Molecular Identification of *Malus hupehensis* (Tea Crabapple) Accessions Using Simple Sequence Repeats

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Abstract. The U.S. National Plant Germplasm System (NPGS) currently holds 36 separate accessions of the ‘Yichang’ clone of *Malus hupehensis* (Pamp.) Rehd. The ‘Yichang’ clone originally entered the United States in 1908 as seed collected for the Arnold Arboretum by E.H. Wilson near Yichang, Hubei Province, China. The original description of *M. hupehensis* omits fruit characters, and botanists frequently augment these omissions with descriptions of the ‘Yichang’ clone. Apomixis occurs in *Malus*, including *M. hupehensis*, and is strongly associated with elevated ploidy levels. Simple sequence repeats (SSRs) were used to characterize 65 accessions of *M. hupehensis*. To check for polyploidy, a set of *M. hupehensis* accessions was evaluated with flow cytometry. The simple sequence repeat phenotypes and ploidy information revealed the ‘Yichang’ clone under various accession names in arboreta. It was neither known nor suspected that the U.S. National Plant Germplasm System held many duplicate accessions of the ‘Yichang’ clone prior to their molecular characterization. Germplasm conservation decisions for *Malus* species can benefit from an increased knowledge of the genetic variation or lack thereof in naturalized populations and ex situ collections.

Malus hupehensis (Pamp.) Rehd. [*M. theifera* Rehd., *Pyrus theifera* Bailey] is naturally distributed in 17 provinces in China (Zhou, 1999) and cultivated as a rootstock and an ornamental. Young leaves of *M. hupehensis* can be dried for brewing as an herbal tea, a use reflected in one of its common names, tea crabapple (Chun, 1921). Dermen (1936), Sax

(1959), and Zhou et al. (1995) consider *M. hupehensis* to be a triploid species with facultative apomixis, although diploid forms have also been reported (Schuster and Buttner, 1995). The use of apomictically derived seeds from *M. hupehensis* and other *Malus* species to propagate uniform, disease-free rootstocks has been investigated, since seed transmission of viral diseases has not been observed in *Malus* (Olien, 1987). While the facultative and polygenic nature of apomixis in *Malus* (Schmidt, 1977) has thus far precluded its use as a commercial rootstock, this germplasm remains an important source of genes for mildew resistance (Batlle and Alston, 1994).

Characterization of germplasm resources is a crucial step toward their maintenance and utilization. Simple sequence repeats (SSRs) or microsatellites are one of several recently developed molecular markers for identifying clones (Guilford et al., 1997; Lamboy and Alpha, 1998; Nybom, 1990) and for quantifying genetic variation within germplasm collections (Hokanson et al., 1998). These SSRs are non-transcribed tandem repeats of two to five base pairs. Methods for detecting length polymorphisms at SSR loci are preferred tools for DNA fingerprinting because SSRs are hypervariable, widely distributed in eukaryotic genomes, and display codominant segregation patterns (Karp et al., 1997).

Curatorial decisions in the U.S. National Plant Germplasm System (U.S. NPGS) *Malus*

(apple) collection have been aided by the analysis of SSR phenotypes to identify cultivar synonyms and misidentified accessions (Hokanson et al., 1998). In a set of 142 accessions representing 23 *Malus* species, two accessions of *M. hupehensis*, PI 588760 and PI 589522, shared SSR fragment length phenotypes at eight loci, strongly suggesting that they were clones (Hokanson et al., 2001). Ideally, wild material should enter a repository with extensive passport information, including the latitude, longitude, and elevation of the collection site, a description of the vegetation and physical attributes of the collection site, and name(s) of the collector(s). None of these collection descriptors was available for PI 588760 or PI 589522.

