

Centuries-Old Viable Fruit of Sacred Lotus *Nelumbo nucifera* Gaertn var. *China Antique*

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Abstract During the Sino-Japanese conflict of the 1920s, Japanese botanist Ichiro Ohga was presented single-seeded fruit of *Nelumbo nucifera* var. *China Antique* collected by a local farmer from a dry lakebed in Northeast China (then,

“Manchuria”). Ohga studied the fruit and published his findings. Years later, we tested the germination of *Nelumbo* fruit from the same locality. The oldest seed sprouted, having a germination time of ~3 days, was radiocarbon dated to be ~1300 years old. These cold- and drought-tolerant seeds exhibited shoot-before-root emergence and a primary green plumule capable of “dim-light” photosynthesis. Such traits and the notable long-term viability of the fruit spurred the interest of Ray Ming, University of Illinois that has now led to the sequencing of the *Nelumbo* genome. Analyses of this genome may provide insight into the biochemistry of *Nelumbo* on wax-biosynthesis genes, and application of aging-related thermostable proteins to the extension of seed-life and improvement of food quality of economic crops. Here, we review the history of these long-lived *Nelumbo* fruit, and their occurrence, discovery, collection, propagation, and methods of seedling care. The robust impermeable wax- and suberin-covered pericarp is a major factor contributing to their remarkable longevity. New findings are presented on the modern and 459- and 464-year-old pericarp anatomy, impermeability to water, and whole fruit and pericarp mechanical properties, and comparison of the mode of fruit weight-gain during imbibition and germination time relative to fruit maturity.

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Abbreviations

AMS Accelerator mass-spectrometry
BP Before Present
IAA Indole-3-acetic acid, auxin
WWII World War Two

Symbols and Units

Gb Gigabase
GPa GigaPascal

kgf kilogram force
mN milli-Newton

Introduction

Cultivated for more than 4,000 years throughout Asia, with virtually all parts of the plant being edible and regarded as having medicinal value, *Nelumbo nucifera* represents a so-called “full body of treasures” (Zhao 1987; Duke 2002; Shen-Miller 2002). A major crop plant in Asia where it is harvested especially for its large rhizomes and seeds, *Nelumbo* embryo axes, imbibed as tea, are reported to provide effective treatment for insomnia and stress (Zhao 1987; Duke 2002).

The Nelumbonaceae, the smallest family of angiosperms, includes only the single genus *Nelumbo* and its two species, *N. nucifera* (Asia, pale pink) and *N. lutea* (North America, light yellow). The two species are easily differentiated by their geographic distribution and the color of their showy flowers (Fig. 1). *Nelumbo* is commonly mistaken to be a water lily (Nymphaeaceae). However, it is neither a water lily nor is it closely related either to the Egyptian sacred blue-lily (the lily-of-the-Nile, *Nymphaea caerulea*; Raven PH, pers. comm. to JMS, 2012) or to *Lotus japonicus* (Leguminosae). The ancient Greeks referred to these plants, and to papyrus, *Nelumbo* and others, as lotus (“*lotos*”), a practice that has led to confusion. A problem further exacerbated by Alfred Lord Tennyson’s poem (1959) “The Lotos-Eaters,” which depicts the eating of lotos fruits by mythical Greeks as causing dreamy indolence (whereas the fruits referred to in the poem are actually those of the mucilaginous tree *Zizphus lotus* of the buckhorn family Rhamnaceae; Webster Ninth New-Collegiate Dictionary, 1985). To avoid such confusion, the term “lotos” will therefore not be used in this paper, and the plant *N. nucifera* will be referred to by its generic name, “Nelumbo,” which for simplicity will not be italicized.

Members of the Nelumbonaceae exhibit characteristics that differ markedly from those of common angiosperms.



Fig. 1 First bloom (July, 2013) of one-yr-old offspring of a 543-yr-old fruit (Fig. 6, Old, OL05-PC4) of *Nelumbo nucifera* var. China Antique at the Wuhan Botanical Garden, China, showing a ~20-cm flower on the 4th day of bloom, its anthers prostrate, fruits (9) pollinated, fruit receptacle turned from yellow to green, and its petals will soon fade and fall

For example, at germination their chlorophyllous green shoots are primed for photosynthesis (Ushimaru et al. 2003) and emerge before the roots. This characteristic provides a “jumpstart” for plant growth and may have been spurred by duplication of the whole *Nelumbo* genome about 65 Ma ago (Ming et al. 2013), the time of the end-Cretaceous mass extinction when it could have served to adapt to dim-light “Nuclear-winter-type” conditions. In addition, the China Antique variety of *Nelumbo* is notable for having a large number of directly ¹⁴C-dated fruit hundreds of years old ($n=22$; Table 1) that exhibit a high germination rate (~84 %) and maintain vigorous shoot-emergence after a short ~3-day imbibition period (Table 2). Finally, appreciable amounts of the seed proteins of the embryo axis and cotyledons of a 549-year-old fruit have been shown to remain soluble at 110 °C (~30 and 75 %, respectively), and one thermostable enzyme tested for protein-repair from a modern fruit retains activity after being heated to 100 °C (Shen-Miller et al. 2013). All of the thermally stable *Nelumbo* proteins thus far identified and analyzed are known to have roles in stress and repair.

These unique traits of *Nelumbo* spurred the interest of Ray Ming, University of Illinois at Urbana-Champaign during a bus ride with JSM to the 2008 American Plant Biologists Conference at Mérida, Mexico; a conversation that led to the sequencing and annotation of the *Nelumbo* genome (Ming et al. 2013).

Annotation of ~86 % of the genome of *Nelumbo nucifera* Gaertn var. China Antique shows it to have a size of ~1 Gb (situated on 16 chromosomes, 2 N) that codes for ~27,000 genes (Ming et al. 2013). Significantly, and perhaps importantly with respect to the long-term viability of *Nelumbo* seeds, the sequenced genome is unlike that of most angiosperms, exhibiting an unusually low content of mutations and a seemingly unique expansion of genes of adaptive value that are involved in cellular protection (Ming et al. 2013; Nelson and Schuler 2013). This genome contains the information that enables *Nelumbo* seeds to remain viable for hundreds of years, documented by their direct ¹⁴C-dating. The oldest of the 22 seeds now dated, all collected from a Holocene dry lakebed in NE China (at Xipaozi Village, near Pulandian, Liaoning Province; Chen et al. 1965), has an age of ~1300 years and viable (Shen-Miller et al. 1995). In addition to *Nelumbo* heat-stable proteins (Shen-Miller et al. 2013) and membrane fluidity (Priestley and Posthumus 1982), the fruit pericarp, the coat that encloses single *Nelumbo* seeds, is a major factor for maintaining seed viability.

This review, besides discussing historical aspects of the occurrence and discovery of these distinctive old fruit and the germination-propagation of seedlings and their cultivation, addresses prime characteristics of these long-living fruit that may have application to crop improvement—their novel embryogenesis, atypical germination- and maturation-

Table 1 Summary of germination and ages of fruits of *Nelumbo nucifera* Gaertn var. China Antique, Xipaozi Village, Pulandian, Liaoning Province, NE China

Fruits (n)	Dwt (g)	¹⁴ C Age (BP)	Age (y)	Germination	
				Days	%
Old (22)	0.82 ± 0.1	40 to 1350	104 to 1288	3.5 ± 1.0	84.2 (n=19)
Modern (27)	0.87 ± 0.9	Modern	Modern	6.1 ± 2.2	92.6 (n=27)

n number tested

Dwt dry weight

BP before present

± standard deviation

Table 2 Imbibition (D1, day1, etc.) and Germination (Germ) of old and modern fruits of *Nelumbo nucifera* Gaertn var. China Antique (Xipaozi Village, Pulandian, Liaoning Province, NE China)

