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The carbon stable isotope composition of pollen

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Abstract

The $\delta^{13}C$ value of plant tissue is increasingly used to infer environmental and ecological conditions in modern and ancient environments. Isolation techniques and morphological descriptions have been established that characterize plant pollen for the greater part of the Phanerozoic eon. If the $\delta^{13}C$ value of fossil pollen could be used as an indicator of the carbon isotope composition of ancient pollinating communities, it could provide insight into ancient paleoenvironments. Previous studies have revealed a correlation between the $\delta^{13}C$ value of pollen and the $\delta^{13}C$ value of bulk plant tissue in a few isolated species; this study sought to quantify the isotopic relationship between modern pollen and stem and leaf tissues across all the major phylogenetic clades of the tracheophytes. One-hundred and seventy-five different species of tracheophytes were collected from 11 arboreta and botanical gardens across the United States and analyzed for $\delta^{13}C$ value of pollen, leaf, and stem tissue. The carbon isotope difference between pollen and leaf tissue values in the same species was $=\pm 3.00‰$ for >90% of data pairs, and the same relationship was true for comparisons between pollen and stem tissue. When the data was examined by phylogenetic clade, it was found that some groups, such as the Cornales and Ericales, exhibited a much closer and more consistent correlation between pollen and stem and leaf tissue. It was also noted that green-stemmed clades tended to show $\delta^{13}C_{stem}<\delta^{13}C_{pollen}$ while woody-stemmed clades showed the opposite ($\delta^{13}C_{pollen}<\delta^{13}C_{stem}$), a difference attributable to the different chemical composition of the two types of stem tissue. The chemical preparation method involving Schulze's solution that is commonly employed to isolate pollen from pre-Quaternary sediments was shown not to affect the carbon isotope composition of pollen by more than 0.23‰. The manual isolation of sufficient pollen from the fossil record for $\delta^{13}C$ analysis of a single species would be prohibitively laborious; however, these results showed that when understood in terms of paleobotanical taxonomy, $\delta^{13}C$ analyses of bulk fossil pollen can be used to infer the $\delta^{13}C$ value of the ancient plant community to within 1.5‰.

Keywords: pollen; carbon stable isotopes; palynology; palaeobotany

1. Introduction

Analysis of the carbon stable isotope composition of plant tissues and plant compounds has a myriad of applications spanning biology, ecology, and geology. The $\delta^{13}C$ value of modern and fossil plant compo-

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nents has been used to elucidate environmental conditions and relationships in current (reviewed in Dawson et al., 2002), as well as ancient (reviewed in Beerling and Royer, 2002), ecosystems. Many recent publications describe the use of carbon stable isotopes to gain a diverse array of insights into plant communities. Some examples in modern plant ecosystems include the use of plant tissue $\delta^{13}C$ value to determine C3 vs. C4 photosynthetic pathway in monocots (Li et al., 1999), to discern the recycling of CO_2 within ecosystems (Buchmann et al., 2002; Sternberg and DeAngelis, 2002), to follow the incorporation of plant matter into soil (Baisden et al., 2002), to elucidate plant water stress (Ward et al., 2002), and to compare plant intercellular CO_2 concentration between species in the same environment (Sandquist and Ehleringer, 2003). Similarly, the recent literature reveals the broad application of carbon stable isotope analysis of plant tissues to problems in paleoclimatology; plant fossil material $\delta^{13}C$ value has been used to determine the shifts from C3 to C4 vegetation in ancient environments (Scott, 2002) and to infer changes in the climate environment that caused such shifts (Boom et al., 2002; Nordt et al., 2002). The $\delta^{13}C$ value of fossil terrestrial organic material has also been used to quantify changes in the carbon isotope composition of CO_2 (Arens and Jahren, 2000; Hasegawa et al., 2003; Jahren, 2002), and to identify what type of photosynthetic organism a fossil once was (Jahren et al., 2003).

A particularly long and rich history exists in the morphological characterization and taxonomic systematization of pollen (Jones and Rowe, 1999; Traverse, 1988). Plant pollen can be retrieved from the fossil record for the entire 400+ million year history of plant macrofossil assemblages; moreover, plant pollen appears in the fossil record many millions of years before plant macrofossils can be found (Strother, 2000). In addition, pollen is commonly isolated from various sediments, analyzed for its morphology, and archived. All of these attributes recommend pollen as an ideal plant–tissue substrate for carbon isotope analysis. However, to date, only a handful of studies have examined the carbon stable isotope composition of pollen, and these studies have focused on a few plant families. Amundson et al. (1997) reported the $\delta^{13}C$ values of pollen from 11 species of Poaceae; Descolas-Gros et al. (2001) reported the $\delta^{13}C$ values of pollen from 33 species of trees, herbs, and grasses; Loader and Hemming (2000) reported the $\delta^{13}C$ values of pollen from five species of plants; Loader and Hemming (2001) reported an in-depth study of the $\delta^{13}C$ value of modern *Pinus sylvestris* (Pinaceae) and its relationship to the climate environment of pollen production. This study sought to characterize the $\delta^{13}C$ value of modern pollen from all the major tracheophyte clades, and to quantify the general isotopic relationship between pollen and other plant tissues. If the claim that the $\delta^{13}C$ value of pollen is "closely related" to that of the parent plant tissue (page 19 of Amundson et al., 1997) holds true for all tracheophytes, pollen could be established as an isotopic substrate and used to elucidate environmental conditions in both modern and ancient ecosystems through the wealth of applications discussed above.

Table 1
Botanical gardens and arboreta of collection

Site abbreviation	Site	Location	Average April–May temperature[a] (°C)
ASU	The Arboretum at Arizona State University	33°14′ N 112°1′ W	21.1
ATH	The State Botanical Garden of Georgia	33°39′ N 84°26′ W	18.3
ATL	The Atlanta Botanical Garden	33°57′ N 83°19′ W	19.2
BTA	Boyce Thompson Arboretum State Park	33°26′ N 112°14′ W	21.4
JHU	The Johns Hopkins University Campus	39°11′ N 76°40′ W	15.6
MA	Morris Arboretum	39°53′ N 75°15′ W	13.5
MIN	Minnesota Landscape Arboretum	44°53′ N 93°13′ W	12.0
NA	The United States National Arboretum	38°51′ N 77°2′ W	15.6
NCA	The North Carolina Arboretum	35°26′ N 82°32′ W	15.8
TBG	Tucson Botanical Gardens	32°7′ N 110°56′ W	21.9
WPA	Washington Park Arboretum	47°32′ N 112°18′ W	11.6

[a] Average monthly temperatures for the months of April plus May based on 90+ years of annual records (Spangler and Jenne, 1990).

2. Methods and materials

Plant tissue and pollen samples were collected from mature plants at 11 arboreta and botanical gardens during late May of 2000, 2001, and 2002 (Table 1). Sites of collection were located in the U.S. states of Arizona, Georgia, Maryland, Minnesota, Pennsylvania, Washington, and in Washington, DC. Site conditions spanned a large variety of climates, with average April–May temperatures ranging from 11.6 to 21.9 °C. At these particular arboreta and gardens, all sampled plants were under the care of arboretum and botanical garden staff, and were offered optimal amounts of water and

Table 2
Stable carbon isotope composition of pollen, leaf, and stem tissues from gymnosperm plants

Plant species	Common name	Pollen $\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	Stem $\delta^{13}C$ (‰)	Site[a] collected
Ginkgophyta					
Family Ginkgoaceae					
Ginkgo biloba	Maidenhair tree	−25.59	−25.54	−26.43	MA
Coniferophyta					
Family Cupressaceae					
Cupressus arizonica	Arizona cypress	−25.33	−27.14	−25.50	BTA
Cupressus sempervirens	Italian cypress	−24.63	−25.26	−23.16	BTA
Juniperus scopulorum	Rocky mountain juniper	−23.54	−25.26	−23.35	BTA
Family Pinaceae					
Abies amabilis	Pacific silver fir	−26.42	−26.73	−25.53	MA
Abies bracteata	Bristlecone fir	−25.87	−29.83	−27.13	MA
Abies bracteata	Bristlecone fir	−25.54	−28.26	−24.90	NA
Abies concolor	White fir	−28.16	−28.50	−27.37	MA
Abies lasiocarpa	Subalpine fir	−28.25	−28.45	−27.39	MA
Cedrus atlantica	Atlas cedar	−31.00	−31.14	−29.18	NA
Cedrus libani	Cedar of Lebanon	−27.12	−31.09	−28.32	MA
Larix decidua	European larch	−27.21	−28.06	−27.24	WPA
Larix kaempferi	Japanese larch	−26.38	−26.99	−25.88	MA
Picea abies	Norway spruce	−26.78	−28.72	−26.09	NA
Picea abies	Norway spruce	−28.29	−27.03	−26.31	WPA
Picea glauca	White spruce	−25.96	−26.02	−24.97	NA
Picea mariana	Black spruce	−25.86	−26.87	−24.69	MA
Picea sitchensis	Sitka spruce	−25.88	−27.43	−25.23	MA
Picea sitchensis	Sitka spruce	−27.81	−27.35	−27.47	NA
Pinus brutia	Eldarica pine	−28.70	−27.57	−26.20	BTA
Pinus brutia	Eldarica pine	−28.40	−28.53	−25.68	ASU
Pinus canariensis	Canary Island pine	−24.24	−24.53	−24.09	ASU
Pinus echinata	Shortleaf pine	−30.20	−30.01	−28.32	ATH
Pinus eldarica	Afghan pine	−25.08	−24.83	−23.89	TBG
Pinus mugo	Mugo pine	−27.38	−30.55	−29.00	NA
Pinus pinea	Italian stone pine	−28.00	−26.28	−26.40	BTA
Pinus resinosa	Red pine	−26.78	−29.19	−28.64	MA
Pinus sylvestris	Scotch pine	−28.58	−30.45	−29.52	NA
Pinus taeda	Loblolly pine	−32.34	−30.39	−29.45	ATL
Pinus thunbergiana	Japanese black pine	−27.19	−25.64	−25.12	BTA
Pinus thunbergiana	Japanese black pine	−27.10	−27.14	−27.00	NA
Tsuga canadensis	Eastern hemlock	−28.27	−26.56	−26.50	ATH
Family Taxaceae					
Torreya taxifolia	Florida nutmeg	−27.28	−27.64	−26.21	ATH
Family Taxodiaceae					
Cryptomeria japonica	Japanese cedar	−26.17	−26.82	−27.11	JHU

[a] Site abbreviation corresponds to locations described in Table 1 (same convention applies to Tables 3–5 and 7).