Molecular characterization of in situ populations or their progeny may aid in setting conservation priorities for unique lineages or habitats supporting diverse populations. Land use changes are inevitable along the Changjiang River in China as an estimated 1.3 million people are resettled in preparation for the flooding needed to operate the Three Gorges Dam (Ash, 1998). Areas of the Yubei District of Chongqing are expected to be inundated after the completion of the dam, with possible impacts on an in situ *M. hupehensis* population. While the Chinese National Academy of Science is taking steps to cultivate rare, endemic plant species of the Changjiang River Valley (Lei, 1998), *M. hupehensis* is neither rare nor highly endemic (Zhou, 1999). Molecular characterization of plants at this site and others would provide base-line data on the level of genetic variation in naturalized populations prior to development efforts.

There are special problems identifying the species boundaries of *M. hupehensis*, which has been described either as highly variable (Rehder, 1916; Zhou, 1999) or extremely uniform (Dermen, 1936; Zimmerman, 1971) depending on the reference. These contrasting views appear to reflect the characterization of plants from multiple hybridization events or the uniform derivatives of apomictic reproduction. The debate as to whether to lump or split groups at the species level can be taken to extremes with apomictic genera that produce a multitude of stable, distinct clonal forms or agamospecies (Ramsey and Schemske, 1998). Molecular characterization may be useful for identifying synonymous agamospecies, estimating the geographic range of a single clone, or detecting clonal substructure in natural populations (Nybom, 1996).

Taxonomy is ill served by original species definitions based on incomplete information. Pampanini (1910) did not include fruit characters in the *M. hupehensis* species description, although the ovary is described as ovate and glabrous, with three styles connate at the base. Filling the vacuum, Rehder (1916, 1949), Chun (1921), and Liberty Hyde Bailey Hortorium (1976) described the fruit as small, globose, and green-yellow with red blush. In contrast, *M. hupehensis* accessions in several European arboreta have true red fruit (Coombes, 1992).

The specific objectives of this study were to: 1) document the SSR fragment length phe-

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notypes of 65 accessions of *M. hupehensis* at eight SSR loci; 2) estimate ploidy by flow cytometry in a set of 42 accessions; and 3) evaluate the resulting data set in light of morphological and historical records. The research was conducted to facilitate more effective in situ and ex situ conservation of this taxon.

Materials and Methods

Plant materials. Leaves were collected from accessions of *M. hupehensis* from several sources (Table 1). Historical records tracing the entry of specific accessions of *M. hupehensis* into arboreta were challenging to unravel. Between 1899 and 1910, E.H. Wilson made several plant expeditions to western China sponsored first by J. Veitch and Son, England, and later sponsored by the Arnold Arboretum, Jamaica Plains, Mass. (Sargent, 1916; Wilson, 1913). *Malus hupehensis* Arnold Arboretum (AA) accession 7241 was received in 1908 from seed collected directly in the wild near Yichang, Hubei Province and represents E.H. Wilson Collection No. 451 (Fig. 1). Sargent (1916) further describes Wilson Collection No. 451 as a bush type 1–4 m tall, flowers white, tinged pink, growing north and

south of Yichang at 1000–1600 m elevation. R6T8-SF in this study was propagated from seed gathered from AA accession 7241 in 1965.

The Arnold Arboretum propagated *M. hupehensis* AA17474-1 from a plant received in 1907 from J. Veitch and Son, England, which was collected by E.H. Wilson in China. The precise locality of the original collection is unknown. R6T1-SF and R3T3-SF in this study were grown from seed gathered in 1965 from AA17474-1.

The Shennongjia seedling used in the current SSR study, and *M. hupehensis* P.I. 590052, P.I. 590053, and P.I. 590057 included in the flow cytometry characterization, were grown from seed gathered in the Shennongjia Forest District, northwest Hubei Province (Fig. 1). American cooperators participating in this plant exploration were unwilling to assign a species name to this accession, but the Chinese botanists were certain this accession could be identified as *M. hupehensis* (Bartholomew et al., 1983). The fruit of these trees are red.