Fruits	D1 Weight (% daily net gain)	D2	D3	D4	D5	D6	Total Net-Gain at Germ (%)	Germ (days)
Old (192-645 year-old)								
OL96-44	25.0	46.0	22.4	5.50	–	–	136.4	4
OL96-50	48.1	60.5	3.33	–	–	–	145.7	3
OL96-52	66.5	42.8	6.94	–	–	–	154.2	3
OL96-53	54.5	48.5	6.06	–	–	–	143.2	3
OL96-57	36.1	71.2	2.59	3.33	–	–	147.0	4
OL05-CB1	83.4	30.0	4.17	–	–	–	148.4	3
OL05-CB2	75.0	38.8	17.3	–	–	–	184.9	3
Ave.	55.5	48.2	8.97	4.15	–	–	151.4	3.28
±σ	19.4	12.7	7.15	0.82	–	–	14.6	0.45
Modern (unknown vintage)								
OL96- 6	34.2	70.5	2.28	0.48	0.89	-0.26	136.8	6
OL96- 9	59.8	56.3	1.50	2.20	–	–	159.2	4
OL96-33	41.6	65.3	1.68	1.35	0.87	0.67	145.0	10
05CB-3	69.9	50.2	2.42	–	–	–	161.4	3
OL96-1	90.8	24.4	1.90	4.23	–	–	151.7	4
OL96-8	93.8	21.0	3.12	1.18	1.13	–	147.4	5
OL96-11	198.5	21.5	1.49	0.43	2.99	–	153.2	5
OL96-13	189.1	5.39	1.98	x	x	–	~135.1	5
OL96-34*	116.4	8.13	1.77	1.16	0.78	0.61	144.3	6
OL96-48	128.7	4.63	x	x	–	–	~153.3	4
05CB-4	138.6	4.65	3.13	–	–	–	157.5	3
Ave.	102.7	30.2	2.12	1.58	1.33	0.34	149.5	5.36
±σ	52.5	24.4	0.55	1.21	0.84	0.42	8.27	1.70
New Harvest*								
06Ca-RLa	19.3	33.4	28.9	8.05	3.35	1.81	136.2	8
06Ca-RLb	21.1	38.4	22.5	7.92	2.08	1.37	137.9	9
06Ca-RLc	20.6	37.1	28.0	5.56	2.76	1.56	140.6	8
06Ca-RLd	47.4	37.3	10.7	2.91	1.10	2.37	138.5	6
06Ca-RLe	27.8	42.0	22.5	7.56	2.07	0.69	149.0	12
Ave.	27.2	37.6	22.5	6.40	2.27	1.56	140.4	8.60
±σ	10.5	2.76	6.49	1.96	0.76	0.55	4.50	1.96

Table 2 (continued)

Fruits	D1 Weight (% daily net gain)	D2	D3	D4	D5	D6	Total Net-Gain at Germ (%)	Germ (days)
1-Year-Old								
02-PRI-1	129.5	7.07	2.14	3.28	–	–	159.2	4
02-PRI-2	129.0	5.20	2.10	2.48	–	–	152.0	4
05Ca-R1*	125.5	2.16	1.87	0.86	–	–	136.7	4
05Ca-R2*	130.8	2.46	2.08	1.95	–	–	146.1	4
Ave.	128.7	4.22	2.05	2.14	–	–	148.5	4
±σ	1.96	2.03	0.10	0.88	–	–	8.24	0
3-Year-Old								
03Pn-b	33.0	45.5	16.0	37.4	x	x	~147.6	6
03Pn-c	27.0	47.4	16.6	x	x	x	~135.4	6
03Pn-d	28.9	46.4	17.3	x	x	x	~150.0	6
03Pn-e	24.3	49.9	19.1	x	x	x	~141.7	6
Ave.	28.3	47.3	17.2	37.4	x	x	~143.7	6
±σ	3.17	1.64	1.16	–	–	–	5.65	0

±σ standard deviation

* OL96-34*, mother fruit of New Harvest* and 1-year-Old*, germinated and cultivated at UCLA

x no data collected

periods, and the anatomy and mechanical properties of their robust water-impermeable pericarp.

Fruit Discovery, Collection, Identification and Cataloguing

Discovery of Old Fruit

The recorded history of discovery of the long-living fruit of *Nelumbo* var. *China Antique* dates from the early 20th century when NE China was under Japanese occupation. By the early 1920s, botanist Ichiro Ohga (Government Botanist of Kwantung Leased Territory, Professor of Botany, Educational Institute, South Manchuria, Dairen; Ohga 1923) had arrived at Xipaozi Village near Pulandian in Liaoning Province (then called “Manchuria”) where a local farmer, Liu U, gave him *Nelumbo* fruit that Liu had collected from the local dry lakebed (Ohga 1923, 1926, 1927). When our team of botanists and geologists first visited this village in 1996, “Farmer Liu,” Ohga’s helper, was still remembered by villagers who vividly described Liu’s appearance—“having a full face of crooked muscles”—at his 1951 execution-by-firing-squad as a Japanese collaborator and traitor to China. During this 1996 visit, the Xipaozi villagers regarded our team of foreigners as somewhat “Suspect,” well-remembering the abhorrent and repeated incursions of Japanese and Russians troops warring over their land and its nearby year-round unfrozen seaport, they were less than fully welcoming.

Using geologic features of the meandering Anzi River, in 1927 Ohga estimated that the *Nelumbo* fruit given him by Liu U might be a few hundred years in age. Later, in the 1950s, radiocarbon dating was developed (Libby 1955), a technique that by the beginning of our studies in the late-1980s could be used to ^{14}C -date germinated specimens but that required a one-gram sample and, thus, incineration of an entire *Nelumbo* fruit (Shen-Miller et al. 1995). By the mid-1990s, use of accelerator mass spectrometry (AMS) had decreased the required sample-size to 10 to 15 mg (Shen-Miller et al. 2002; Tele Images-Nature 2003), an advance that has permitted us to germinate such fruit, to radiocarbon-date their removed pericarp (dead tissue), and to cultivate the seedlings from ancient parentage into mature plants (Fig. 2). The resultant ^{14}C -ages in BP (Before Present) are converted to ages in years (Table 1; Stuiver and Becker 1993; Shen-Miller et al. 2002).

Of the seven old Xipaozi fruit that we first germinated at UCLA, presented to JSM by the Beijing Institute of Botany, Academia Sinica, the oldest was radiocarbon dated to be ~1300 years (Shen-Miller et al. 1995). Earlier, in 1950s, one such fruit, given by Ohga to botany professor Ralph W. Chaney of the University of California, Berkeley, had been ^{14}C -dated to be at least a thousand years old (No. C629, Libby 1955). In 1978, Chinese botanists dated a fruit from Xipaozi to have a radiocarbon age of 915 BP (Chang 1978), and a fruit of *China Antique* germinated by Priestley and Posthumus (1982) was shown to have a ^{14}C -age of 430 BP.

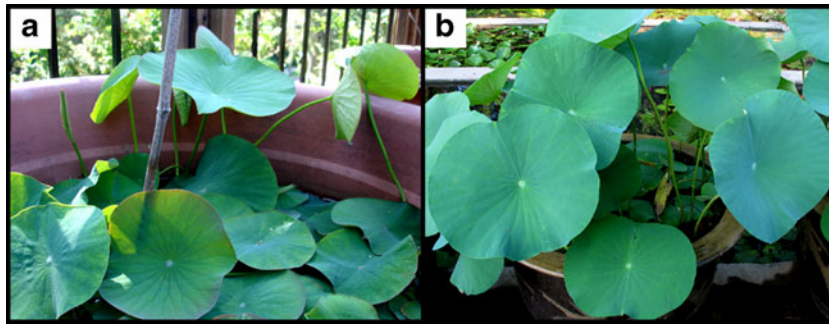


Fig. 2 Offspring of centuries-old fruit from Xipaozi. **a** The offspring of 408-year-old *Nelumbo* var. *China Antique* fruit OL96-50 ~3 months after breaking winter-dormancy, cultivated at UCLA (Los Angeles, CA, USA). **b** The offspring of 432-year-old *Nelumbo* var. *China Antique*

fruit OL05-PC1 (shown at the far left in Fig. 6), ~4 months after planting, a seedling planted 19 days after germination, root emergence and extension, and cultivated at the Wuhan Botanical Garden (Wuhan, China)

Two additional *Nelumbo* fruit given by Ohga to Chaney were presented to the Kenilworth Aquatic Gardens in Washington, D.C. where they have been cultivated since 1951 (Wester 1973; Shen-Miller et al. 1995).

In our initial studies, we used fruit harvested from the Chaney-derived Kenilworth plants to study germination and cultivation. In 1996, we journeyed to NE China to collect additional specimens. With considerable awe our team arrived at the village of Xipaozi and set foot on the same soil that Ohga had tread some 70 years earlier (Shen-Miller et al. 2002). We since have made five additional trips to the area, most recently in 2010.

Centuries ago, much of what is now the village and farmland of Xipaozi was a large lake, cultivated with *Nelumbo* by Buddhist monks who regarded it as a sacred and symbolic plant—emerging from the humble origin of lake-bottom mud to rise high above the water, its showy flowers radiant and its large disc-shaped leaves spotless and verdant (Shen-Miller et al. 1995). A purity-echoing reference to *Nelumbo* is recorded in a classic essay by the Song Dynasty Philosopher Zhou DunYi (1017–1073) as “Soaring through the muddied earth, untainted” a trait that to Zhou symbolized the most valued quality in man and a phrase still used today by Chinese scholars.