Table 3
Stable carbon isotope composition of pollen, leaf, and stem tissues from Rosid Angiosperm plants

Plant species	Common name	Pollen $\delta^{13}C$ (‰)	Leaf $\delta^{13}C$ (‰)	Stem $\delta^{13}C$ (‰)	Site collected
Magnoliopsida (Dicotyledons)					
Rosid Clade					
Family Aceraceae					
Acer buergeranum	Trident maple	−27.18	−28.78	−29.76	ATH
Acer ginnala	Amur maple	−27.09	−28.41	−27.46	ATH
Acer palmatum	Japanese maple	−26.55	−28.62	−28.93	ATH
Family Anacardiaceae					
Pistacia chinensis	Chinese pistachio	−24.76	−27.92	−25.30	BTA
Pistacia vera	Pistachio nut	−26.94	−25.80	−25.53	BTA
Family Betulaceae					
Betula alleghaniensis	Yellow birch	−24.27	−28.49	−27.95	WPA
Betula borealis	Northern birch	−27.41	−26.34	−28.47	WPA
Betula nigra	River birch	−29.57	−30.01	−29.61	ATH
Betula nigra	River birch	−28.70	−26.09	−28.91	ATL
Betula papyrifera	Paper birch	−26.91	−28.34	−29.25	MA
Betula platyphylla	Asian white birch	−29.70	−28.95	−29.52	WPA
Betula uber	Virginia roundleaf birch	−26.27	−26.07	−27.00	ATL
Betula uber	Virginia roundleaf birch	−28.30	−27.41	−28.96	MA
Family Casuarinaceae					
Casuarina cunninghamiana	River sheoak	−25.70	−27.52	−26.78	BTA
Family Crossosomataceae					
Crossosoma bigelovii	Ragged rockflower	−24.59	−25.34	−25.79	BTA
Family Fabaceae					
Acacia berlandieri	Guajillo	−24.59	−27.65	−27.10	BTA
Acacia farnesiana	Sweet acacia	−26.14	−27.97	−29.51	BTA
Acacia greggii	Catclaw acacia	−25.54	−27.13	−27.82	ASU
Acacia redolens	Bank catclaw	−24.71	−26.38	−26.09	BTA
Acacia roemeriana	Roundflower catclaw	−24.51	−25.59	−25.92	BTA
Acacia schaffneri	Schaffner's wattle	−24.89	−26.45	−27.49	BTA
Bauhinia tomentosa	St. Thomas tree	−25.63	−27.82	−26.82	TBG
Bauhinia variegata	Mountain ebony	−28.69	−28.84	−27.80	ASU
Caesalpinia gilliesii	Bird-of-paradise shrub	−23.75	−25.87	−26.75	BTA
Calliandra eriophylla	Fairyduster	−23.74	−27.67	−26.87	BTA
Gleditsia triacanthos	Honeylocust	−25.70	−26.62	−26.05	ASU
Parkinsonia florida	Blue paloverde	−26.53	−25.48	−28.07	ASU
Prosopis glandulosa	Honey mesquite	−24.85	−25.74	−26.58	BTA
Prosopis velutina	Velvet mesquite	−26.83	−25.34	−26.79	TBG
Sophora secundiflora	Mescal bean	−24.12	−27.01	−26.16	BTA
Family Fagaceae					
Fagus grandifolia	American beech	−28.55	−27.33	−28.38	JHU
Quercus alba	White oak	−28.38	−26.49	−27.43	MA
Quercus bicolor	Swamp white oak	−30.68	−27.63	−28.56	MA
Quercus falcata	Southern red oak	−25.74	−24.70	−27.15	ATH
Quercus lyrata	Overcup oak	−28.15	−25.90	−28.38	MA
Quercus macrocarpa	Bur oak	−29.50	−27.68	−28.40	MA
Quercus macrocarpa	Bur oak	−28.06	−26.01	−27.70	MIN
Quercus nigra	Water oak	−24.58	−24.88	−25.75	JHU
Quercus palustris	Pin oak	−27.08	−25.67	−26.74	JHU
Quercus phellos	Willow oak	−26.68	−26.14	−26.44	JHU
Quercus prinus	Chestnut oak	−27.39	−25.37	−28.07	MA
Quercus rubra	Northern red oak	−26.31	−25.48	−27.07	JHU
Quercus virginiana	Live oak	−25.69	−26.95	−26.88	BTA

Table 3 (*continued*)

Plant species	Common name	Pollen $\delta^{13}C$ (‰)	Leaf $\delta^{13}C$ (‰)	Stem $\delta^{13}C$ (‰)	Site collected
Family Geraniaceae					
Geranium maculatum	Spotted geranium	−26.47	−28.41	−28.40	MIN
Geranium sanguineum	Bloody geranium	−28.38	−25.93	−25.89	ATL
Family Hippocastanaceae					
Aesculus pavia	Red buckeye	−28.65	−30.18	−28.88	ATH
Aesculus sylvatica	Painted buckeye	−30.39	−28.85	−30.23	ATL
Family Juglandaceae					
Carya alba	Mockernut hickory	−26.69	−26.74	−29.04	NA
Pterocarya stenoptera	Chinese wingnut	−27.11	−27.77	−26.99	WPA
Family Malvaceae					
Sphaeralcea ambigua	Desert globemallow	−25.04	−27.59	−28.83	BTA
Family Myrtaceae					
Callistemon rigidus	Bottlebrush	−25.74	−27.61	−27.50	ASU
Eucalyptus camaldulensis	Red river gum	−27.68	−28.31	−26.66	ASU
Eucalyptus citriodora	Lemonscented gum	−25.12	−25.95	−26.22	BTA
Family Onagraceae					
Calylophus berlandieri	Berlandier's sundrops	−28.96	−28.15	−28.19	BTA
Calylophus hartwegii	Hartweg's sundrops	−27.63	−27.96	−28.17	BTA
Family Oxalidaceae					
Oxalis hirta	Tropical woodsorrel	−28.34	−29.92	−29.07	ATL
Family Punicaceae					
Punica granatum	Pomegranate	−27.52	−28.11	−27.81	ASU
Family Rosaceae					
Amelanchier arborea	Downy serviceberry	−24.22	−25.35	−25.08	ATH
Chaenomeles speciosa	Flowering quince	−27.81	−28.39	−27.81	NCA
Crataegus viridis	Green hawthorne	−27.13	−28.82	−26.76	ATL
Potentilla arguta	Tall cinquefoil	−28.65	−28.56	−27.89	MA
Prunus laurocerasus	Cherry laurel	−28.24	−29.68	−27.86	ATL
Prunus serotina	Black cherry	−26.80	−28.02	−27.00	ATH
Prunus serotina	Black cherry	−26.26	−24.86	−27.74	JHU
Prunus subhirtella	Eureka weeping	−29.58	−29.97	−30.06	WPA
Pyracantha coccinea	Scarlet firethorn	−26.23	−26.76	−25.32	BTA
Rhodotypos scandens	Jetbead	−27.63	−25.69	−27.49	MA
Family Rutaceae					
Geijera parviflora	Australian willow	−23.39	−26.80	−24.64	BTA
Poncirus trifoliata	Hardy-orange	−25.23	−24.89	−27.60	ATH
Family Simmondsiaceae					
Simmondsia chinensis	Jojoba	−25.91	−27.71	−25.19	BTA
Family Staphyleaceae					
Staphylea bolanderi	Sierra bladdernut	−28.97	−29.20	−26.98	WPA
Staphylea trifolia	American bladdernut	−25.92	−26.35	−28.52	ATH
Staphylea trifolia	American bladdernut	−28.20	−27.77	−27.63	MA
Family Ulmaceae					
Celtis occidentalis	Common hackberry	−26.71	−25.36	−26.30	JHU
Family Zygophyllaceae					
Larrea tridentata	Creosote bush	−23.00	−26.35	−26.32	BTA

fertilizer, and were shielded from all forms of stress.

Samples of pollen, leaf, and stem tissue were taken from 174 different species of C3 plants and from 1 species of C4 plant. Gymnosperms (usually conifers) comprised 27 of the C3 species (Table 2), while angiosperms comprised the other species sampled. *Ginko biloba* of the Ginkgoaceae was sampled, while

Table 4
Stable carbon isotope composition of pollen, leaf, and stem tissues from Asterid Angiosperm plants