Sixteen *M. hupehensis* seed lots, Geneva Malus (GMAL) 4423–4439, were germinated from seeds collected in 1997 on a hillside in the Yubei District of Chongqing (Fig. 1). A

separate GMAL number was assigned to the progeny of each of the 16 maternal trees; therefore the seedlings in one lot are at least half-sibs. This site was designated Field Collection Population Number 7 by the U.S. Dept. of Agriculture (USDA)–Southwest Agricultural Univ. Joint Plant Exploration of Sichuan Province. Global positioning system readings for this population were lat. 29°54′44.1″N, long. 106°40′45.5″E at an elevation of 870 m. Leaves from 34 *M. hupehensis* trees were collected and dried for DNA extraction and SSR characterization, whether or not the trees were bearing fruit. Maternal trees growing in China and a field planting of seed-derived progeny at USDA-ARS, Plant Genetic Resources Unit, Geneva, N.Y., displayed remarkable morphological uniformity.

The accession identified as Cornell Univ. Arboretum *M. hupehensis* grows near the entrance to the arboretum in Ithaca, N.Y. The Cornell Arboretum acquired this tree from a commercial nursery (Table 1).

SSR characterization. Genomic DNA was extracted from ≈100 mg fresh or 45 mg dried leaf tissue using the protocol described by Lamboy and Alpha (1998), which was devel-

Table 1. *Malus hupehensis* accession sources, morphological characters, and SSR fragment length phenotypes.

Accession	Source	Fruit color	Vegetative traits	SSR phenotype ^a
Long Ashton seedling	USDA, ARS Fruit Laboratory, Beltsville, Md. From A.I. Campbell, Long Ashton Research Sta.	Red	---	A
Shennongjia seedling	Grown from seed gathered by 1980 Sino-American Botanical Expedition to Western Hubei Province, China	Red	---	B
Tissue culture regenerant	Dr. Suman Singha, Original plant from Mellinger's Nursery, Ohio	Green-yellow with red cheek	---	C
9988(1)	Dr. B. Suszka. Kórnik Arboretum, Poland. Kórnik received as seed in 1950 from Wageningen	---	Lvs. elliptical, entire	D
9988(2)	Dr. B. Suszka, as above.	---	Lvs. elliptical, entire	D
9988(3)	Dr. B. Suszka, as above.	---	Lvs. elliptical, entire	D
'Normal Triploid'	H. Schmidt through F. Dunemann, Institute for Ornamental Plant Breeding, Ahrensburg, Germany	Dull red ^a	Thorny juvenile growth ^{a, w}	D
Sarstedt/Dahlem	H. Schmidt through F. Dunemann, as above.	Red ^x	Lobed lvs. ^x	E
80BS34301	Agricultural Univ., Wageningen, The Netherlands	---	---	F
92BS-WAG17	Agricultural Univ., Wageningen, The Netherlands	---	---	G
Rootstock breeding line	Dr. S.Y. Wan, Huazhong Agricultural Univ., Wuhan, Hubei Province	Yellow with red cheek ^a	Lvs. entire, serrulate margin	H
Commercial line	F.W. Schumacher Co. Sandwich, Mass.	---	---	I
82BS28204	Agricultural Univ., Wageningen, The Netherlands	---	Lvs. entire, ovate, serrulate margin	J 'Yichang type'
R6T1-SF	USDA, ARS Fruit Laboratory, Beltsville, Md. Grown from seed gathered from AA 17474-1	Green-yellow with muddy purple cheek	---	J 'Yichang type'
R6T8-SF	USDA, ARS Fruit Laboratory Grown from seed gathered from AA 7241	Green-yellow with muddy purple cheek	---	J 'Yichang type'
R3T3-SF	USDA, ARS Fruit Laboratory Grown from seed gathered from AA 17474-1	Yellow	Lvs. entire, ovate, serrulate margin	K 'Yichang + type' ^v
Plant Introduction (PI) 588760	USDA, ARS Plant Genetic Resource Unit, Geneva, N.Y. Received from Oregon State Univ.	Green-yellow with red-brown cheek	Lvs. entire, ovate, serrulate margin	J 'Yichang type'
<i>M. hupehensis</i> 'Rosea'	F.W. Schumacher Co. Sandwich, Mass.	---	Entire lvs.	L 'Yichang + type' ^v
Cornell Univ. Arboretum <i>M. hupehensis</i>	Schmidt and Son Nursery Co., Boring, Ore.	Green-yellow, with red-brown cheek	Lvs. entire, ovate, serrulate margin	J 'Yichang type'
34 trees, Population 7	Yubei District of Chongqing, China	Green-yellow with red-brown cheek	Lvs. entire, ovate, serrulate margin	J 'Yichang type'
12 seedlings GMAL 4435-4437	Seedlings grown from the Yubei District. collection at the USDA, ARS Plant Genetic Resource Unit, Geneva, N.Y	Juvenile trees	Lvs. entire, ovate, serrulate margin	J 'Yichang type'