The striking cleanliness of *Nelumbo* leaves results from their wax-covered surface that is peppered by protruding nanostructures that produce the so-called “self-cleaning lotus effect” (Barthlott and Neinhuis 1997; Nelson and Schuler 2013) that has been used in Europe to develop an industrial water-repellent paint. The basic mechanism of the cleansing effect exhibited by the *Nelumbo* leaves may someday be further adapted to healthcare (e.g., dentistry), laboratory, and space crafts.

Burial Lake of *Nelumbo* Fruit

Originating in India, Buddhism later spread into China and by 372 A.D. had been introduced into the Korean peninsula via

China’s Liaoning Province, the source of the centuries-old *Nelumbo* fruit (Fig. 3) where the plant had been cultivated in the large lake that is now the site of Xipaozi Village (“Paotsutun,” Ohga 1927). Interestingly, the three Chinese characters for this village, *Xi*, *Pao*, and *Zi*, literally mean “Steeped Seeds of the West,” in reference to a lake filled with seeds of an aquatic plant, *Nelumbo*, which had been introduced from western regions. As a result of centuries of historically recorded earthquakes in the Xipaozi region (Shen-Miller et al. 2002, Table 1), the *Nelumbo*-populated lake ultimately drained into the Bo Hai Sea (Fig. 3) converting the former lakebed to arable land.

At the time of our team’s first visit, in May 1996, the dry lakebed had been recently planted with new crops (Fig. 4a). After a night of spring rain, we found more than a dozen shiny *Nelumbo* fruit glistening on the soil of farmer Liu U’s former land (on the southwest side of the lakebed). Such fruits and their associated lakebed peat, the organic-rich sediment of the originally *Nelumbo*-laden lake, had been brought to the surface at the time of Mao ZeDong’s 1958 “Great Leap Forward” when the local farmers overturned the deposit to expose the underlying peat for use as fuel (Anon 1962; Shen-Miller et al. 2002). Use of this farm-soil peat as fuel was proudly demonstrated to us by a local farmer who ignited it by the strike of a match. This peat layer, in the middle of the lakebed, is estimated to be ~1-m thick (Ohga 1927). Over the years, the local farmers, aware of the centuries-old *Nelumbo* fruit and their potential worth, have routinely found and saved them as they tilled the land.

During a subsequent visit in 2002, when we participated in the filming of an hour-long *Nelumbo*-based television documentary (Tele Images-Nature 2003), we were surprised to discover a newly built paved road running south-to-north through the middle of the farmlands of the Xipaozi former lakebed that traversed a newly constructed stone bridge, *LianXinQiao* (“*Nelumbo*-Heart Bridge”) and that to the north led past rows of freshly painted houses recently built

Fig. 3 Map of Liaoning and neighboring provinces of NE China. The arrow denotes Xipaozi Village where the centuries-old fruit of *Nelumbo nucifera* var. *China Antique* were collected. *Inset*: map of China showing the location of Liaoning Province (red circle)



by the increasingly prosperous farmers. On subsequent visits, we found that a superhighway to Xipaozi from the large port city of Dalian, ~80 km south, had been completed (Fig. 3) and that the now-bustling Xipaozi village had become transformed into a center of light industry, housing projects, hotels, gardens, offices, and tourism. Except for a few hot houses at the periphery of the former lakebed (Fig. 4), farming had all but disappeared.

Thriving stands of *Nelumbo* var. *China Antique*, derived from rhizomes and fruit of the lakebed, can still

be found at Xipaozi in a tributary of the Anzi River (Fig. 4b) and near Pulandian, a few km south of Xipaozi Village, at the *GuLianWen* Water Park (in which “GuLian” means “Ancient Nelumbo,” Fig. 5). This impressively large water park, operated by the husband-and-wife team of Xu Gang and Guan Li, is a popular site for festivities and tourism (Fig. 5). In 2010, during our latest visit to Xipaozi, we were dismayed to see heavy machinery rolling across the former lakebed to construct a NW-SE “Trans-Nelumbo Superhighway”.

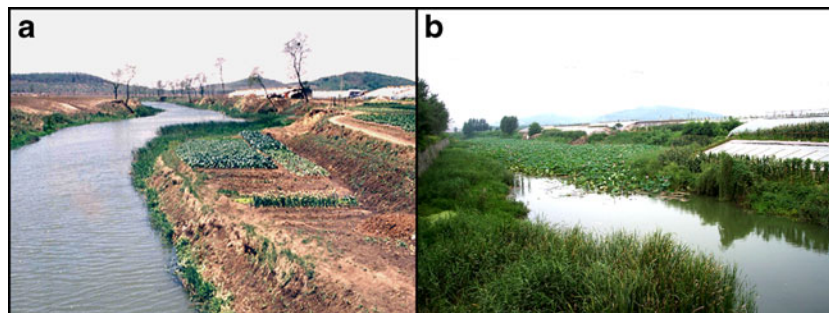


Fig. 4 Xipaozi Village, Liaoning Province, NE China, in photos taken at *LianXingQiao* (Nelumbo-Heart Bridge), looking toward the west (rather than north as is indicated in Shen-Miller et al. 2002, Fig. 2) and showing a tributary of the Anzi River that extends westward from the center of the former Nelumbo-containing lake. **a** A photo taken in May 1996 showing the tributary and *MaJiaSan* (Horse-Riding Hill) in

the distant background. **b** A comparable image acquired in August 2010, showing on the right bank (north bank) of the tributary a dense patch of *Nelumbo* var. *China Antique* derived from fruits and rhizomes originally embedded in peat of the former lakebed; the tent-like structures at the right are hothouses



Fig. 5 *GuLianWan* (Water Park of Ancient Nelumbo) at Pulandian, Liaoning Province, NE China showing var. *China Antique* in bloom taken on 17 August 2010 during festivities of the Third Exhibition of Ancient Nelumbo

From now on, the search for centuries-old Nelumbo at Xipaozi Village will be a fruitless venture.

Identification of Old Fruit

In addition to the initial gift of old-lakebed *China Antique* fruit provided by the Academia Sinica Beijing Institute of Botany and those gathered during our 1996 trip to Xipaozi, a few of the old fruit in our collection were given to us by, and most were purchased from the farmers of Xipaozi Village who were eager sellers. Most such fruit could be judged immediately to be old, indicated by the shiny “pock-marked” appearance of their pericarp and their lack of a terminal “nipple-like” stigmata remnant (cf. Shen-Miller et al. 1995, Fig. 1). The shiny pericarp of the old fruit is a result of the degradation of their outer epidermis and exposure of the underlying palisade layer of water-impervious suberin-impregnated lamellae (Figs. 8c and 11a, below; Shaw 1929). Modern fruit have an opaque cuticle surface and a prominent stigmata nipple (cf. Shen-Miller et al. 1995, Fig. 4). The appearance of the pericarp surface and the absence or presence of a stigmata remnant have proven reliable for distinguishing the old fruit from the modern; of 23 fruit thus assessed, only one did not match expectations.

For germination to occur, one end of a Nelumbo fruit must be filed to permit water-penetration and imbibition, a factor that presents an additional characteristic by which to distinguish between old and modern fruit. The filed ends of old-fruit pericarps fray into shreds within minutes of soaking (Fig. 6, old fruit), whereas those of modern fruit (cf. Fig. 6, center fruit) maintain their integrity of intact edges throughout germination.

Five criteria can be used to distinguish old fruit from their modern counterparts. Of these, listed below, criterion 4 is the most reliable. The centuries-old Nelumbo fruit from Xipaozi typically:

- (1) Exhibit a shiny rather than dull surface (Fig. 1, Shen-Miller et al. 1995);
- (2) Have a surface that is generally pock-marked rather than smooth and opaque (Fig. 1, Shen-Miller et al. 1995); and
- (3) Lack the apical stigmata remnant characteristic of modern fruit (Figs. 1 and 4; Shen-Miller et al. 1995).

In addition:

- (4) The filed edges of the pericarps of the old fruits rapidly disintegrate during imbibition (Fig. 6, old fruit), rather than remaining intact (Fig. 6, modern); and
- (5) The old fruits exhibit a more-or-less gradual net weight-gain during the first 2 days of imbibition preceding germination, whereas the weight-gain of the modern is more variable (Table 2).

The visual distinctions between old and modern fruit were not initially recognized. The first batch of purchased fruit, claimed by a Xipaozi villager to be old, were later shown by radiocarbon analyses to be modern, containing ^{14}C “bomb carbon” derived from post-WWII thermonuclear weapon tests (an unfortunate if useful lesson that resulted in the entire lab being scrubbed and checked for ^{14}C contamination).

