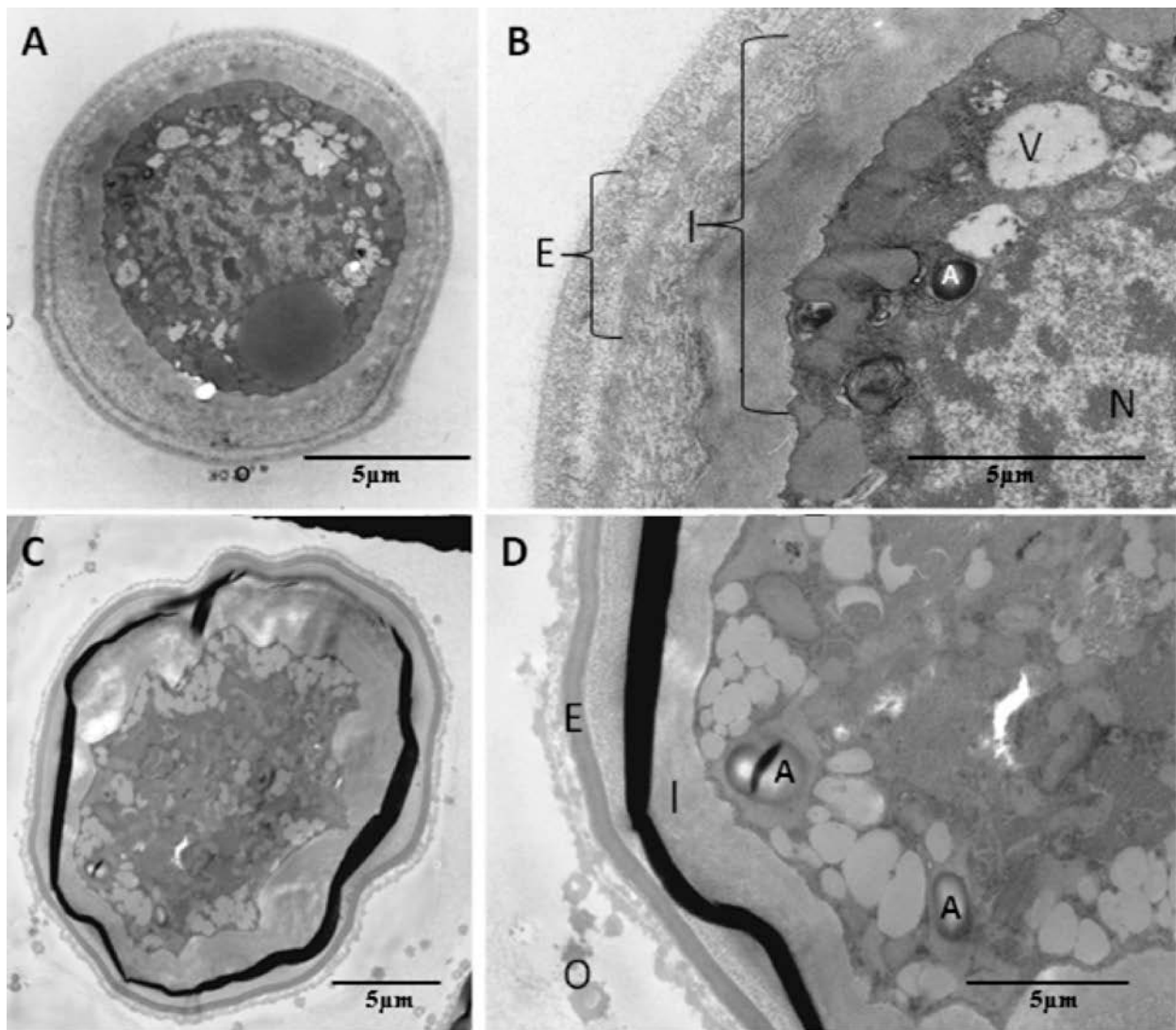


Figure 2. Transmission electron micrographs of intact pollen grains. (A-B) *Juniperus ashei* intact pollen grain and (C-D) *Juniperus pinchotii* pollen with nucleus, lipid bodies, amyloplasts with starch grain, and vacuole. Images show a double-layered exine, a triple-layered intine and orbicules on the exine surface.