^aDifferent letters denote unique SSR phenotypes.

^wData not collected.

^xData provided by cooperator.

^v*Malus* does not develop true thorns. The numerous aborted branchlets on some wild and cultivated varieties are referred to as thorns.

^vSSR phenotype includes all the fragments from the 'Yichang' phenotype and additional fragments.

Fig. 1. *Malus hupehensis* collection sites in Southern China: 1) Yichang, vicinity of the E.H. Wilson collection, 1908; 2) Shennongjia Forest District (pattern area), 1980 Sino-American Botanical Expedition; 3) Chongqing Administrative Zone, inclusive of the Yubei District, 1997 Southwest Agricultural Univ. collection.

oped for leaf samples rich in PCR-inhibiting polysaccharides and polyphenolics. To increase DNA yield from dried samples, the protocol was modified by overnight rehydration of ground leaf tissue in buffer solution.

Szewc-McFadden et al. (1995) designed the eight SSR primer pairs used in this study from a guanine-cytosine enriched genomic DNA library of *M. domestica* Borkh. 'Golden Delicious' (Table 2). To allow use of an automated fluorescence detection system, forward primers were labeled with dyes TET, HEX, or 6-FAM (PE Applied Biosystems, Foster City, Calif.). Reagents for PCR described by Hokanson et al. (1998) were modified for reactions primed by single primer pairs. Although the primer pairs were designed to accommodate multiplex reactions, single primer pair reactions yielded higher signal with less noise. Primer pairs GD 12, 15, 96, 100, and 103 were placed in separate 25- μ L reaction mixtures containing 25 to 50 ng of template DNA, 10–60 pmol of primer, 0.2 mM of dNTPs, 1 $\times$ ThermoPol reaction buffer (New England BioLabs, Beverly, Mass.), and 0.2 units of Deep Vent polymerase (New England BioLabs). Primer pairs GD 142, 147, and 162 were placed in separate 20- μ L reaction mixtures containing 30 ng of template DNA, 20 to 24 pmol primer, 5 mM $MgSO_4$, 0.2 mM of dNTPs, 1 $\times$ ThermoPol reaction buffer, and 0.2 units of Deep Vent polymerase. A Perkin-Elmer Cetus 9600 thermocycler (Perkin-Elmer, Boston, Mass.) was used to achieve the PCR conditions described by Hokanson et al. (1998). Negative controls were run for all primer pairs by substituting filtered, autoclaved, deionized H_2O for the DNA template.

Prepared PCR products were separated by electrophoresis using an ABI Genescan 377 or an ABI PRISM 310 (PE Applied Biosystems). Fragment sizes in mobility units were assigned using GeneScan Analysis Software Version 3.1 or ABI Genescan 672 software version 1.2 (PE Applied Biosystems). The Fortran program 'Binning' written by Warren Lamboy was used to assist conversion of data

in mobility units to whole integer base pair sizes. ABI Genescan 672 output for R6T1-SF, GMAL 4435.C, and PI 588760 were scored independently by two laboratory workers (Tables 1 and 3).