Fruit Cataloguing and Storage

After their acquisition at Xipaozi, Nelumbo fruit were given a sink-float water test to predict viability. This proved to be a reliable indicator of germination, floaters



Fig. 6 Old and modern Nelumbo fruit after 2 days of imbibition. The fruit in the center (OL05-PC3) was shown to contain ^{14}C bomb carbon and, thus, to be modern; like that of all 27 modern fruit analyzed (Table 1), the end of the fruit filed to facilitate germination was unfrayed during imbibition. In contrast, the filed ends of the other four

fruit frayed and shredded (occurring minutes after imbibition). From left to right: old fruit OL05-PC1 (432 year), OL05-PC2 (448 year), modern fruit (OL05-PC3), and old fruit OL05-PC4 (543 year) and OL05-PC-6 (566 year)

invariably being unable to sprout. Each old fruit was then stored individually at 4 °C in a glass bottle having a perforated loosely-fit cap labeled by specimen number, dry weight, date and site of acquisition, seller or collector, and its sink/float result. Records of the catalogued information were further stored in a notebook. About 100 such catalogued fruit are now available for study. Fruit of modern *Nelumbo* var. *China Antique* bought or collected at Xipaozi Village, GuLianWen Water Park and the Kenilworth Aquatic Gardens (plus miscellaneous others) were catalogued and stored in brown paper bags at 4 °C and used for comparative studies.

Fruit Propagation, Imbibition, Weight-gain and Cultivation

Nelumbo normally propagates by rhizomes rather than the much slower germination of seeds. Water does not penetrate a *Nelumbo* fruit until the fruit-coat pericarp is disrupted (Ohga 1923; Shen-Miller et al. 1995). Shaw (1929) observed water penetration into *Nelumbo* fruit only after treatment with acetone or xylene. In one experiment, Chinese botanists soaked fruit for a year without obtaining germination, a problem solved by consulting a centuries-old book on

Nelumbo cultivation relating that germination would occur if the pericarp were to be broken by a sharp-edged roof-tile (Chang 1978). In nature, germination of such fruit occurs after physical abrasion in lakes and ponds or by their passage through the intestines of water fowl or other animals.

A developing immature single-seeded *Nelumbo* fruit is green, having a chlorophyllous pericarp, cradled in a “shower-head-like” fruit-filled receptacle (Fig. 7d). Cultivars bred for fruit production can yield up to 38 g-sized fertile fruit per flower (Ni 1987). *Nelumbo* fruit are sold fresh (Fig. 7d) or are processed, their pericarps having been removed and the embryo axes and cotyledons then dried and packaged for marketing. The pericarp of an immature fruit is green, soft, and easily peeled (Fig. 7d) and is unlike the two peanut-sized fresh cotyledons of the seed which are a creamy white (Fig. 7b). At maturity, the pericarp becomes dark brown and hardens (Fig. 7a) and the embryo axis is a chlorophyllous green (Fig. 7b).

Fruit Propagation

For germination in the lab, *Nelumbo* fruit were mechanically filed at the “dimple-end,” the site of fruit attachment to the receptacle and the location of the cotyledon

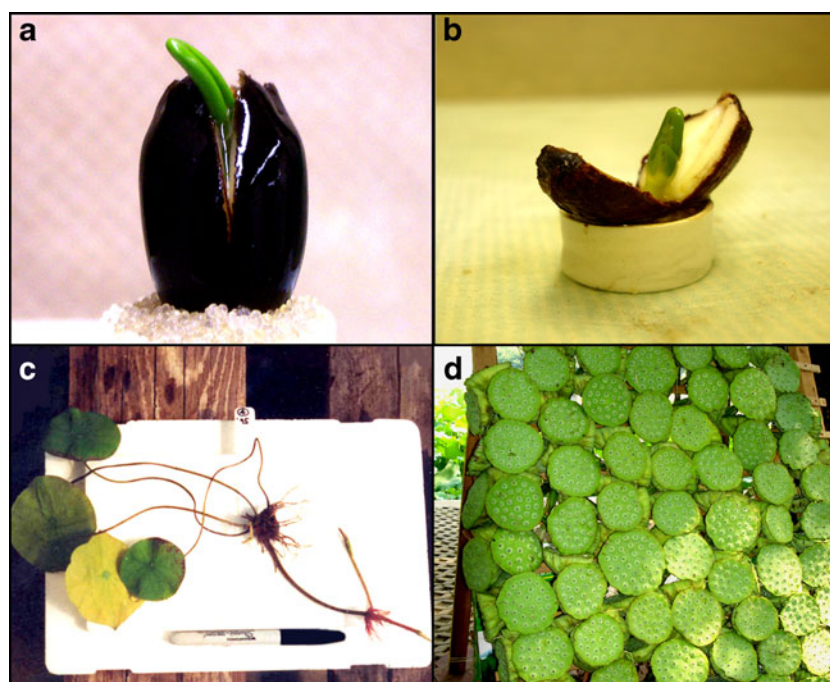


Fig. 7 A recently germinated old *Nelumbo* fruit, a mature modern fruit, a seedling, and fruit receptacles on a rack ready for market. **a** A 482-year-old *Nelumbo* fruit (OL96-57) on the second day of germination after 4 days of imbibition, showing its “shoot-before-root” germination; its dark brown pericarp has split longitudinally along the cotyledon halves, the curvature of its emerging green plumule evidencing gravitropism. **b** A pre-soaked, mature, manually opened modern *Nelumbo* fruit having two creamy cotyledons enclosed by brown seed coat; the centrally situated embryo axis consists of two green plumules and a meristem and is surrounded by the cotyledons which are attached

to the base of the pale yellow radicle of the embryo axis. **c** A *Nelumbo* seedling that exhibits four plumules (embryonic leaves); after emergence of the plumules, the fibrous roots of this seedling emerged at the base of the radicle followed by formation and extension of a rhizome, the emergence of a new shoot (first true leaf) and fibrous roots at the first rhizome node, and extension of a second rhizome (the apex of the second node is situated at the bottom right). **d** Shower-head-like *Nelumbo* fruit receptacles, each containing numerous plump green fruit ready for consumption, for sale at a fruit stand at DongTing Lake, Wuhan, China

tips (cf. Shen-Miller et al. 1995, Figs. 3 and 6), resulting in removal of ~15 mg of the pericarp before the fruit was soaked in chlorine-free tap water (using water from copper pipes, rather than from those that contain plasticizers that inhibit seedling growth). Until planting of seedlings, water was changed daily and the weight of imbibed fruit was recorded. As noted below, differences in daily weight-gain are a useful index to differentiate between old and modern fruit (Table 2). Germination begins with the splitting of the pericarp along the two halves of the cotyledons (Fig. 7a) followed by the emergence of a dark green plumule.

Shown in Fig. 7b is a pre-soaked, manually opened mature *Nelumbo* fruit having the green plumule of its embryo axis flanked by two creamy cotyledons and enclosed by its brown seed coat (cf. Fig. 10, Shen-Miller et al. 1995). The cotyledons comprise about two-thirds of the dry weight of a *Nelumbo* fruit (~600 mg). A pale yellow radicle is present at the blunt end of the embryo axis where it is attached to the base of the two cotyledons (Figs. 7b and 10, Shen-Miller et al. 1995). At maturity, *Nelumbo* fruit have green plumules that are maintained over hundreds of years. In a dry state, the embryo axis (from plumule tip to radicle base) fills the central trough between the two cotyledons (Fig. 10, Shen-Miller et al. 1995). In such specimens, the axis typically was ~1.0 cm long, ~3 mm wide and had a dry weight of ~25–30 mg.

Imbibition and Weight Gain

In the following, we use the term “weight-gain” (rather than “water-uptake”) since it more accurately reflects the effects of imbibition on the overall development of *Nelumbo* fruit over sequential days of soaking.

Viable mature modern fruit from Xipaozi Village had an average dry weight of $\sim 0.87 \pm 0.9$ g and germinated in ~6.1-days (Table 1). In comparison, old fruit from the Xipaozi lakebed, 104- to 1288-year in age, weighed $\sim 0.82 \pm 0.1$ g and germinated in ~3.5 days. The centuries-old fruit tended to have a more-or-less gradual net weight-gain during the first 2 days of imbibition, followed by germination on the third or fourth day (Table 2). Their net weight-gains averaged 56 % on day-1 and 48 % on day-2. In contrast, “Modern” *Nelumbo* fruit of unknown vintage, as well as those of “1-Year-Old” fruit (Table 2) exhibited a rapid net weight-gain on day-1 (103 % and 130 %, respectively); tapered off to 30 % and 7 %, respectively, on day-2; and germinated after ~4–5 days (Table 2). A slower rate of weight-gain is evident in fruit of “New Harvest” and “3-Year-Old” fruit (Table 2) that had germination times prolonged to ~6 to 9 days. Regardless of their vintage, all modern fruit required a longer germination time than the old fruit (Tables 1 and 2).