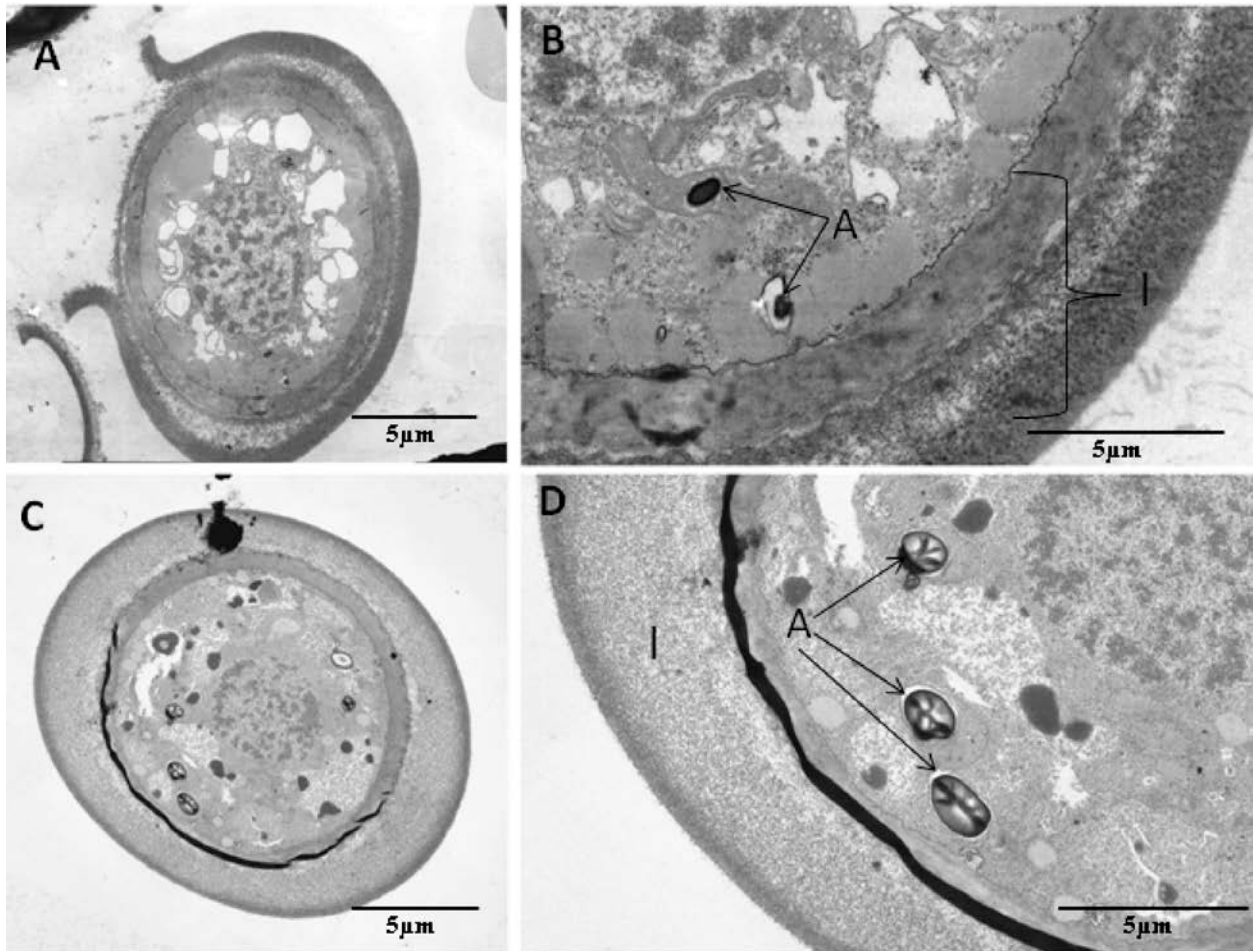


Figure 3. Transmission electron micrographs of hydrated pollen grains lacking exine. (A-B) *Juniperus virginiana* pollen grain and (C-D) *Juniperus pinchotii* pollen grain lacking exine. Amyloplasts with starch grain and triple-layered intine are evident.