Plant species	Common name	Pollen $\delta^{13}C$ (‰)	Leaf $\delta^{13}C$ (‰)	Stem $\delta^{13}C$ (‰)	Site collected
Magnoliopsida (Dicotyledons)					
Asterid Clade					
Family Acanthaceae					
Justicia brandegeeana	Shrimpplant	−23.15	−24.47	−26.29	TBG
Justicia californica	Beloperone	−26.34	−26.46	−28.02	TBG
Justicia spicigera	Mohintli	−27.65	−28.20	−28.30	TBG
Ruellia occidentalis	Western wild petunia	−23.63	−26.73	−26.49	BTA
Family Apiaceae					
Zizia aurea	Golden zizia	−29.57	−27.88	−27.38	MIN
Family Aquifoliaceae					
Ilex myrtifolia	Myrtle dahoon	−26.43	−28.29	−27.47	ATH
Ilex vomitoria	Yaupon holly	−26.31	−27.37	−26.81	ATL
Family Asteraceae					
Ambrosia deltoidea	Triangle burr ragweed	−27.19	−26.64	−28.49	BTA
Baileya multiradiata	Desert marigold	−26.67	−27.83	−27.04	BTA
Echinacea purpurea	Eastern purple coneflower	−28.01	−28.49	−28.45	MA
Gaillardia spathulata	Western blanketflower	−28.39	−28.72	−28.59	TBG
Osteospermum ecklonis	Blue and white daisybush	−26.73	−27.81	−26.84	BTA
Packera aurea	Golden ragwort	−30.78	−31.35	−30.16	ATH
Viguiera parishii	Parish's goldeneye	−26.28	−28.52	−28.08	TBG
Family Bignoniaceae					
Bignonia capreolata	Crossvine	−28.23	−30.69	−28.73	ATL
Chilopsis linearis	Desert willow	−26.86	−27.71	−27.90	ASU
Tecoma stans	Yellow trumpet bush	−25.66	−25.65	−27.91	TBG
Family Boraginaceae					
Cordia parviflora	Small-leaf geigertree	−24.87	−25.39	−25.37	BTA
Mertensia virginica	Virginia bluebells	−30.71	−31.75	−30.65	MIN
Family Caprifoliaceae					
Lonicera maackii	Amur honeysuckle	−23.72	−24.47	−24.93	BTA
Viburnum acerifolium	Mapleleaf viburnum	−26.88	−28.45	−26.75	MA
Viburnum dilatatum	Linden arrowwood	−25.97	−25.76	−27.17	ATH
Viburnum obovatum	Small-leaf arrowwood	−24.19	−25.55	−26.65	ATH
Viburnum plicatum	Japanese snowball	−28.76	−28.88	−29.71	ATH
Viburnum prunifolium	Blackhaw viburnum	−24.38	−24.33	−27.13	ATH
Viburnum setigerum	Tea viburnum	−24.27	−27.19	−27.78	MA
Viburnum suspensum	Viburnum	−27.85	−27.53	−27.64	MA
Family Cornaceae					
Cornus florida	Flowering dogwood	−25.97	−25.31	−29.53	ATH
Family Ericaceae					
Rhododendron canescens	Sweet mountain azalea	−27.57	−28.22	−29.12	ATH
Rhododendron macrophyllum	Pacific rhododendron	−27.10	−28.01	−27.73	WPA
Family Hydrangeaceae					
Hydrangea quercifolia	Oakleaf hydrangea	−25.01	−25.70	−25.61	MA
Family Lamiaceae					
Salvia clevelandii	Fragrant sage	−26.47	−27.71	−26.42	BTA
Salvia greggii	Autumn sage	−25.98	−27.68	−28.72	ATH
Salvia microphylla	Baby sage	−28.88	−28.10	−28.13	ATL
Teucrium fruticans	Shrubby germander	−24.66	−26.20	−27.73	BTA
Family Lauraceae					
Sassafras albidum	Common sassafras	−27.96	−28.68	−30.28	ATH
Family Myoporaceae					
Eremophila maculata	Spotted emu bush	−25.53	−26.63	−25.68	BTA

Table 4 (*continued*)

Plant species	Common name	Pollen $\delta^{13}C$ (‰)	Leaf $\delta^{13}C$ (‰)	Stem $\delta^{13}C$ (‰)	Site collected
Family Oleaceae					
Forsythia suspensa	Weeping forsythia	−32.22	−30.14	−30.77	WPA
Jasminum officinale	Poet's jasmine	−26.36	−28.53	−27.00	ATL
Osmanthus fragrans	Sweet osmanthus	−22.93	−28.22	−28.13	ATH
Syringa vulgaris	Common lilac	−29.19	−29.74	−30.82	ATL
Syringa vulgaris	Common lilac	−27.13	−27.95	−27.20	MA
Family Pittosporaceae					
Pittosporum confertiflorum	Ho'awa	−23.93	−27.23	−25.56	BTA
Family Polemoniaceae					
Phlox divaricata	Wild blue phlox	−30.64	−30.01	−28.95	ATH
Phlox stolonifera	Creeping phlox	−29.84	−29.28	−28.47	ATH
Family Scrophulariaceae					
Paulownia tomentosa	Princess tree	−29.61	−27.43	−26.96	ATH
Russelia equisetiformis	Fountainbush	−23.35	−26.52	−28.79	TBG
Rehmannia glutinosa	Chinese foxglove	−27.39	−27.37	−25.55	ATL
Family Styracaceae					
Styrax americanus	American snowbell	−27.38	−27.66	−27.81	ATL
Family Theaceae					
Camellia japonica	Camellia	−31.84	−30.36	−29.95	WPA
Family Valerianaceae					
Centranthus ruber	Red valerian	−30.46	−31.02	−29.45	BTA
Family Verbenaceae					
Aloysia gratissima	Whitebrush	−27.39	−26.97	−26.01	BTA

four families of the Coniferophyta (Cupressaceae, Pinaceae, Taxaceae, and Taxodiaceae) were sampled. The Pinaceae were the most extensively sampled of the Coniferophyta, with six genera represented in the study. Within the angiosperms, 70 Rosid (Table 3), 51 Asterid (Table 4), and 27 other angiosperm plants (Table 5) were sampled. This roughly corresponded to the present-day proportions of different types of C3 plants currently on Earth. Fifty-five angiosperm families are represented in the study, with seven families represented by more than seven species (Asteraceae, Betulaceae, Caprifoliaceae, Fabaceae, Fagaceae, Magnoliaceae, and Rosaceae). The study sampled most diversely across the Fabaceae, including tissues from eight different genera.

Tissue and pollen collection was performed during late May because the greatest number of species in the gardens were actively producing pollen during this time. Plants were first identified to species-level using arboreta or garden designations, and confirmed using basic plant guidebooks (Little, 1980a,b; Niering and Olmstead, 1979; Spellenberg, 2001). In order to homogenize the minor isotopic differences that may be present between plant individuals, each sample contained the contribution of at least three plant individuals of the same species. These individuals were often located in very close proximity, since most arboreta and botanical gardens are laid out to showcase subgardens of similar species. Care was taken to sample only stem and leaf tissue that had been produced since the beginning of the most recent spring, thus ensuring that pollen, leaf, and stem samples all represented tissue produced by the species during the same growing season. Leaf and stem tissues were clipped from the selected plants and packed as tightly as possible in 22-ml borosilicate vials. Vials were tightly closed with teflon-sealed caps, labeled, and wrapped in parafilm to secure the caps. Vials containing plant tissues were stored on ice in the field and kept at ~3 °C in the laboratory to prevent microbial decomposition. Upon return to the laboratory, samples were lyophilized for 24 h and then ground to a fine uniform powder in preparation for stable isotope analysis.

Pollen was collected using a Mariotte bottle of distilled, deionized water and thoroughly washing the pollen-bearing stamens or cones (Fig. 1) while collecting the runoff in a 50-ml centrifuge tube.

Table 5
Stable carbon isotope composition of pollen, leaf, and stem tissues from non-Rosid, non-Asterid Angiosperm plants

Plant species	Common name	Pollen $\delta^{13}C$ (‰)	Leaf $\delta^{13}C$ (‰)	Stem $\delta^{13}C$ (‰)	Site collected
Liliopsida (Monocotyledons)					
Family Araceae					
Arisaema triphyllum	Jack in the pulpit	−29.26	−30.28	−29.99	MIN
Chamaerops humilis	European fan palm	−27.57	−27.94	−27.35	ASU
Family Iridaceae					
Sisyrinchium angustifolium	Narrowleaf blue-eyed grass	−26.72	−27.97	−27.61	ATL
Family Liliaceae					
Trillium grandiflorum	Snow trillium	−27.31	−28.04	−27.11	ATH
Trillium grandiflorum	Snow trillium	−28.22	−27.29	−25.63	MIN
Uvularia grandiflora	Largeflower bellwort	−28.93	−28.84	−27.97	MIN
Magnoliopsida (Dicotyledons)					
Family Cactaceae					
Pediocactus simpsonii	Simpson hedgehog cactus	−14.70	−14.14	−12.55	BTA
Family Fumariaceae					
Dicentra eximia	Turkey corn	−27.90	−28.18	−26.90	MIN
Lamprocapnos spectabilis	Bleeding heart	−27.72	−28.38	−27.21	ATL
Family Hamamelidaceae					
Fothergilla gardenii	Dwarf witchalder	−28.00	−27.07	−27.53	ATH
Fothergilla major	Large fothergilla	−29.00	−28.40	−29.66	ATH
Liquidambar styraciflua	Sweetgum	−27.10	−27.03	−28.31	JHU
Family Magnoliaceae					
Liriodendron tulipifera	Tuliptree	−25.76	−26.71	−26.02	JHU
Magnolia acuminata	Cucumber tree magnolia	−27.76	−27.66	−28.90	MIN
Magnolia fraseri	Mountain magnolia	−24.92	−24.53	−28.83	MA
Magnolia grandiflora	Southern magnolia	−28.47	−24.54	−24.46	MA
Magnolia liliflora	Decorative magnolia	−25.63	−25.92	−25.72	ATH
Magnolia portoricensis	Puerto Rico magnolia	−26.95	−26.39	−25.17	MA
Magnolia stellata	Star magnolia	−25.21	−27.54	−26.18	MA
Family Menispermaceae					
Cocculus orbiculatus	Queen coralbead	−26.47	−26.01	−25.48	ASU
Family Nymphaeaceae					
Nymphaea odorata	American white waterlily	−29.14	−26.11	−27.17	JHU
Family Papaveraceae					
Stylophorum diphyllum	Celandine poppy	−30.79	−28.78	−28.28	ATL
Stylophorum diphyllum	Celandine poppy	−27.23	−27.88	−27.57	MIN
Family Polemoniaceae					
Phlox divaricata	Wild blue phlox	−30.64	−30.01	−28.95	ATH
Phlox stolonifera	Creeping phlox	−29.84	−29.28	−28.47	ATH
Family Ranunculaceae					
Aquilegia canadensis	Red columbine	−28.30	−28.36	−27.58	ATL
Aquilegia canadensis	Red columbine	−28.13	−28.54	−28.29	MIN
Aquilegia chrysantha	Golden columbine	−26.84	−28.76	−28.84	TBG
Aquilegia formosa	Western columbine	−26.12	−28.09	−27.81	TBG
Family Saxifragaceae					
Tiarella cordifolia	Heartleaf foamflower	−28.38	−30.01	−28.18	MIN

Tubes were then tightly closed, labeled, and stored on ice until returned to the laboratory. In the laboratory, the contents of each centrifuge tube were decanted into large petri dishes, extensively examined, and any large flower parts (e.g., petals), insects, or other obvious impurities were removed with forceps. Although this step was laborious, it was advantageous since these impurities were much