The patterns of weight-gain of fruit of the “Modern (unknown vintage)” evidently comprise two distinct groups (Table 2). The four fruit listed at the top of this category (viz.,

OL96-6, -9, -33 and 05CB-3) exhibited a pattern of net weight-gain similar to that of fruit of the “Old” category. However, all four fruit in this group were shown to require a longer germination period and having modern ages by ^{14}C dating (≤ 50 yr, based on the presence of residues of bomb-carbon generated either by atomic bombs detonated over Japan in 1945 or the above-ground weapon tests of the 1950s). The larger, remaining group in the “Modern (unknown vintage)” category, listed below the data in boldface in Table 2, exhibited a pattern of weight-gain similar to the “1-Year-Old” fruit.

The five fruit listed as “New Harvest*” and the two in the “1-Year-Old” category, 06Ca-R1* and -R2*, were direct descendants of OL96-34* (Table 2; modern unknown vintage). These two sets of offspring, of the same vintage but of differing age, exhibited differing patterns of weight-gain (Table 2). Seed maturation has been shown to correlate directly with seed germination (Rajjou et al. 2012). Additional net weight-gain data on fruit of differing vintages are needed to elucidate these differences and to determine, if these patterns are signs of *Nelumbo* fruit maturity, what mechanisms govern their increase in the seed weight during imbibition.

Whether the patterns of weight-gain upon imbibition are related to fruit maturity or to fruit damage over maturation and aging, or both, also needs to be clarified. Intracellular repair of damage to nucleic acids, proteins and organellar components during maturation can occur during imbibition (Bewley and Black 1994; Rajjou et al. 2012). The greater such damage, the longer the time that may be required for germination (cf. OL96-33 and 06Ca-RLe, Table 2).

Two *Nelumbo* fruit (2-year- and 4-year-old specimens harvested from the Kenilworth Aquatic Gardens in 1996) that had been successfully checked for potential germination by the sink/float test required 33 and 58 days of imbibition, respectively, before germination. The fruit exhibited 103 % (the 2-year-old) and 61 % (the 4-year-old) net weight-gain on day-1 and, respectively, 11 % and 14 % gains on day-2, a pattern similar to that of the “Modern (unknown vintage)” fruit listed in Table 2. These two fruit had an unusually prolonged phase of imbibition from day-3 onward, fluctuating between days of minimal net weight-gain and weight-loss until they germinated on days 33 and 58. When germinated, their total net weight increases were 143 % and 185 %, respectively, not appreciably different from ~150 % total net gain in weight of other *Nelumbo* fruit (Table 2). These results suggest that the prolongation of germination may have been related to repair of cellular damage that occurred during or after fruit ripening.

Given their relatively large seed-size (~1 g) and exceptional longevity, *Nelumbo* fruit can be a model system for studies of seed maturation and repair. Taken together, the data in Table 2 suggest that the patterns of weight-gain in *Nelumbo* fruit during imbibition most likely reflect fruit maturity, the old

fruit tending to gain weight at a more-or-less gradual rate, especially during the first 2 days of imbibition, and the less mature fruits exhibiting a more variable pattern.

Nelumbo fruit in “Old,” “Modern (unknown vintage),” “New Harvest,” “1-Year-Old” and “3-Year-Old” all showed a total net weight-gain of ~150 % on the day of germination (Table 2), regardless of the period required for germination and the pattern of net weight-gain. This result is similar to the finding of Bewley and Black (1994) who report that the total water-uptake in most germinated seeds is two to three times their initial dry weight.

Cultivation of Seedlings

Before the emergence of a true leaf from the node of the first rhizome, the Nelumbo seed sprouts four plumules, embryonic leaves (Fig. 7c). In the cultivation of such seedlings, care of the first three emergent plumules was absolutely necessary, as they rapidly blacken and withered if grasped. Planting is best done when the seedling had formed basal roots that extended ~2–3 cm (Fig. 7c; Vegetable Research Institute, Wuhan, Hupei, and Qindao China Water-Lily World, Shangdong, 2010, pers. comm. to JSM).

For radiocarbon dating of Nelumbo fruit, the pericarps from imbibed fruits were peeled before seedling planting and cultivation (Fig. 2). Figure 2 shows two healthy offspring cultivated from ancient fruit, 408- and 432-year in age. Clay-rich pond mud was the preferred potting media, such mud or “paddy soil” having been shown to be effective at botanical gardens in China. At UCLA, seedlings were planted in pots (~50-cm diameter, ~60 cm high; Fig. 2a). Water level was initially shallow and gradually increased to a depth of ~20 cm. Potted plants were placed in full sunlight. Other techniques are also effective: at China’s Wuhan Botanical Garden, potted Nelumbo plants are set in a water-filled concrete-paved pond during the growing season and moved into a greenhouse during winter.

After the appearance of standing leaves that emerged from the rhizome nodes, fertilizer (time-release pellets of N-P-K/16-9-12; Laguna Plant Growth, Hagen Inc., Canada) was applied. The fertilizer pellets in two or three capped perforated tubes (~1.5 × 18 cm) were inserted into the potting soil. Direct dispensing of loose fertilizer into the water resulted in an unwanted algal bloom. Rather than using such pellets, many of the Nelumbo-cultivating gardens in China rely on organic fertilizer (“green manure”), the decomposing oil-extracted residues of soybean meal applied before and after flowering. Except in the tropics, new plantings may not flower in the first season, becoming dormant in the fall (even in the temperate climate of southern California). Infesting algae should be removed manually from the pots or by application of PondCare AlgaeFix (polyoxyethylene di-dimethylimino

ethylene dichloride, Aqua Mart Inc.). Senescing leaves and dead or decaying tissues should also be removed to eliminate nutrient for algal blooms.

Ambient temperature may not be as critical in farm ponds of China—Nelumbo can survive a temperature of minus 30 °C if the rhizome-containing pond-bottom mud remains unfrozen (Huang 1987). The unique thermal-regulation mechanism exhibited during pollination by Nelumbo flowers (Grant et al. 2008) merits investigation in other organs, for example in rhizomes where it could serve to offset cold-stress, particularly in light of the expanded array of wax/lipid biosynthetic genes recently documented in the Nelumbo genome (Ming et al. 2013; Nelson and Schuler 2013) which may provide a substrate for the uncoupling protein (UCP) of thermogenesis (Lowell and Spiegelman 2000; Rasmusson et al. 2009).

Spring season re-potting by removal of excess rhizome clumps proved important to ensure vigorous growth and flowering. Clumps, removed from the pot, can be most effectively propagated that contained at least two or three rhizome segments (≥0.5 cm diameter) having intact roots, exhibited newly emergent leaves, and most importantly, having one or two healthy rhizome apices. In China and much of Asia where Nelumbo is an important dietary component, large rhizomes (~8 cm diameter; cf. Shen-Miller et al. 1995, Fig. 16) are being routinely harvested in late fall from lakes and ponds (e.g., HongHu, Hupei Province) and processed for distribution (Tele Images-Nature 2003).

Fruit and Pericarps

The architecture of the pericarp of Nelumbo fruit is designed to preserve the longevity of the seed, as they are typically subjected to repeated periods of cold, drought and other stresses. This architecture is robust and tightly sealed, impermeable to water. In the following, we present findings of water-repellent anatomy of the pericarps of modern immature (green) Nelumbo fruit collected at the Kenilworth Aquatic Gardens and modern mature and centuries-old fruit from the lakebed at Xipaozi, and mechanical properties of whole fruit of Nelumbo and modern and old fruit-pericarps.