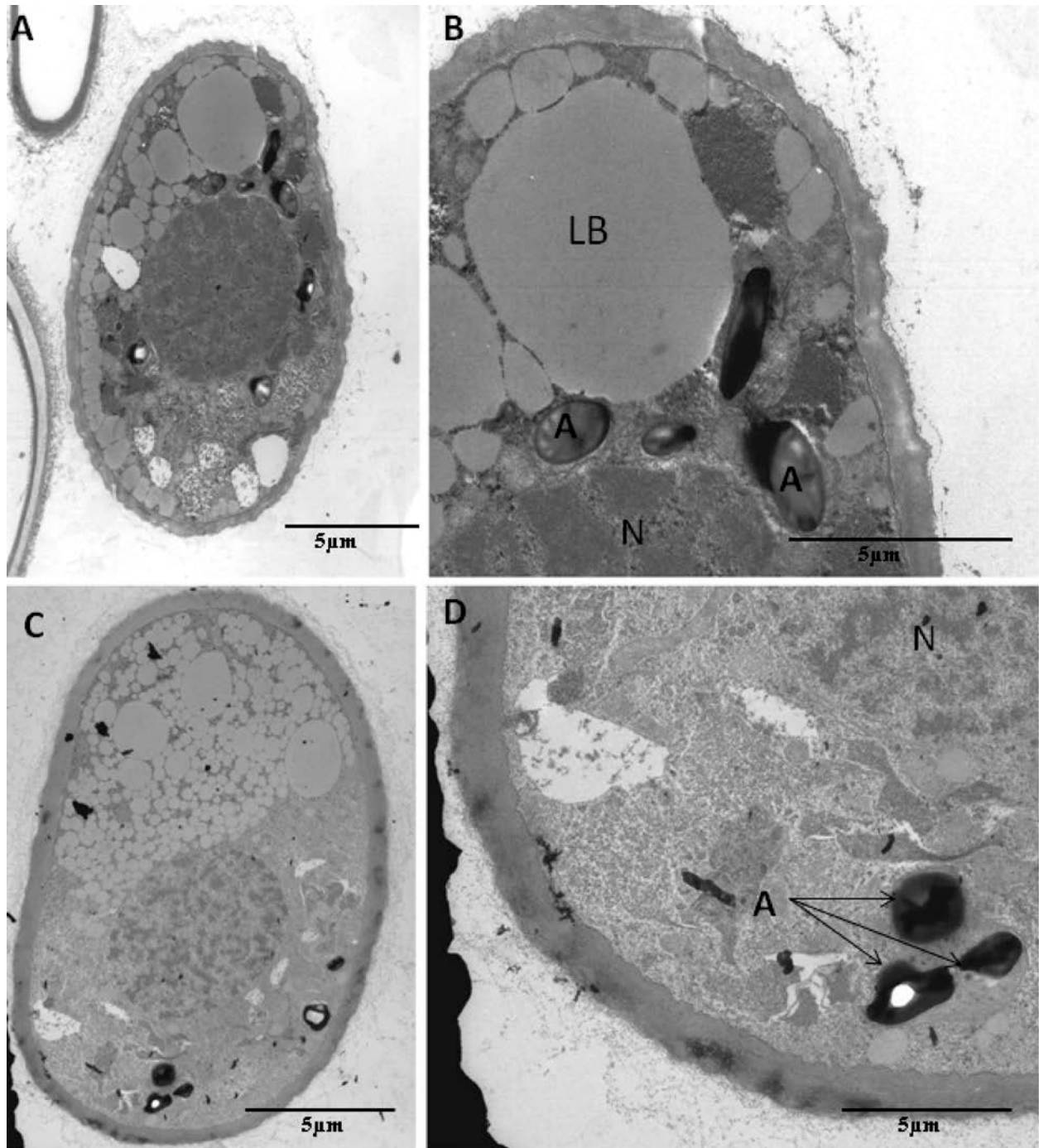


Figure 4. Transmission electron micrographs of pollen grains lacking exine and intine. (A-B) *Juniperus ashei* pollen grain and (C-D) *Juniperus pinchotii* pollen grain lacking exine and most of the intine. It represents nucleus, lipid bodies and amyloplasts with starch grains. A: amyloplasts, E: exine, GB: golgi bodies, I: intine, LB: lipid bodies, N: nucleus, O: orbicules, P: protoplast, V: vacuoles.