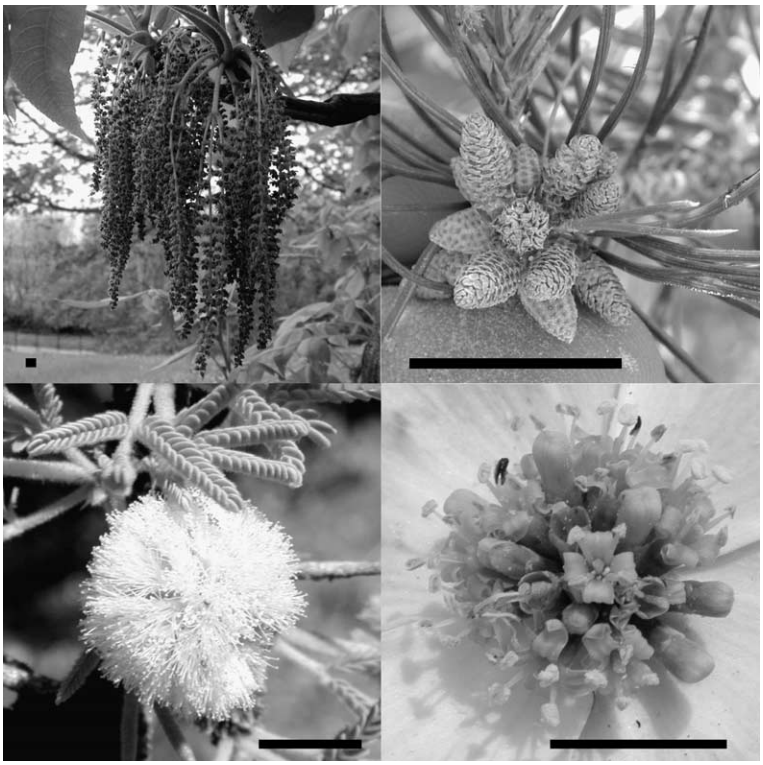

Fig. 1. Light photographs of representative species sampled for pollen, stem, and leaf tissue (scale bars=1 cm). Upper left: staminate catkins of *Carya alba* (Juglandaceae); upper right: microsporangiate strobili of *Pinus eldarica* (Pinaceae); lower left: radial corolla with exserted staminal tubes of *Acacia schaffneri*(Fabaceae); lower right: petals and stamens in the flower of *Cornus florida* (Cornaceae).

easier to remove prior to centrifugation, which tended to compact impurities into the pollen or even break the impurities into finer pieces (e.g., detached insect legs). After large impurities were removed, the liquid was transferred into clean 50-ml centrifuge tubes and centrifuged at high speed for 5 min. For >95% of the samples, this resulted in all solid material settling to the bottom of the centrifuge tube. In these cases, at least 40 ml of the supernatant were discarded, the tube was recapped, and the remaining material was agitated back into suspension. This reduced amount of liquid was subjected to a multiple-sieve technique, which employed stackable micro-mini-sieves with eight different-sized mesh inserts (25, 35, 45, 68, 80, 120, 170 and 230 mesh per linear inch; Fisher Catalog #37846-0000). If centrifugation did not result in complete settling, the entire volume of liquid was put through the stackable sieves. The sieves were stacked with the coarsest mesh at the top, grading to finer mesh toward the bottom, all set within a large petri dish that served as a catch pan. The stacked sieves were thoroughly wetted with distilled, deionized water prior to use. The fluid that collected in the bottom petri dish was transferred to a clean 50-ml centrifuge tube and centrifuged at high speed for 5 min. After centrifugation, pollen either settled to the bottom of the tube or formed a distinct floating layer at the top. In either circumstance, pollen was then transferred to a medium-sized petri dish for visual verification of its purity. The pollen was visually checked for uniformity and purity at low magnification (40×) under a binocular microscope. A random subsample of this material was smeared onto a glass slide and its purity was verified at higher magnification (1000×) using transmitted light microscopy, or alternatively, by Au coating and subsequent scanning electron microscopy (Fig. 2). Once the purity and homogeneity of the pollen was verified, the sample was transferred

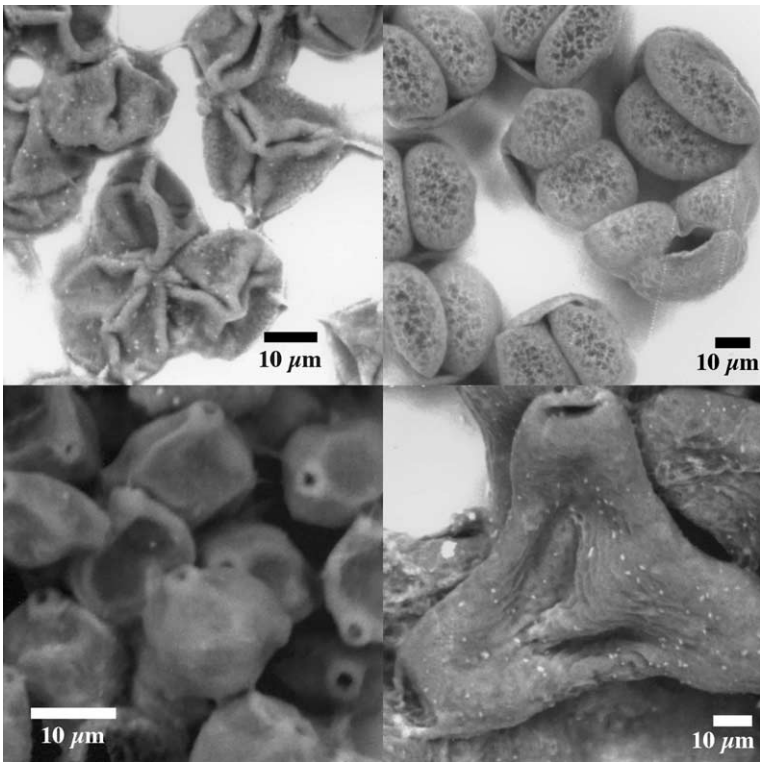

Fig. 2. SEM micrographs of pollen isolated from sampled plants illustrating the purity and homogeneity of the isotopic substrates. Upper left: tricolporate pollen grains of *Camellia japonica* (Theaceae); upper right: *Pinus echinata*(Pinaceae) pollen grains with saccae; lower left: polyporate pollen grains of *Betula nigra* (Betulaceae); lower right: triporate pollen grains of *Calylophus berlandieri* (Onagraceae).

to a 2-ml centrifuge tube and centrifuged at very high speed, then stored at ~3 °C prior to stable isotope analysis. No chemical pretreatment was performed on any of these samples, since the purpose of this portion of the study was to assess the carbon stable isotope relationship between modern pollen and other tissues of mature plants. Note especially that no solvent extraction was performed, as is occasionally employed in order to remove lipids from pollen and spores prior to analysis.

Pollen, leaf, and stem tissues were analyzed for $\delta^{13}C$ value using a Eurovector automated combustion system in conjunction with an Isoprime stable isotope mass spectrometer at the Johns Hopkins University. All samples were introduced to the combustion system in pure tin capsules; pure pollen was transferred to tin capsules by repeatedly injecting the capsules with 25-μl aliquots of pollen suspended in water, and allowing the water to evaporate within a sterile hood. Tables 2–5 present the carbon stable isotope values of pollen, leaf, and stem tissues sampled from all plants involved in the study. Stable isotope values are reported in standard δ-notation: $\delta=(R_{sample}-R_{standard}/R_{standard})1000$ (‰). The reporting standard is Pee Dee Formation limestone (PDB) with $R={}^{13}C/{}^{12}C=0.011237$. Analytical uncertainty associated with each measurement was ±0.05‰. Values presented represented the average of three replicate capsules; the average isotopic variability of replicate capsules was ±0.12‰ for pollen, ±0.13‰ for stem tissue, and ±0.12‰ for leaf tissue. Combustion also resulted in a quantification of %C in each sample: pollen uniformly consisted of 52%C by mass; the average value of stem tissue was 46%C; the average value of leaf tissue was 45%C (all values ±1%C analytical uncertainty).

3. Results

Carbon stable isotope analysis of the samples resulted in 189 pairs of pollen and stem tissue values, and 189 pairs of pollen and leaf tissue values (Fig. 3). Pollen values varied between −32.34‰ and −14.70‰; stem tissue varied between −30.82‰ and −12.55‰; leaf tissue varied between −31.75‰ and −14.14‰ encompassing the entire carbon isotope range first reported for land plants (Smith and Epstein, 1971). Overall, the data formed a cluster very close to the 1:1 line for both the relationship between leaf tissue and pollen, and the relationship between stem tissue and pollen. The carbon isotope difference between the pollen and leaf tissue values in the same species ($\Delta_{pollen-leaf}$) varied between −3.93‰ and +5.29‰ with >90% of data pairs within $\Delta_{pollen-leaf}=\pm 3.00$‰. Similarly, the carbon isotope difference between the pollen and stem tissue values in the same species ($\Delta_{pollen-stem}$) varied between −4.01‰ and +5.44‰ with > 90% of data pairs within $\Delta_{pollen-stem}=\pm 3.00$‰. In order to compare these results to findings for C4 plants and other data sets, Fig. 3 includes 16 pairs of plant tissue and pollen δ^{13}C values determined for 11 C3 and C4 Poaceae species (closed circles) by Amundson et al. (1997). In contrast, $\Delta_{pollen-bulk}$for the Poaceae varied between −6.10‰ and +5.60‰, although as Amundson et al. (1997) mentioned, the Poaceae range decreased somewhat if only pollen and tissue taken from the same plant individual were considered.