Materials and Methods—Pericarps and Whole fruit

The pericarps from green Nelumbo fruit of Kenilworth Aquatic Gardens were peeled and removed, fixed in 2 % glutaldehyde-paraformaldehyde with 0.5 M PIPES buffer, post-fixed in 2 % osmium tetroxide, dehydrated in a graded acetone series, and embedded in Spurr’s firm-resin (Dannenhoffer and Shen-Miller 1993). The resin-embedded pericarps were then sectioned with a glass knife and the mounted sections were stained with aqueous 0.5 % basic Fuchsin (Johansen 1940) and examined by light microscopy

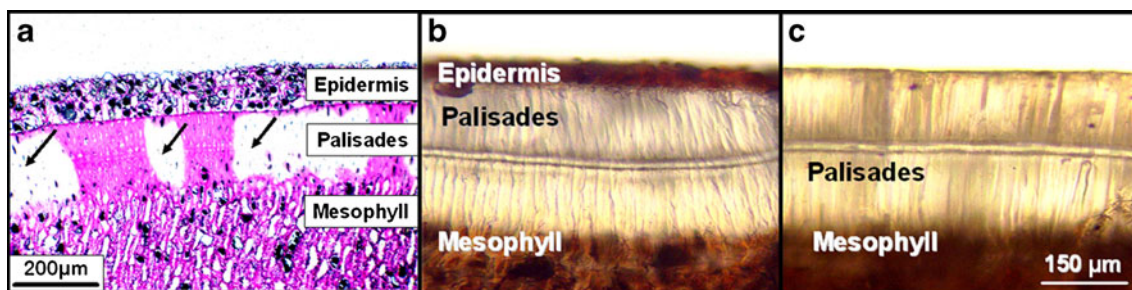


Fig. 8 Pericarps of immature (*green*) and mature modern *Nelumbo* fruit compared with that of a centuries-old *Nelumbo* fruit. **a** A vertical section of the immature green pericarp of a modern fruit from the Kenilworth Aquatic Gardens, Washington, DC, showing its epidermis, palisades, and mesophyll sclerenchyma. The white regions, denoted by *arrows*, are intact suberin-rich palisade cells not stained by the aqueous dye whereas neighboring cells, their water-repellent suberized lamellae having been cut away during sectioning, stained pink; the “zipper-like” macrocleride cellular band dividing the palisades into two zones and

discernible in the pink-stained areas of this immature pericarp is more distinct in palisades of mature fruit (**b** and **c**). **b** An unstained comparable section of a mature modern pericarp of China Antique from Xipaozi showing its three cell layers (outer epidermis, palisades, mesophyll sclerenchyma); the palisade layer exhibits a prominent zipper-like band of macrosclereids that subdivides it into parallel zones. **c** The unstained pericarp of a 549-year-old fruit of China Antique from Xipaozi that, unlike modern fruit, lacks an outer epidermal layer

(Leitz Ortholux II; Fig. 8a). For comparison with these sections of green pericarps, the moistened, hard pericarps of a modern mature fruit and a 549-year-old fruit var. China Antique collected at Xipaozi were sectioned by use of a razor blade, mounted on glass slides, and the unstained sections were studied by light microscopy (Fig. 8b, c).

Initial mechanical tests of the hardness of the tissues of whole fruit of modern China Antique were carried out in duplicate at the USDA-ARS Postharvest Quality and Genetic Research Unit (Parlier, CA). Measurements were made by use of an Instron-4200 instrument having a steel pin-punch probe that applied force at a constant speed as it penetrated through the pericarp on one side of a fruit, traveled through the cotyledons and embryo axis, and exited through the pericarp on the opposite side of the fruit (Aung et al. 1996). The results are shown in Fig. 9 in which the measured load is plotted in units of kilogram force, kgf, recorded over the travel-time of the probe.

The mechanical properties of the pericarps of a modern and a 464 year-old *Nelumbo* fruit were analyzed in triplicate at the Nanomechanics Research Laboratory, Hysitron Inc. (Minneapolis, MN). Nano-indenting instruments (a 3D Omni Probe, and the Berkovich probe of a 300–500 nm TI 900 Triboindenter; Hysitron Inc. 1997) and a “load-controlled partial-unloading” technique that achieved a maximum load of 1000 mN (milliNewton) were used to measure the reduced modulus (an indicator of elastic stiffness) and hardness at various depths within the pericarps (Fig. 10). Optical images of the surfaces of the modern and old pericarps were acquired before and after indentation (Fig. 11).

Results—Pericarps and Whole Fruit

Anatomy—Green Pericarp

The exterior region of vertical sections of *Nelumbo* green pericarps peeled from immature fruit harvested at the Kenilworth Aquatic Gardens was shown to be composed of three principal layers: an outermost epidermis; an immediately underlying palisade layer of compact elongate cells divided in half by a “zipper-like” band into two parallel cellular zones; and a more interior mesophyll sclerenchyma (Fig. 8a). Interior to these, the entire pericarps included two additional layers, a vascular layer (~1.0 to 0.50-mm thick) and an inner epidermis (0.10 mm thick).

In such green pericarps, the fleshy mesophyll sclerenchyma layer was typically the most prominent, varying from ~2.0 to ~0.43 mm in thickness. However, the zipper-like band of macrosclereids (Ohga 1926) that subdivided the ~0.27- to ~0.05-mm-thick palisade layer was less evident than in comparable sections of mature modern fruit (Fig. 8b). The pericarps both of immature and mature modern *Nelumbo* fruit were enclosed by a well defined cutin-rich outer epidermis (Figs. 8a, b). The thickness of the epidermis of the green fruit varied, being typically ~0.04-mm-thick but thicker near the stigmata end ~0.4-mm (Figs. 4 and 5, Shen-Miller et al. 1995).

The immature green pericarps contained patches of white palisade cells not stained by the aqueous dye (Fig. 8a, arrows), indicating that their suberin-covered lamellae around the cell wall (Shaw 1929) were impervious to water, whereas neighboring cells, cut during preparation and devoid of such suberin-coated lamellae, stained pink. This differential staining

evidences the water-impermeability of *Nelumbo* pericarps, a major factor contributing to the long-term integrity of *Nelumbo* seeds and their lack of germination prior to abrasion.

In addition to the occurrence of epidermal waxy cutin and water-resistant suberin-lined palisades and macrosclereids (robust, secondarily lignified fibrous cells; Ohga 1926; Shaw 1929; Nelson and Schuler 2013), a further water-repellent feature of the *Nelumbo* pericarp is the presence of latex. Both species of *Nelumbo* produce latex, an important component not only of the fruit-coat pericarp but of underground rhizomes, petioles, leaves and flowers (Esau and Kosakai 1975). The abundance of such latex is vividly illustrated in the *Tele Images-Nature* (2003) documentary in which a freshly snapped *Nelumbo* stem from a tributary of Anzhi River at Xipaozi Village (Fig. 4) is shown to ooze with latex from its broken ends, reminiscent of a classical Chinese phrase—“The embracing silk of a broken rhizome”—in reference to determined lovers who though separated by distance remain connected, their bond unbroken.

The latex-forming cells (laticifers) of *Nelumbo* are most prominent in the vascular bundles, but they occur also in ground tissues (Esau and Kosakai 1975). The laticifers of *Nelumbo*, like those of latex-rich *Hevea brasiliensis*, have been shown to contain dense uniform-sized particles of rubber (Esau and Kosakai 1975), a nonpolar and highly viscoelastic material. In addition to being impervious to water, latex is reported to provide protection from insects and fungi (Agrawal and Konno 2009).

Anatomy—Mature Pericarp

The pericarp was shown to comprise approximately one-third of the dry weight of a mature fruit of *Nelumbo* China Antique. Comparison of the anatomy of the pericarp of a mature modern fruit (Fig. 8b) and a 549-year-old fruit from Xipaozi (Fig. 8c) showed that both contained mesophyll sclerenchyma and palisade layers but that the old fruit lacked a cuticle-covered exterior epidermis. The lack of this outer epidermal layer, typical of such centuries-old fruit, gave them a shiny exterior (cf. Shen-Miller et al. 1995, Fig. 1), a characteristic that, as discussed above, was useful in distinguishing old fruit from their modern counterparts. The lack of occurrence of this layer in old fruit studied (e.g., Figs. 8c and 11b; Fig. 1, Shen-Miller et al. 1995) suggests that its absence reflects disintegration of the cutin-rich epidermis during aging. Regardless of the cause, its absence in the old fruit indicates that the robust suberin-rich and macrosclereid-containing palisade layer, infused with latex, is the primary water-repellent barrier that protected the interior of such fruit from becoming water-logged and decayed over hundreds of years of quiescence, thereby enabling the long-term viability of the fruit.

Mechanical Properties of Modern Whole-Fruit

As is graphically presented in Fig. 9, the hardness of whole fruit of modern *Nelumbo* was measured by use of a test probe that entered each fruit at a constant speed from the proximal side of a specimen (“the outer pericarp,” “Probe Outside-In →”) and punctured the pericarp (at a peak-force of ~2.72 kgf=27.2 Pa). As the probe traversed the interior of the fruit the load rapidly decreased to ~0.5 kgf (~5 Pa) and remained approximately constant as it passed through the cotyledons and embryo-axis. When the probe then encountered and punctured the pericarp at the distal side of the fruit (Fig. 9, the “inner pericarp,” “Probe Inside-Out→”) the load rapidly increased to a breakpoint of ~2.25 kgf (~22.5 Pa).