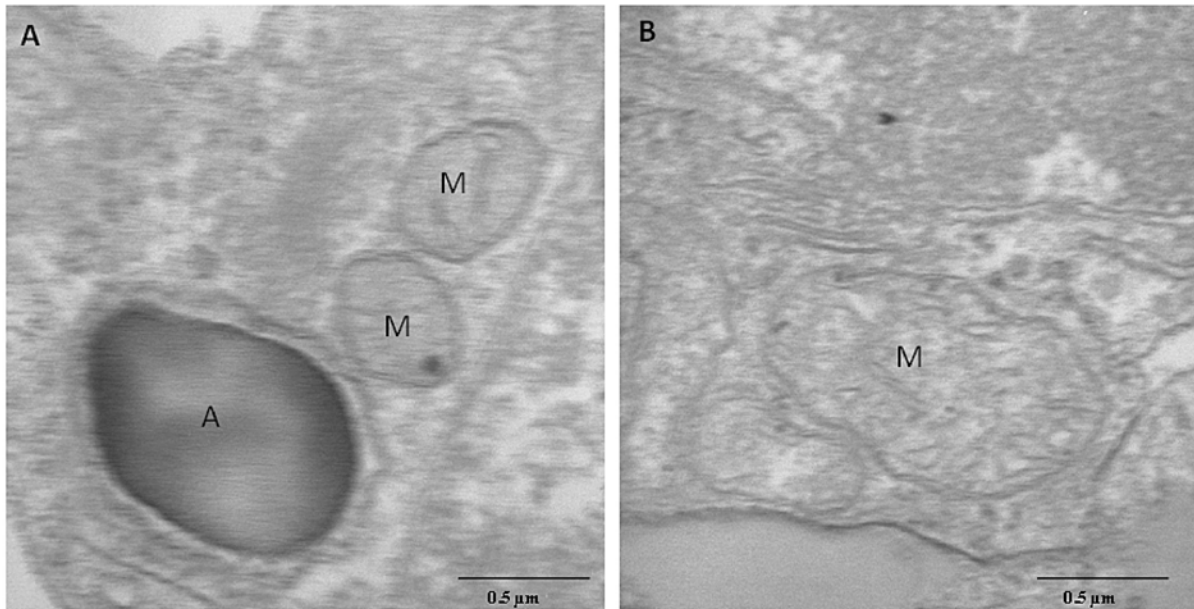


Figure 5. Transmission electron micrographs of *Juniperus ashei* pollen grains. (A-B) *Juniperus ashei* pollen grains with both amyloplasts and mitochondria. A: amyloplasts, M: mitochondria.

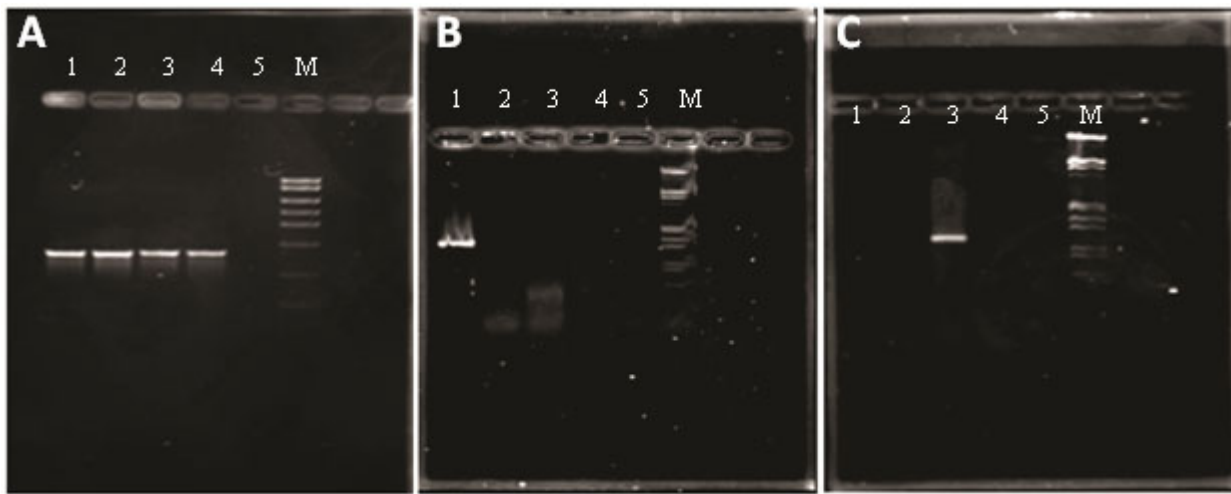


Figure 6. Agarose gel electrophoresis of amplified plastid genes. (A) *Juniperus ashei*, *J. pinchotii*, *J. virginiana* and a positive control pollen DNA amplified by *rbcL* primers (B) *Juniperus ashei* pollen DNA amplified by species-specific *matK* primers. (C) *Juniperus virginiana* pollen DNA amplified by species-specific *matK* primers. NOTE: Lane 1: *Juniperus ashei* pollen DNA, Lane 2: *Juniperus pinchotii* pollen DNA, Lane 3: *Juniperus virginiana* pollen DNA, Lane 4: Positive control in (A) and blank in (B) and (C), Lane 5: Negative control, Lane 6: λ -DNA ladder as marker (M).