In order to further examine the isotopic relationship between C3 pollen and stem and leaf tissue sampled in this study, the data was separated by tracheophyte clade, according to the ruling paradigm of tracheophyte phylogeny based on morphological characters, as well as on *rbcL*, *atpB*, and 18S nuclear ribosomal nucleotide sequences (Fig. 4 and Angiosperm Phylogeny Group, 1998; Pryer et al., 1995). Separation by major clade allowed for the

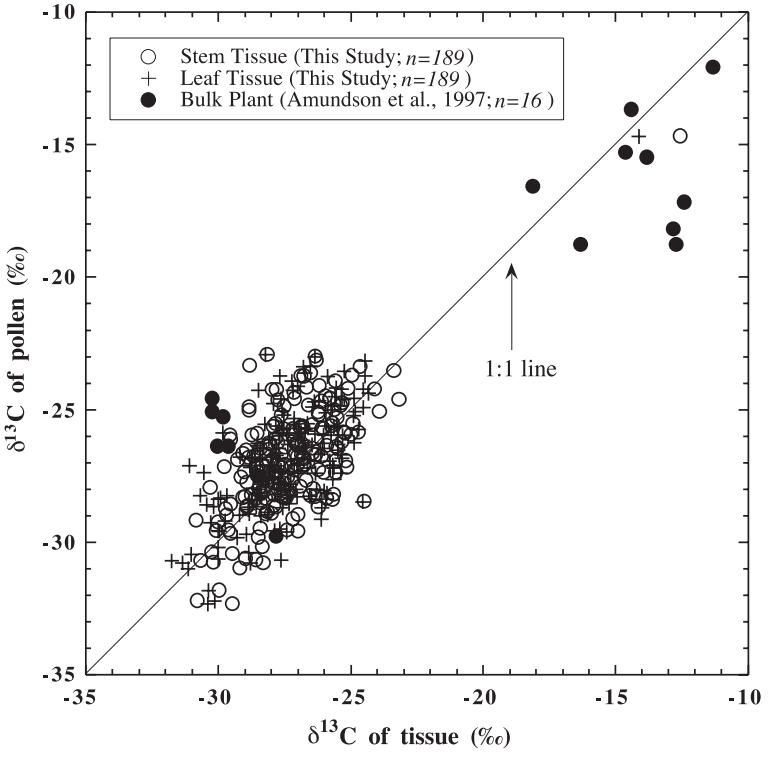

Fig. 3. The relationship between the δ^{13}C value of stem and leaf tissue and that of pollen from 174 different species of C3 plant and from 1 species of C4 plant (189 sets of measurements total; open circles and crosses). Sixteen sets of measurements on Poaceae bulk tissue (usually leaf) and pollen reported by Amundson et al. (1997) are included for comparison (closed circles).

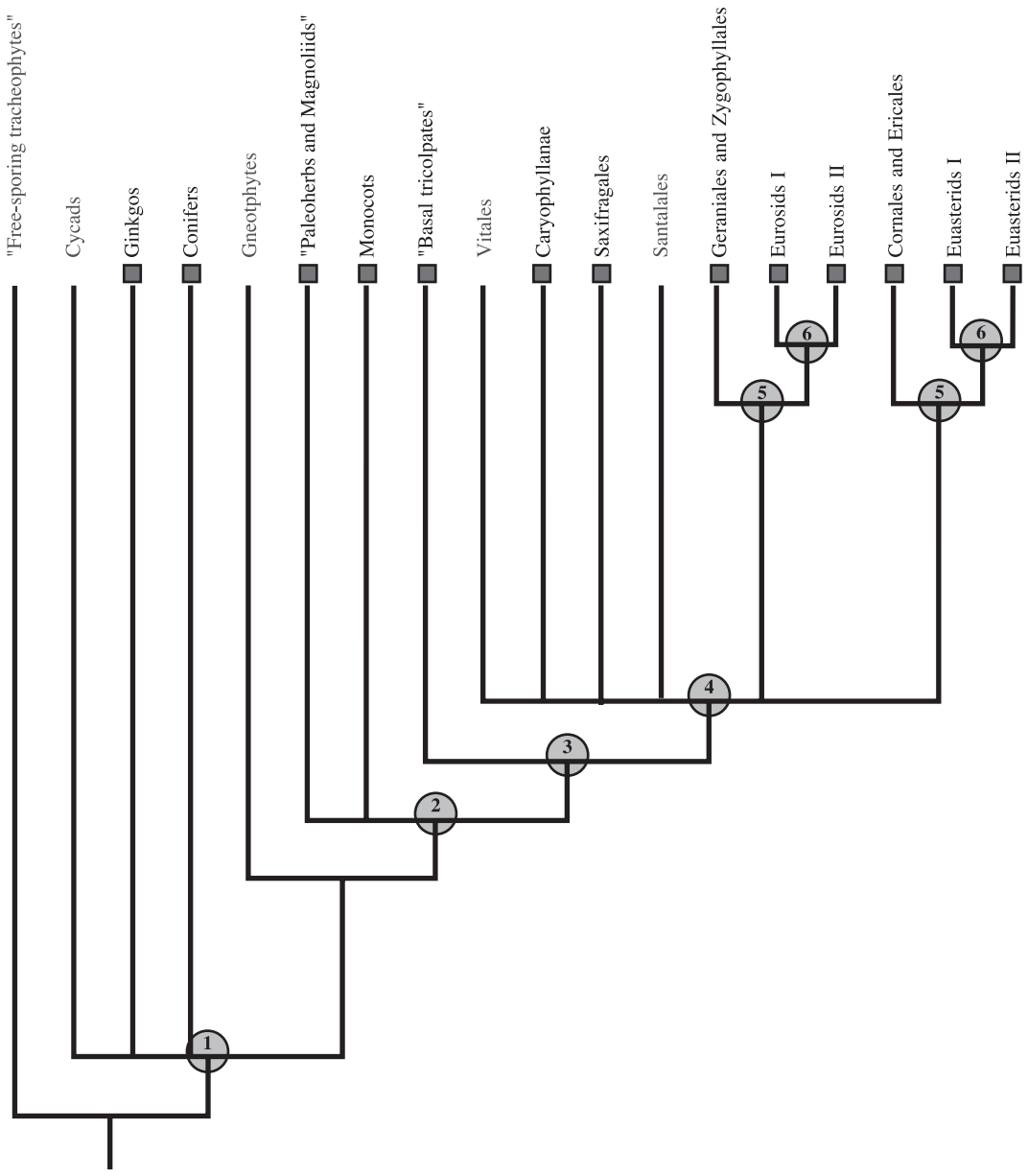

Fig. 4. Simplified diagram of phylogenetic relationships among major groups of tracheophytes, with emphasis on major angiosperm clades. Modified from results based on characters derived from morphology, *rbcL*, *atpB*, 18S nuclear ribosomal nucleotide sequences reported by the Angiosperm Phylogeny Group (1998) and Pryer et al. (1995). Quotation marks indicate that the group, although commonly regarded as a clade (Judd et al., 1999), is actually paraphyletic according to this phylogeny. Clades preceded by a black box were sampled in this study. Nodes are numbered according to the total number of branching points that they are distant from the root; only nodes of differentiation that result in sampled clades were included in this count.

comparison of pollen–tissue relationships at various stages of differentiation during tracheophyte evolution; nodes of Fig. 4 that are branching points that include species sampled in the study are numbered in accordance with their distance to the root of the tree. According to this paradigm, the differentiation

that led to the evolution of the Conifer clade preceded the differentiation that led to the evolution of the Monocot clade. Similarly, the evolution of the Erosid I clade resulted from several more differentiation events than did the evolution of the Conifer clade. By comparing the $\delta^{13}C$ values of pollen, stem and leaf tissue of different major tracheophyte clades, insight may be gained into changing chemical relationships between pollen and bulk plant tissues during the history of plant evolution.

Fig. 5 presents the isotopic relationship between pollen, stem, and leaf tissue in species that resulted from differentiation at the "lower" nodes of tracheophyte phylogeny (i.e., nodes 1, 2, and 3 of Fig. 4). It was notable that in three of these four clades—the

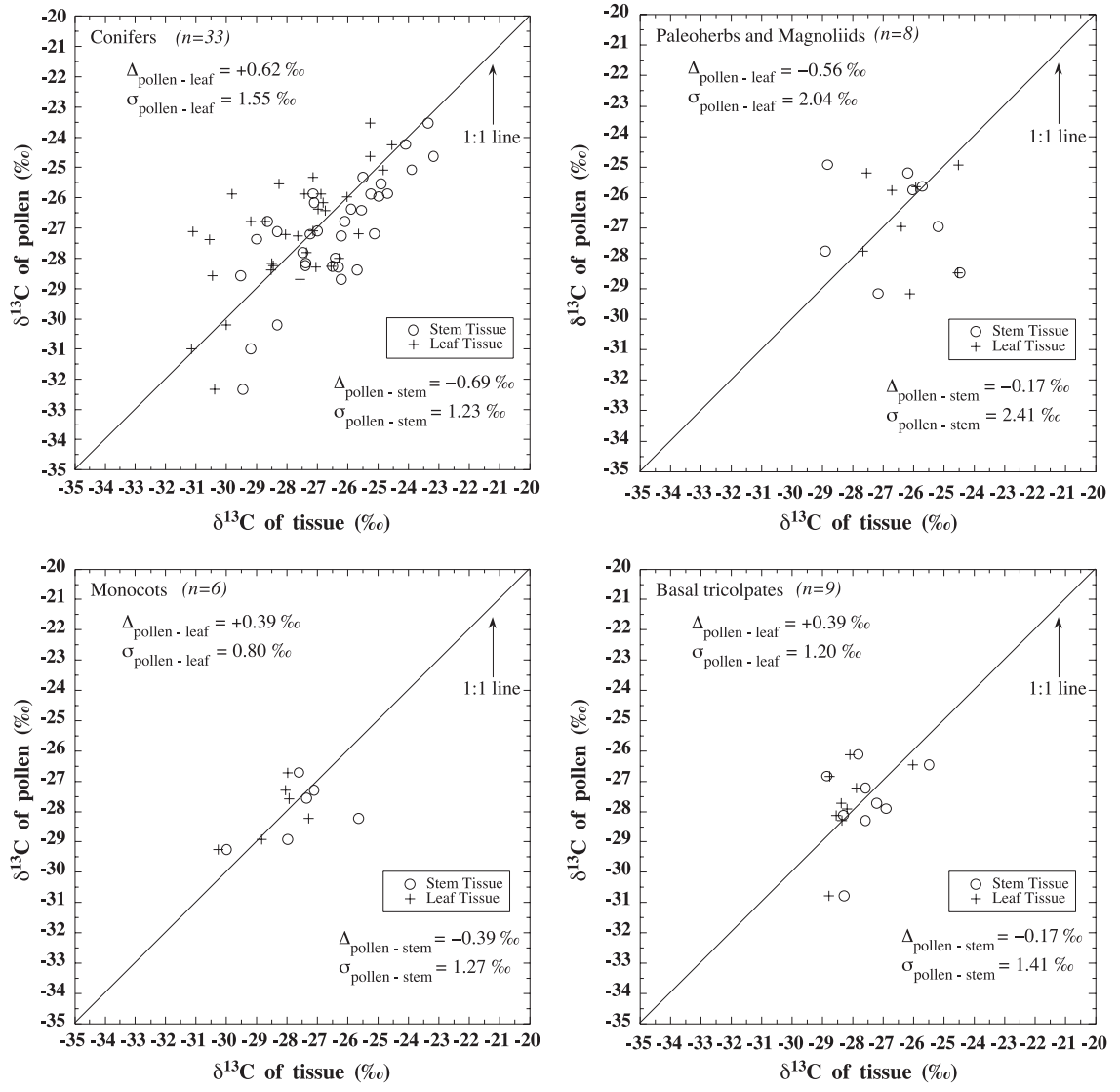

Fig. 5. The relationship between the $\delta^{13}C$ value of stem and leaf tissue and that of pollen from the Conifers, "Paleoherbs and Magnoliids", Monocots and Basal tricolpates major tracheophyte clades. Average distance (Δ) from 1:1 relationship is included, both between stem tissue and pollen, and between leaf tissue and pollen. Also included is the standard deviation (σ) of all distances.