Results obtained from two sets of analyses were similar (Fig. 9), specimen 2 having a stronger shell pericarp that took a bit longer and needing a higher load to be punctured (“breakpoint”) from the outer pericarp (Fig. 9). Essentially similar load-levels were required on the tissues of the two fruit for reaching the breakpoints of the pericarp from outside and from within, respectively ~2.72 kgf and ~2.25 kgf. The small difference presumably reflected the cracking and weakening of the fruit during initial penetration. The results also showed that the hardness of the exterior pericarp (~2.7 kgf) of such fruit is about five times greater than that of their interior seed tissues (~0.5 kgf).

Micro-mechanical Properties of Old and Modern Pericarps

The pericarp is the first line of defense that protects the much fragile *Nelumbo* embryo axes and cotyledons (Fig. 9) from damage, and a major factor responsible for the exceedingly long-term viability of such fruit. Not only does its impermeability to water provide a primary protective shield but it also contains procyanidins, condensed tannins that function as strong antioxidants and free-radical scavengers (Ling et al. 2005), consistent with the analyses of Van Bergen et al. (1997). The presence of such antioxidants is a likely contributor to the centuries-long viability of *Nelumbo* fruit.

Some properties of the pericarps of the old fruit from Xipaozi have changed over hundreds of years of aging. Unlike modern fruit, the old fruit lack an exterior epidermis and cuticle (Fig. 8c) and, after being filed at one end to promote imbibition for germination, the pericarp at the filed edge of old fruit rapidly frayed when soaked in water (Fig. 6, old fruit) whereas this tissue remained intact in similarly treated modern fruit (Fig. 6, center).

Age-related change in the robustness of the pericarps of the old fruit appeared not to have affected the water impermeability of the fruit coats or the viability of their enclosed seeds. The filed and imbibed fruit illustrated in Fig. 6, representative of 19 old and 27 modern fruit tested for

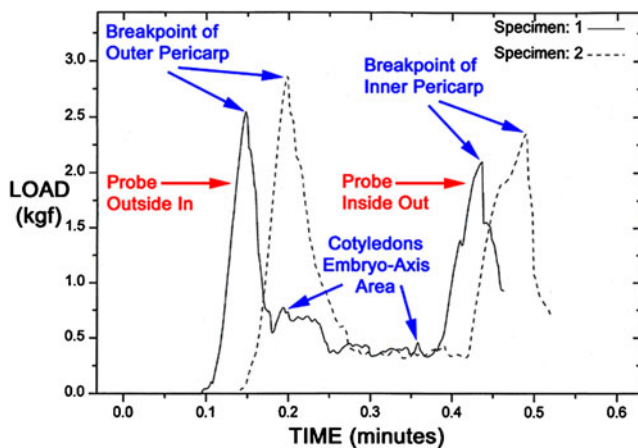


Fig. 9 Mechanical properties of whole fruit of modern *Nelumbo* var. China Antique, analyzed in duplicate by use of an Instron-4200 probe, showing measurements of hardness in units of kgf (kilogram force) recorded over the travel-time of the probe as it passed through fruit tissues; see text for explanation and discussion

germination (Tables 1 and 2). The degree of fraying of the pericarp of the old fruit during imbibition was somewhat variable. Of these four specimens (Fig. 6), the fruit second from the left (OL05-PC-2), having an age of ~448 year, and that at the far right (OL05-PC-6), ~556-year-old, were the most severely frayed. Though both of these fruit were brought to germination (OL05-PC-2 in 3 days, and PC-6 in 7 days), neither survived planting. Both of the somewhat less frayed old fruit shown in Fig. 6—the fruit at the far left (OL05-PC-1), ~432 year-old, and that second from the right (OL05-PC-4), ~543-year-old—germinated in 3 days and developed into healthy plants (Fig. 2b, OL05-PC1). Though a reliable indicator of an old rather than modern age, the data available are too few from the fruit analyzed to conclude that the degree of pericarp-fraying upon imbibition, the degradation of the fruit coat that it evidently reflects, is a reliable indicator of seedling vigor.

Results from analyses of the reduced modulus and hardness of the mature pericarps of an old and a modern *Nelumbo* var. China Antique fruit, analyzed in triplicate, are shown in Fig. 10. The probe contact-depths vs. the reduced modulus (Fig. 10a) measured the elastic stiffness of the specimens whereas Fig. 10b shows the relation between the contact-depth and hardness of these pericarps. Because differences in anatomy between the old and modern pericarps (Fig. 8b, c), and perhaps coupled with their degree of dehydration, a higher probe pressure was initially required to penetrate to comparable depths the palisade-covered 464-year-old fruit pericarp than the epidermal-covered tissues of its modern counterpart.

The elastic stiffness (~24 GPa) and hardness (~0.45 GPa) of the palisade-covered 464-year-old fruit pericarp (Fig. 10) were twice as great as those of its epidermis-covered modern counterpart (~10 GPa and ~0.25 GPa, respectively). Thus, the

pericarp of the old fruit was both stiffer and harder than the modern fruit. These characteristics may be a result of its resilient chemistry, anatomy and, perhaps, dehydration over centuries of aging. Interestingly, these measurements of the mechanical properties of the water-impermeable encompassing coat of the old fruit showed it to be comparable in stiffness and hardness to the antlers of elk—for the old fruit, having a reduced modulus of ~24 GPa vs. ~20 GPa for elk, and a hardness of ~0.45 GPa vs. ~0.54 GPa for elk (Chen PY, 2008, pers. comm. to JSM). The epidermis-lacking pericarp of the old *Nelumbo* fruit was not only impermeable to water but physically robust.

Optical Images of Surfaces of Old and Modern Pericarps

Optical images of the fruit-coat surfaces of these old and modern *Nelumbo* fruit before and after micro-mechanical indentation provided additional information about their anatomical and physical properties. As is shown in Fig. 11a, b, the epidermis-lacking surface of the pericarp of the 464-year-old fruit was composed of shiny smooth palisade cells (Fig. 8c; cf. Shen-Miller et al. 1995, Fig. 1) in which the pock-like circular pores were evidently suberin-infused stomates (Fig. 11a; Shaw 1929), and the triangular impression (Fig. 11b, arrow) marking the site of micro-indentation. In contrast, the pericarp of the modern fruit (Fig. 11c, d) is enveloped by a cutin-rich waxy epidermal layer (Fig. 8b; cf. Shen-Miller et al. 1995, Fig. 4). Evidently, the greater elasticity of the epidermis and overlying cuticle of the modern specimen erased evidence of the indentation by which it was analyzed (Fig. 11d). These images, consistent with those of vertical sections of old and modern *Nelumbo* pericarps (Fig. 8), further document the differing properties of such tissues.

General Discussion

Shoot-before-Root Germination

Seed-germination in most plants begins with the protrusion of roots (Bewley and Black 1994; Rajjou et al. 2012), whereas the shoot-before-root germination exhibited by *Nelumbo* occurs rarely in common crops (Dakshini and Tandon 1970; Kordan 1977; Wijte and Gallagher 1996). In *Nelumbo*, the emergence of fibrous roots from the basal radicle of its embryo axis occurs only 1 to 2 weeks after shoot emergence, the protrusion and extension of a primordial rhizome from the radicle occurring still later. Subsequently, roots and leaf-bearing shoots arise from successive nodes of its rhizomes (Fig. 7c) and the youngest apical nodes extend new growth at the rhizome apex. The unusual shoot-before-root germination of *Nelumbo* may reflect an atypical hormonal feedback between auxin (IAA, indole-3-acetic acid) and cytokinin (Muller and Sheen 2008; Moubayidin et al. 2009) not exhibited by most other angiosperms.