The Fortran program "SSRs" written by Warren Lamboy was used to assist data analysis of identical and nonidentical accession pairs, alleles per locus, and polymorphic information content (PIC) (Röder et al., 1995). The PIC was calculated by the formula $\Sigma[1-(P_i)^2]$, where P_i represents the frequency of each phenotype for the data set under investigation with the 'Yichang' clone represented by a single sample.

Ploidy characterization by flow cytometry. The internal standards *Zea mays* L. 'Ultimate' (5.5 pg DNA/2C) or *Oryza sativa* L. 'Taipei 309' (0.95 pg DNA/2C) were germinated from single seed lots. Genome sizes are conventionally expressed in terms of Swift's constant, C, which measures nuclear DNA content without reference to ploidy level. Leaf tissue of an angiosperm sporophyte is generally 2C, while sexually derived gametes are 1C.

The youngest available fresh leaf tissue was gathered from *M. hupehensis* accessions (Table 4). About 3 to 4 mm² of sample (*M. hupehensis*) and standard (corn or rice) leaf tissue were placed in a petri dish on ice. Sample preparation followed the protocol of Dickson et al. (1992), modified by the use of

plant standards or the unpublished protocol of Kazuo Watanabe. In the Watanabe protocol, leaf tissues were flooded with 1 mL freshly prepared buffer solution (10 mM $MgSO_4 \cdot 7H_2O$ pH 8, 10 mM dithiothreitol, 50 mM KCl, 4.6 mM HEPES, 0.25% Triton X-100®, 1% PVP-40) and chopped with a razor blade. Nuclei liberated into the solution were pipetted through a 10–60 μ m nylon filter and stored on ice. About 30 min before assaying, 5 μ L of 7.5 mM propidium iodide was added to each sample as a fluorochrome. Samples were processed on an EPICS PROFILE (Coulter Electronics, Luton, U.K.) or FACSCalibur (Becton-Dickinson, Franklin Lakes, N.J.) flow cytometer set to monitor red fluorescence (FL 2). Data acquisition was continued for each sample until a minimum of 10,000 events were recorded. FACSCalibur output was processed with CellQuest 3.1 (Becton-Dickinson) software. Sample DNA content was calculated by dividing the mean sample fluorescence by the mean standard fluorescence, then multiplying by the standard pg DNA/2C.

Results and Discussion

Molecular characterization of 65 accessions of *M. hupehensis* revealed 12 unique SSR fragment length phenotypes (Table 1). All pairs of accessions with known morphological differences displayed unique fragment length phenotypes with 3 to 13 alleles per locus (Table 2). A length polymorphism at one SSR locus is sufficient to declare two accessions unique, but the number of indistinguishable loci needed to be confident that two accessions are probably clones depends on knowledge of the allele frequencies in a relevant reference population and linkage among loci. DNA fingerprinting at eight SSR loci appears reasonable. Six unlinked loci in this data set had PIC values exceeding 0.80. Values of PIC range from 0 to 1, increasing as heterozygosity increases, but with less weight given to rare alleles. The probability of matching a pair of accessions at all loci by chance alone is 0.3573×10^{-7} for the 105 possible pairs of accessions used in this analysis.

The 'Yichang' clone. Fifty-one accessions from in situ and ex situ sources displayed SSR fragment length phenotypes identical with that of R6T8-SF (Table 3), a tree whose provenance can be traced to E.H. Wilson collection No. 451 gathered near Yichang, Hubei Province, China. Hereafter, plants matching the

Table 2. Primer pairs used for PCR amplification of *M. hupehensis* total genomic DNA.