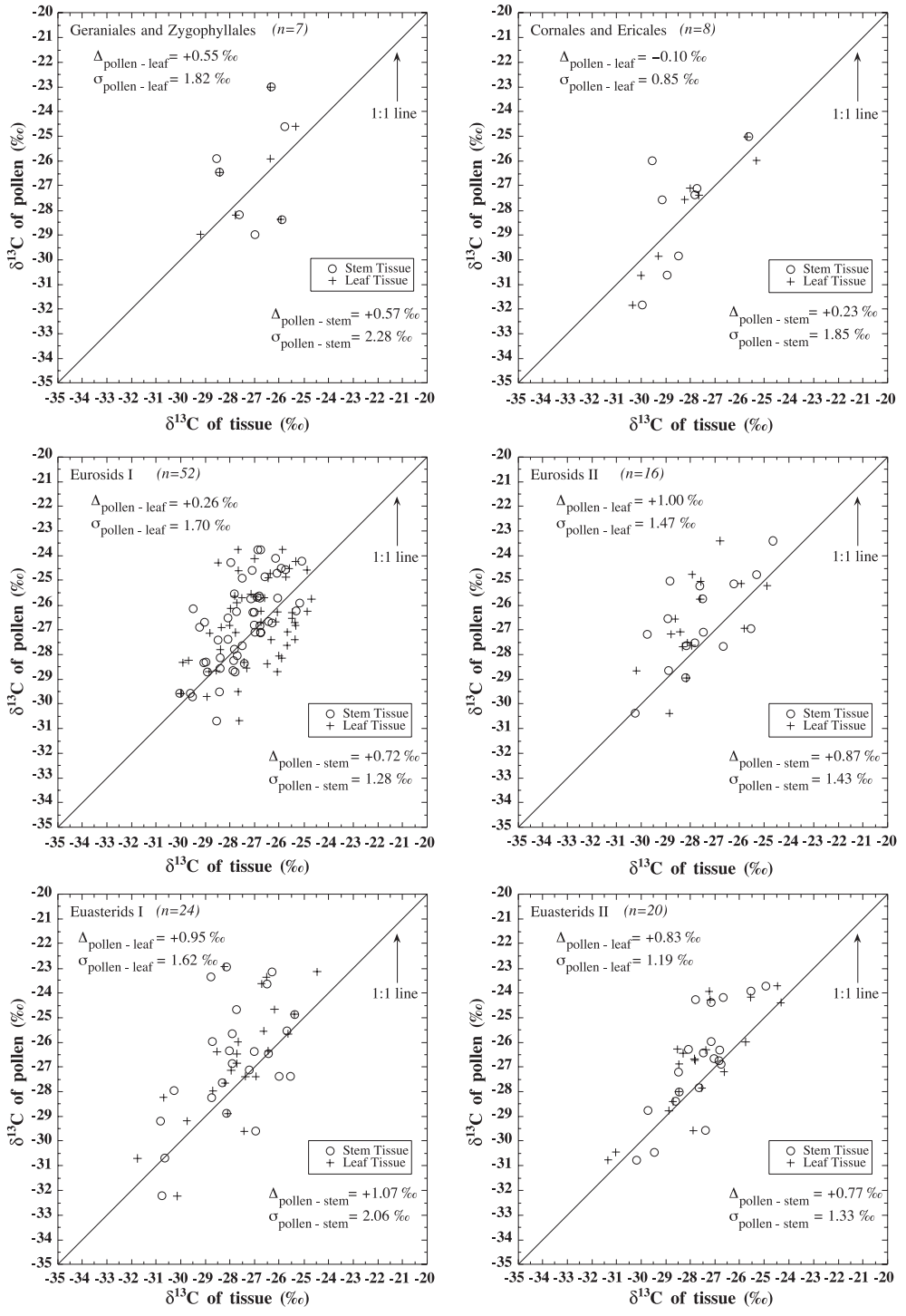

Fig. 6. The relationship between the $\delta^{13}C$ value of stem and leaf tissue and that of pollen from the Geraniales and Zygophyllales, Cornales and Ericales, Eurosids I and II, and Euasterids I and II major tracheophyte clades. Average distance (Δ) from 1:1 relationship is included, both between stem tissue and pollen, and between leaf tissue and pollen. Also included is the standard deviation (σ) of all distances.

Conifers, Monocots, and Basal tricolpates clades—the mean value of $\Delta_{pollen-leaf}$ was >0, while the mean value of $\Delta_{pollen-stem}$ was <0. In each of these clades, the standard deviation (σ) seen in calculated Δ values was low to average compared to all other data of the study ($\sigma_{pollen-leaf}$=0.80–1.55‰ and $\sigma_{pollen-stem}$=1.23–1.41‰). This meant that within these clades, leaf tissue had a lower isotopic value (i.e., was less enriched) than pollen, and that stem tissue had a higher isotopic value (i.e., was more enriched) than pollen, on average. In contrast, Fig. 6 presents the isotopic relationship between pollen, stem, and leaf tissue in species that resulted from differentiation at the "higher" nodes of tracheophyte phylogeny (i.e., nodes 4, 5, and 6 of Fig. 4). In five of these clades—the Geraniales and Zygophyllales, Eurosids I and II, Euasterids I and II—the mean value of $\Delta_{pollen-leaf}$ and $\Delta_{pollen-stem}$ were both >0. Therefore, both leaf and stem tissues within these clades had a lower isotopic value (i.e., were isotopically lighter) than pollen, on average. In each of the clades discussed above, the standard deviation (σ) seen in calculated Δ values was low to average compared to all other data of the study ($\sigma_{pollen-leaf}$=1.19–1.82‰ and $\sigma_{pollen-stem}$=1.28–2.28‰).

Two of the ten clades studied proved exceptions to these overall trends: within the Paleoherbs and Magnoliids clade, standard deviation (σ) of both $\Delta_{pollen-leaf}$ and $\Delta_{pollen-stem}$ was very high (σ>2 in both cases) illustrating the relative lack of correlation between the isotopic composition of all tissues in this particular clade. Conversely, the Cornales and Ericales clade showed very high correlation between the isotopic composition of pollen and leaf tissue, as well as between pollen and stem tissue. The resulting data for this clade diverged very little from the 1:1 line (Fig. 6) and exhibited low to average standard deviation (σ) of both $\Delta_{pollen-leaf}$ (σ=0.85‰) and $\Delta_{pollen-stem}$ (σ=1.85‰).

4. Discussion

4.1. Isotopic differences between stem, leaf, and pollen tissue

The above trends in $\Delta_{pollen-leaf}$ and $\Delta_{pollen-stem}$ can be explained in terms of differences in plant tissue composition across the clades sampled. Stem and leaf tissue are primarily composed of polysaccharides (65–90% by mass), lignin (9–30% by mass), lipids (1–5% by mass), and various secondary compounds, including secondary metabolites and amino acids ($\ll$1% by mass). In contrast, all pollen is composed primarily of sporopollenin (Brooks and Shaw, 1968), which is based on long-chain alkyl moieties in combination with aromatic units including ester- and ether-bound cinnamic acids, such as *p*-coumaric acid (van Bergen et al., 1993, 1995; Wehling et al., 1989). It has long been known that the major components of plant tissues can have widely different carbon isotope signatures. Cellulose, the main polysaccharide found in plant tissues, is enriched in ^{13}C relative to bulk plant tissue (O'Leary, 1988): $\delta^{13}C$ values up to 4‰ higher than bulk tissue have been reported for isolated cellulose (Marino and McElroy, 1991). In contrast, lignin has been found to have lower $\delta^{13}C$ value than the bulk tissue from which it was isolated: $\delta^{13}C$ values up to 4.2‰ lower than bulk tissue have been reported for isolated lignin (Benner et al., 1987). Although lignin is often associated with wood, both green stems and leaves also contain significant amounts of lignin. Lipids are among the isotopically lightest compounds found in plant tissues: Values of mixed plant lipids were found to range from 5‰ to 10‰ less than bulk tissue $\delta^{13}C$ values (Chikaraishi and Naraoka, 2003; Freeman and Collarusso, 2001; Hobbie and Werner, 2004; Park and Epstein, 1961; Rieley et al., 1991). Lipids perform diverse functions within plants; lipids are used for defense and energy storage, as well as for the construction of membranes. In addition, epicuticular waxes are composed of various lipids; the relatively low carbon isotope value of these lipids contributes to the general observation that $\delta^{13}C_{leaf}<\delta^{13}C_{stem}$ within a plant. Plant internal processes contribute as well: leaf cellulose has been shown to have $\delta^{13}C$ values 2–4‰ lower than stem cellulose in the same plant (Leavitt and Long, 1982), indicating that biochemical processes at the site of synthesis may play a role. The general trend that $\delta^{13}C_{leaf}<\delta^{13}C_{stem}$ was evidenced in this dataset: average $\Delta_{leaf-stem}$=−0.12‰ (n=189; σ=1.38‰).

The previous section reported that species of the "lower" nodes of tracheophyte phylogeny showed $\delta^{13}C_{leaf}<\delta^{13}C_{pollen}<\delta^{13}C_{stem}$ while species of the

"higher" nodes showed $\delta^{13}C_{leaf}<\delta^{13}C_{pollen}$ and $\delta^{13}C_{stem}<\delta^{13}C_{pollen}$. This is best explained by the observation that bark-covered woody stems dominated the species of the "lower" nodes (all the Coniferophyta, as well as the *Magnolia* spp.), whereas species of the "higher" nodes (both trees and herbs) were dominated by green flexible stems. Large carbon isotope differences (up to 6‰) have been observed when comparing lipids from shade and sun tissues in living plants; these differences were attributed to enhanced maintenance of protective lipids and/or enhanced photosynthetic rates in sun-adapted tissues (Lockheart et al., 1997). From this, it might be expected that green stems would have lower carbon isotope values, on average, than woody stems since all green plant tissues are capable of photosynthesis. The relatively low $\delta^{13}C$ values of the green stem tissue of the "higher" nodes resulted in the $\delta^{13}C$ value of pollen being greater than that of both leaf and stem tissues measured in these species.