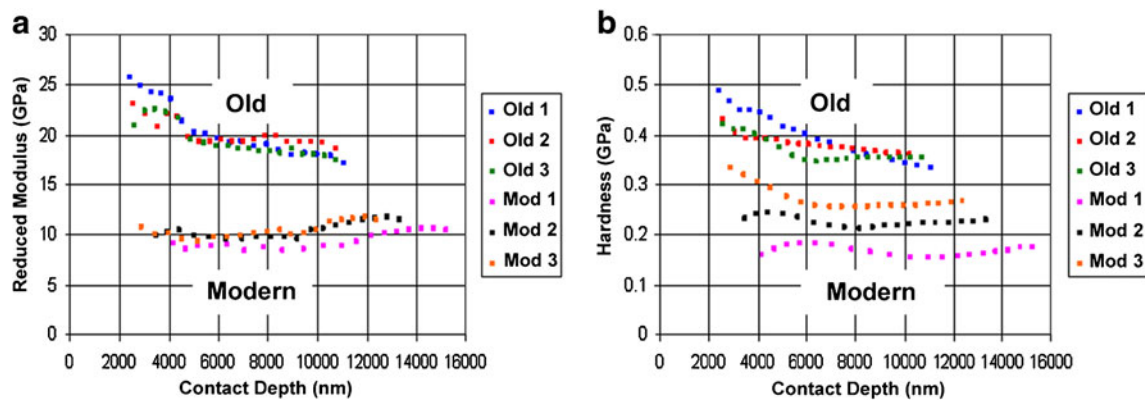


Fig. 10 Mechanical properties of the pericarps of a 464-year-old and a modern fruit of *Nelumbo* var. China Antique, analyzed in triplicate by use of a Hysitron Triboindenter 900 instrument, showing measurements

of the reduced modulus (**a**) and hardness (**b**) in units of GPa (GigaPascal); see text for explanation and discussion

Green Shoot at Germination

The germination of *Nelumbo* fruit also differs from that of most other angiosperms by commencing with the sprouting of a chlorophyllous green plumule (Fig. 7a), a trait resulting in immediate photosynthesis of adaptive value. *Nelumbo* thus differs distinctly from most crop plants, especially monocots in which the primordial leaves are typically yellow. Although so-called “stay-green seeds,” like those of *Nelumbo*, are not especially uncommon (Janzen 1982; Thomas and Smart 1993), mature *Nelumbo*-like seeds that contain green embryo axes are rare. The “green seeds” reported from other plants

have been shown invariably to be immature (Ruska et al. 2004), seeds that prior to germination become apochlorotic or dedifferentiated (Casadoro et al. 1980). Of the few reported taxa having mature seeds that exhibit green embryo axes, most are xerophytes and/or halophytes subjected to drought and high salinity (Janzen 1982; Raghavan 2002; Zhang et al. 2010; Wu et al. 2012), plants adapted to settings in which embryonic photosynthesis at low light intensities is of selective advantage (Zhang et al. 2010).

Photosynthesis of the *Nelumbo* plumule begins upon germination (Ushimaru et al. 2001). Light-harvesting chlorophyll a/b, cytochrome c, chlorophyll-binding protein 1, and a/b-

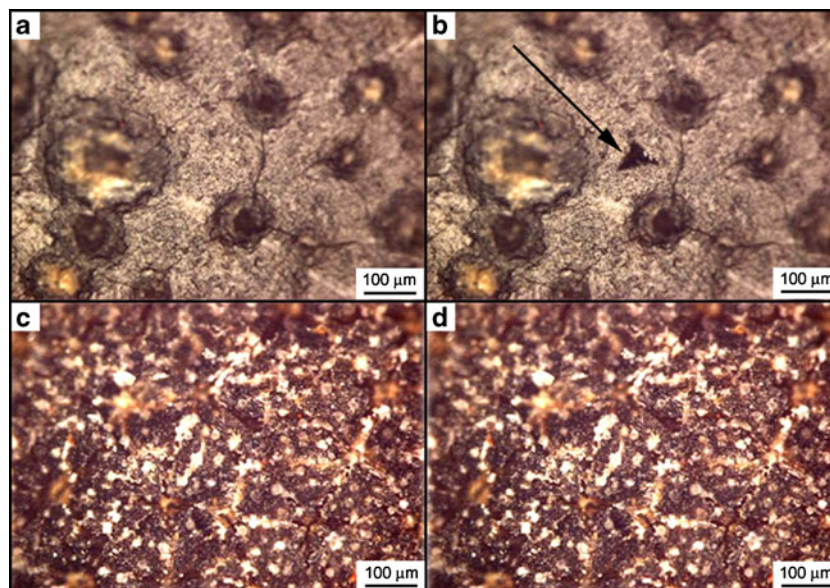


Fig. 11 Optical photomicrographs comparing the pericarp surface of old and modern mature *Nelumbo* var. China Antique fruit before (**a** and **c**) and after (**b** and **d**) micro-indentation. **a** and **b** The pericarp of a 464-year-old fruit from Xipaozi lacks an epidermis and is pock-marked by circular subrinsealed stomatal pores (cf. Shen-Miller et al. 1995, Fig. 1). (**c** and **d**) The

pericarp of a modern fruit from Xipaozi has a smooth flexible cutical/epidermal surface (cf. Shen-Miller et al. 1995, Fig. 4). The *arrow* in (**b**) points to the probe-mark produced by micro-indentation of the pericarp of the old fruit, a mark not discernible in the image of the modern pericarp (**d**)

binding proteins have been detected in the dry green tissue of mature *Nelumbo* embryo axes (Ushimaru et al. 2003). The plumules of dry *Nelumbo* seeds have been shown to contain chloroplasts that exhibit a rudimentary lamellae stack (the components of a “prolamellar body”) as well as large starch-grain amyloplasts. Even in dark-grown *Nelumbo* seedlings the chlorophyll a/b ratio is ~1, although their total a/b content is less than that of a comparable light-grown seedling (Ushimaru et al. 2003). After germination in total darkness, the chloroplasts of a 5-day-old *Nelumbo* seedling have been shown to undergo differentiation into orderly thylakoids, complete with well-defined grana and starch granules (Ushimaru et al. 2003). These data suggest that mature chloroplasts can develop in seedlings of *Nelumbo* grown in darkness or very low light, conditions not dissimilar from those experienced by the sediment-enclosed centuries-old Xipaozi fruit (Fig. 7a). This unusual characteristic of *Nelumbo* chloroplasts may have resulted from the whole-genome duplication ~65 million-yr ago (Ming et al. 2013), an adaptation that would have bolstered survival under the “dim skies” produced worldwide by the massive impact event that marks the Cretaceous-Tertiary boundary (Fawcett et al. 2009).

Because of the presence of chlorophyll in embryo axes of the seeds of China Antique and concern that photosynthetically incorporated ¹⁴C-bearing CO₂ could affect their radiocarbon ages, the initial batch of old fruit analyzed (Table 1) was germinated in total darkness and observed under a green safe-light (Shen-Miller, et al. 1995). Subsequent use of highly sensitive AMS to radiocarbon dating the peeled non-chlorophyllous pericarps (dead tissues) of mature *Nelumbo* fruit showed this precaution to be unnecessary (Shen-Miller et al. 2002; Tele Images-Nature 2003).

Nelumbo Fruit

The comparatively prolonged germination period of the modern *Nelumbo* fruit may be a result of their relative immaturity and/or time used for cellular repair (Table 2). The lack of maturity in modern fruit is further evidenced in the lower heat-stability of proteins in their embryo axes and cotyledons (Shen-Miller et al. 2013, Table 1).

The germination rate of 84 % for the centuries-old fruit, like the 92 % rate for their modern controls (Table 1), is similar to that of many 2- to 3-year-old modern seeds (Priestley 1986). Like other centuries-old germinating *Nelumbo* fruit, the 482-year-old (OL96-57) illustrated in Fig. 7a on day-2 exhibited a green plumule that was curved, implying a gravitropic response. This response indicated that after nearly five centuries of aging the physiochemistry of this fruit had remained intact, capable of sensing and responding to gravity and to the plant-growth hormone IAA (Naqvi and Gordon 1966; Davies 2004).

Conclusions

The genetics of humans and other animals are widely regarded to be a prime determinant of lifespan (Grady et al. 2013), the remainder being related to environment and lifestyle. For plants, given their sessile habit, genetics may play an even greater role. The recently sequenced and annotated genome of *Nelumbo* (Ming et al. 2013) has shown it to exhibit an exceptionally low content of nucleotide mutation (perhaps evidencing unusually effective nucleotide-repair mechanisms or superior gene construct) and to evidence a unique gene expansion (particularly of genes related to the biosynthesis of waxes [Nelson and Schuler 2013] and adaptation to aquatic environment). Such traits can be expected to help elucidate the centuries-long viability of its fruit and, perhaps, to contribute to the breeding (genetic engineering) of other crop plants and enhancement of their seed shelf-life and food quality.

Nelumbo exhibits numerous unusual characteristics: the presence of a structurally and chemically water-impervious robust pericarp that protects the enclosed embryo; a prolonged period of maturation and repair prior to germination; shoot-before-root germination; a green embryo axis at maturity capable upon germination of dim-light photosynthesis; a unique mechanism of thermal-regulation in flowers primed for pollination that in other organs might be important for stress abatement; and the maintenance of fruit-viability for hundreds of years under conditions of cold, drought and compaction in anoxic sedimentary environments. Such characteristics, combined with the recent documentation of the *Nelumbo* genome (Ming et al. 2013), suggest that this long-cultivated crop plant could provide a pivotal role in improvement of the quality, stress-adaptation and seed-longevity of other agricultural species.

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