4.2. Dependence of pollen $\delta^{13}C$ value on site temperature

Recent work by Loader and Hemming (2001) showed that the $\delta^{13}C$ value of *Pinus sylvestris* pollen was highly correlated with mean annual temperature, particularly when organisms with a similarly timed period of pollen development were compared. Samples of modern *P. sylvestris* pollen from 28 sites throughout Europe exhibited the following relationship with site temperature: $\delta^{13}C_{pollen}=0.61\times DMT-34.21$ ($R^2=0.68$), where DMT was the developmental period temperature (°C). The sampling plan for this study allowed for the comparison of pollen $\delta^{13}C$ values from five conifer species and nine angiosperm species produced under two different values of DMT; only 9 of the 14 pairs were more isotopically different than measured within-site $\delta^{13}C$ variability (=±0.25‰). Of these nine pairs, five pairs exhibited increasing $\delta^{13}C$ value of pollen with increasing DMT, similar to the relationship determined by Loader and Hemming (2001). Interestingly, four pairs exhibited decreasing $\delta^{13}C$ value of pollen with increasing DMT. These results suggested that in many tracheophyte species, the carbon isotope composition of pollen was not straightforwardly controlled by environmental temperature, and that the strong positive correlation between $\delta^{13}C_{pollen}$ and DMT reported by Loader and Hemming (2001) for *P. sylvestris* may be specific to that species or applicable only to similarly widespread conifers.

One might expect temperature to be negatively correlated with plant tissue $\delta^{13}C$ value based on the observation that as environmental temperature increases, vapor pressure deficit also increases, further provoking an increase in stomatal conductance. An increase in stomatal conductance could, in turn, cause the ratio of intercellular to atmospheric pCO_2 to increase. An increase in the amount of intercellular CO_2 relative to atmospheric CO_2 could affect the $\delta^{13}C$ value of plant tissue according to Farquhar et al. (1980), which described carbon assimilation in C3 plants in the following way:

$$\delta^{13}C_i = \delta^{13}C_a - a - (b-a)c_i/c_a \quad (1)$$

The above description has been established as the dominant conceptual model of carbon isotope fractionation during C3 photosynthesis (Farquhar et al., 1989). Within Eq. (1), $\delta^{13}C_i$ is the isotopic composition of plant tissue derived from C3-photosynthetic carbon assimilation, $\delta^{13}C_a$ is the composition of the atmospheric CO_2 (global average= −7.85‰; Ciais et al., 1995); a is the isotopic discrimination dominated by a simple diffusivity comparison of $\delta^{13}CO_2$ vs. $\delta^{12}CO_2$ in air (=4.4‰; Craig, 1953) and does not depend on stomatal density or conductivity; b is the isotopic discrimination imparted during carboxylation (=27‰; O'Leary, 1988; O'Leary, 1989) mainly through the initial carbon-fixation enzyme in C3 plants, RuBisCO; and c_i/c_a is the ratio of intercellular to atmospheric pCO_2 expressed in parts per million. Eq. (1) predicts that when stomatal conductance is high relative to CO_2-fixation capacity, c_i/c_a is high and $\delta^{13}C_i$ tends toward lower values. Validation of this relationship between $\delta^{13}C_i$ and c_i/c_a has been gained from laboratory studies that correlated gas exchange measurements with leaf $\delta^{13}C_i$ values and short-term fractionation measurements (Bowman et al., 1989; Brugnoli et al., 1988). However, validation of a relationship between vapor pressure deficit and the stable isotope composition of plant tissues has not been straightforwardly evidenced by field experiments (e.g., Day, 2000; McDowell et al., 2004; Mora and Jahren, 2003).

4.3. Effects of chemical preparation on fossil pollen $\delta^{13}C$ value

Recent studies have detailed a progressive alteration in the chemical composition of fossil organic matter through geologic time (Briggs et al., 2000) and the potential effects of diagenesis on carbon stable isotope composition (Poole et al., 2002; van Bergen and Poole, 2002). Fossil and modern pollen is mainly composed of sporopollenin, a polymer that is extremely resistant to alteration; as such, pollen emerges as an ideal organic substrate for stable isotope analysis. However, even sporopollenin has been reported to alter chemically as the result of diagenesis (van Bergen et al., 1993), implying that the geologically younger the substrate, the more likely that the pollen $\delta^{13}C$ value has not been altered. Unfortunately, the isolation and examination of fossil pollen from geologic sediments often employs extensive chemical preparation techniques, which are designed to remove other organic matter through oxidation. Quaternary pollen is often prepared for examination using acetic acid and/or acetic anhydride (e.g., Bush et al., 1992; Bush and Rivera, 1998). Amundson et al. (1997) demonstrated that these acetolysis techniques generally decrease the $\delta^{13}C$ value of Poaceae pollen, often by as much as 5‰. Loader and Hemming (2000) improved upon this by developing an acid-digestion technique that avoids carbon-bearing compounds in favor of concentrated sulfuric acid. This method was documented to lower *Pinus sylvestris*, *Populus tricocarpa*, and *Zea mays* pollen $\delta^{13}C$ values by an average value of 2.5‰ (Loader and Hemming, 2000) and was employed in the investigation of temperature-dependency in *P. sylvestris* pollen $\delta^{13}C$ value (Loader and Hemming, 2001) discussed in the previous section.

Studies that involve sediments older than Quaternary age commonly employ the dissolution of the rock matrix in hydrofluoric acid, followed by digestion in hydrochloric acid to remove fluorite (CaF_2), followed by maceration in Schulze's solution (composed of equal parts of 70% HNO_3 and a saturated solution of $KClO_3$). In addition, an additional digestion in hydrochloric acid is sometimes used to start the preparation process. These steps, hereafter referred to in total as "Treatment 1", have been extensively used to extract pollen from Phanerozoic rocks (e.g., Eble, 2002; Nichols, 2002). For this reason, it is important to assess the effect of these treatments on the carbon stable isotope composition of pollen. Towards this, four species of Pinaceae pollen were collected in particular abundance during the course of this study: *Cedrus deodara* from the Johns Hopkins University campus, *Pinus coulteri* and *P. rigida* from the United States National Arboretum and *Pinus echinata* from the State Botanical Garden of Georgia. Aliquots of modern pollen that had been purified using the micro-sieve methods described earlier in this paper were subjected to the three chemical treatments listed in Table 6. One of the treatments was "Treatment 1" described above, while the other two involved oxidation in H_2O_2 and NaOH, two chemicals commonly employed to simulate diagenesis in chemical alteration studies (Jahren et al., 1997). However, it should be noted that chemical treatment of modern samples has been shown to inadequately mimic the actual fossilization process (Bierstedt et al., 1998), and therefore the

Table 6
Change in pollen carbon isotope composition resulting from chemical treatment

Treatment	$\Delta\delta^{13}C$ *Cedrus deodara* (‰)	$\Delta\delta^{13}C$ *Pinus coulteri* (‰)	$\Delta\delta^{13}C$ *Pinus echinata* (‰)	$\Delta\delta^{13}C$ *Pinus rigida* (‰)	Mean $\Delta\delta^{13}C$ (‰)
24 h in 30% H_2O_2	−0.15	−0.17	−0.42	−0.68	−0.36
24 h in 17% NaOH	+0.79	+1.22	+1.55	+0.96	+1.13
"Treatment 1"	−0.11	−0.04	−0.26	−0.52	−0.23
24 h in 49% HF					
24 h in 1 M HCl					
24 h in Schulze's solution[a]					

[a] Schulze's solution is composed of equal parts of 70% HNO_3 and a saturated solution of $KClO_3$.

substrates produced by these treatments represent only an imperfect effort to roughly simulate the effects of diagenesis.

For each of the three treatments, 5 mg of pure, concentrated pollen from each of the four species was transferred to a 50-ml polypropylene centrifuge tube and subjected to various solutions within a fume hood rated for HF. All reactions were carried out at 23 °C for 24 h. Specifically, "Treatment 1" involved the addition of 10 ml of 49% hydrofluoric acid to each sample, followed by gentle stirring, and then being allowed to sit, loosely covered, for 24 h. Afterwards, each tube was centrifuged at low speed (1000 rpm) for 10 min, washed six times with 45-ml aliquots of deionized water; each sample was centrifuged and the supernatant was decanted after each wash. Next, 10 ml of 1 M HCl were added to each sample, again followed by gentle stirring, and being allowed to sit, loosely covered, for 24 h. As with the previous step, each tube was centrifuged at low speed for 10 min, but washed only three times with 45-ml aliquots of deionized water; each sample was centrifuged and the supernatant was decanted after each wash. In the final step, the samples were transferred to glass centrifuge tubes and 10 ml of Schulze's solution were added to each sample. This was followed by gentle stirring, and the samples were then allowed to sit, loosely covered, for 24 h. Afterwards, the samples were centrifuged at low speed for 10 min, and the reaction was stopped by washing three times with 45-ml aliquots of deionized water, centrifuging and decanting the supernatant after each wash. In the second chemical treatment, 10 ml of 30% H_2O_2 were added to each 5-mg aliquot of pure, concentrated pollen. In keeping with the other procedures, this was followed by gentle stirring, and each sample was then allowed to sit, loosely covered, for 24 h; the samples were then centrifuged at low speed for 10 min, and washed three times with 45-ml aliquots of deionized water, centrifuging and decanting supernatant after each wash. In the third chemical treatment, 10 ml of 17% NaOH were added to each 5-mg aliquot of pure, concentrated pollen. This was followed by gentle stirring, and each sample was afterwards allowed to sit, loosely covered, for 24 h; the samples were then centrifuged at low speed for 10 min, and washed three times with 45-ml aliquots of deionized water, centrifuging, and decanting the supernatant after each wash. In all cases, the substrate resulting from the chemical treatment was prepared for isotopic analysis via transfer to preweighed pure tin capsules using 25 μl glass pipettes and desiccated in a 100 °C drying oven. When fully dry, capsules were crimped shut, reweighed, and analyzed for $\delta^{13}C$ value.

Pollen from the four species treated was also examined before and after each treatment using light microscopy in order to evaluate changes in appearance caused by the chemical treatments. Photomicrographs of *Pinus echinata* before and after each treatment are presented in Fig. 7; these images showed that only the treatment involving NaOH substantially altered the appearance of the pollen, and even in that case the pollen was thoroughly identifiable.

Fig. 8 shows the effect of the three chemical treatments listed in Table 6 on the carbon stable isotope composition of four species of conifer pollen; Table 6 also summarizes the change in $\delta^{13}C$ value seen in each species, as well as the average change for each treatment. Both the H_2O_2 and "Treatment 1" chemical preparations lowered the isotopic value of pollen by a small amount; the average change in $\delta^{13}C$ value across species was −0.36‰ and −0.23‰, respectively. In contrast, the NaOH chemical preparation increased the isotopic composition of pollen by 1.13‰ on average. This change in stable isotope composition may have resulted from the removal of the cellulose intine and outer polysaccharide layer (Fig. 7 and Loader and Hemming, 2000); in addition, ^{13}C-depleted parts of the sporopollenin (e.g., ester-bound cinnamic acids; van Bergen et al., 1993) may have also been removed. Therefore, if a study requires the isolation of chemically and isotopically unaltered pollen, a preferred method of oxidation might be digestion in H_2O_2, based on these results. It is encouraging to note that the very commonly used technique involving Schulze's solution led to only very minor alteration of the carbon isotopic signature of pollen, although these results may be specific to the chemical exposure time and the type of pollen used in the analyses presented here. Similarly, acidification of organic matter in hydrochloric acid, a necessary treatment to remove carbonate prior to carbon isotope

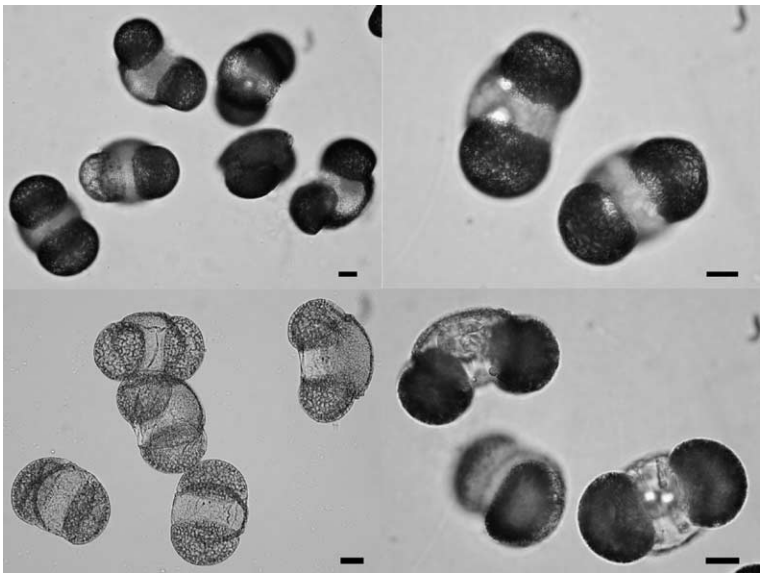

Fig. 7. Light micrographs of *Pinus echinata* pollen before and after application of the three chemical treatments described in Table 6 (scale bars=10 μm). Raw pollen (upper left) appears very similar to pollen that has been subjected to H_2O_2 (upper right) and "Treatment 1", which includes a digestion in Schulze's solution (lower right). Only treatment with NaOH (lower left) affects the appearance of the pollen, probably by removing the outer polysaccharide layer and the cellulose intine.

analysis, has been found to result in no significant alteration of organic matter $\delta^{13}C$ value under most conditions (Midwood and Boutton, 1998).

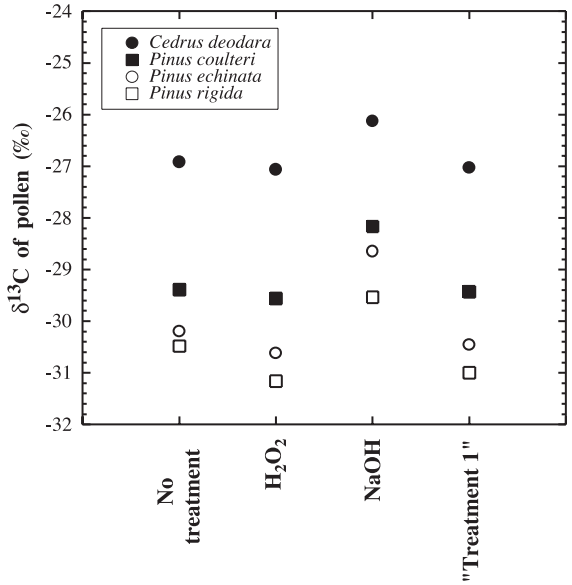

Fig. 8. Effects of the chemical treatments described in Table 6 on the carbon stable isotope composition of four species of conifer pollen. Each data point represents the average of three replicate analyses; total variability in replicate analyses was less than 0.10‰.

5. Conclusions

These results suggest that pollen isolated from Phanerozoic rocks via "Treatment 1" may have a carbon isotope composition that is within 3.0‰ of the value of the whole-plant tissues from which the pollen originated, provided that the chemical composition of sporopollenin has not changed extensively due to diagenesis (Collinson et al., 1994; van Bergen et al., 1993). This resolution is more than enough to distinguish C3 from C4 plant populations on the basis of the carbon isotope composition of bulk fossil pollen. For Cretaceous and younger sediments, the isotopic difference between fossil pollen and the pollinating plant community might be further constrained based on the close correlation between pollen $\delta^{13}C$ value and stem and leaf $\delta^{13}C$ values seen in many angiosperm clades. For example, if recovered bulk pollen was evaluated to be dominated by certain clades, such as the Cornales and Ericales, it could be argued that the isotopic composition of the pollen was within 1.5‰ of the value of the plant community, based on these results. Such resolution of the carbon stable isotope composition of the pollinating plant community may be sufficient to test hypotheses regarding the onset or

alleviation of water stress through geologic time, for example.

Although the morphology of pollen is often taxonomically distinctive at the species level, measuring the carbon isotope composition of individual species within an ancient community using $\delta^{13}C$ analysis of fossil pollen is prohibitively labor-intensive. A medium-to-large pollen grain measures 25 μm in diameter; assuming a spherical shape and a composition of 50% carbon by mass, each pollen grain contains about 2×10^{-3} μg of carbon. Using current automated combustion methods, approximately 2500 pollen grains would have to be manually isolated for one carbon isotope measurement that could be trusted without replication. However, it has been noted that some sediments contain fossil pollen assemblages with > 95% of the pollen coming from a single species (Graham, 1999); such pollen assemblages could be isotopically analyzed in order to infer the $\delta^{13}C$ value of that species within the sedimentary context. Also, analysis revealed that for the 20 different Families sampled, the average variation in

Table 7
Within-family variation in pollen $\delta^{13}C$ value at each site of collection

Site collected	Plant family	Pollen mean $\delta^{13}C$ value (‰)	Range in pollen $\delta^{13}C\pm\sigma$[a] (‰)	n, number of species[b]
ASU	Fabaceae	−25.92	3.15±1.45	4
	Myrtaceae	−26.71	1.94±1.37	2
	Pinaceae	−26.32	4.16±2.94	2
ATH	Aceraceae	−26.94	0.63±0.34	3
	Caprifoliaceae	−25.83	4.57±2.11	4
	Hamamelidaceae	−28.50	1.00±0.71	2
	Pinaceae	−29.24	1.93±1.36	2
	Polemoniaceae	−30.24	0.80±0.57	2
	Rosaceae	−25.51	2.58±1.82	2
ATL	Betulaceae	−27.49	2.43±1.72	2
	Oleaceae	−27.78	2.83±2.00	2
	Rosaceae	−27.69	1.11±0.78	2
BTA	Anacardiaceae	−25.85	2.18±1.54	2
	Asteraceae	−26.86	0.52±0.28	3
	Cupressaceae	−24.50	1.79±0.90	3
	Fabaceae	−24.59	2.40±0.73	9
	Lamiaceae	−25.57	1.79±1.28	2
	Onagraceae	−28.30	1.33±0.94	2
	Pinaceae	−27.96	1.51±0.76	3
JHU	Fagaceae	−26.64	3.97±1.43	5
MA	Betulaceae	−27.61	1.39±0.98	2
	Caprifoliaceae	−26.33	3.58±1.85	3
	Fagaceae	−28.82	3.29±1.29	5
	Magnoliaceae	−26.34	3.55±1.65	4
	Pinaceae	−26.75	2.39±0.93	9
	Rosaceae	−28.14	1.02±0.72	2
MIN	Liliaceae	−28.58	0.72±0.50	2
NA	Pinaceae	−27.52	5.46±1.71	8
TBG	Acanthaceae	−25.71	4.50±2.31	3
	Asteraceae	−27.33	2.12±1.49	2
	Fabaceae	−26.23	1.20±0.85	2
	Ranunculaceae	−26.48	0.72±0.51	2
WPA	Betulaceae	−27.13	5.43±2.73	3
	Pinaceae	−27.75	1.08±0.76	2

[a] σ is the standard deviation of the range seen in n species sampled at the site.
[b] n is the number of species sampled within the Family specified.

pollen $\delta^{13}C$ value was =2.33‰ (Table 7) and no systematic difference in $\delta^{13}C$ value could be attributed to number of species within Family sampled. Because this average range in pollen $\delta^{13}C$ value was relatively small, compared to the total range in pollen $\delta^{13}C$ value seen across all species sampled (total range=17.64‰; total range=9.41‰ with the exclusion of *Pediocactus simpsonii*), isolation and analysis of Family-specific pollen from the fossil record might lead to a reduction in labor and successful inference of the ancient $\delta^{13}C$ value of plant tissue for the Family sampled. This is further reinforced by the relatively low variability between Family mean pollen $\delta^{13}C$ values across different sites, e.g., range in mean pollen $\delta^{13}C$ value for Pinaceae=2.92‰ for six different sites (Table 7).

This study revealed that modern pollen showed a close and consistent isotopic relationship with the bulk plant tissue from which it was derived, and that the variability in this relationship can be further understood in terms of tracheophyte phylogeny. The carbon isotope composition of fossil pollen can be used to infer the $\delta^{13}C$ value of ancient pollinating plant communities, provided that the basic taxonomy of the pollen assemblage is known, and the method of chemical preparation was appropriately chosen.

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