Locus	Repeat motif ^a	Linkage group ^b	Alleles per locus	Sample PIC ^c
GD 12	(CT) ₃₂	10	10	0.814
GD 15	(AGC) ₅	NI ^w	3	0.658
GD 96	(TC) ₂₂	1	13	0.839
GD 100	(GA) ₁₂	6	13	0.891
GD 103	(GA) ₂₀	2	4	0.347
GD 142	(TC) ₁₉	3	11	0.8133
GD 147	(AG) ₇	4	11	0.833
GD 162	(GA) ₂₃	5	10	0.855

^aInformation from Hokanson et al. (1998).

^bInformation from Hemmat et al. (1999).

^cPolymorphic information content as described by Röder et al. (1995).

^wNI = Not included in Hemmat et al. (1999).

R6T8-SF SSR fragment length phenotype are referred to as the ‘Yichang’ clone. There are minor variations in the morphological descriptions compiled for the ‘Yichang’ clone (Table 1). The fruit overcolor has been described as red, red-brown, and muddy purple, but fruit overcolor changes in response to canopy shading and light exposure. The different descriptions may reflect phenotypic plasticity in different environments, observations made at different times in the growing season, and different choices of words by botanists. The general morphological similarity among trees of the ‘Yichang’ clone is reflected in the observations compiled by botanists at different locations over the years (Table 5). Distinctive features of the clone include straw-colored triangular seeds inside globose fruit ≈ 1 cm in diameter with prominent calyx scars (Fig. 2). The leaves are thick, with a V-shaped tertiary

structure, foliar stipules that abscise with the leaves, and petioles channeled on the upper surface. In comparison with other crabapples, the ‘Yichang’ clone produces a tree with attractive foliage and moderately early, showy flowers (Fig. 3).

In situ concerns. All 34 trees sampled in the Yubei District of Chongqing and 12 of their seedling progeny displayed SSR fragment length phenotypes matching the ‘Yichang’ clone. Flow cytometry data showed that 35 seedling progeny from 16 different maternal trees growing in the Yubei District were triploid (Table 4). Shared, identical SSR fragment length phenotypes and triploidy are consistent with an apomictic mode of reproduction. Identifying *M. hupehensis* in the Yubei District as members of the ‘Yichang’ clone links these trees to a sample taken over 500 km east along the Changjiang River (Fig. 1). A

tree grown from seed sampled in the Shennongjia Forest District away from the Changjiang possessed a unique SSR fragment length phenotype (Table 1). Three additional accessions germinated from seed collected in the Shennongjia Forest District, PI 590052, PI 590053, and PI 590057, were diploid (Table 4). *Malus hupehensis* has a history of human use and the Changjiang has served as a trade route since ancient times. These two factors make it difficult to dismiss the possibility that people have influenced the distribution of the ‘Yichang’ clone. As the *in situ* population in the Yubei District shows strong clonal genetic structuring, efforts to ameliorate the environmental impact of the Three Gorges Dam will be better focused on aquatic species and rare, endemic plants of the Changjiang River Valley rather than on *M. hupehensis*.

Ex situ concerns. Germplasm curators and

Table 3. SSR phenotypes defined by allele lengths at eight loci for putative members of the ‘Yichang’ clone of *M. hupehensis*

Plant SSR phenotype/accession(s)	Fragment length (bp)							
	GD 12	GD 15	GD 96	GD 100	GD 103	GD 142	GD 147	GD 162
‘Yichang’ type J 51 accessions including R6T8-SF (Table 1)	157, 170	148	147, 151, 162	229, 240	null	138	118, 120, 122	199, 209, 211
‘Yichang’ + type K ‘Rosea’	157, 170	148	147, 151, 162	229, 240	null	138, 158	118, 120, 122, 130	199, 209, 211
‘Yichang’ + type L R3T3-SF	157, 170, 180	140, 148	147, 151, 162, 180	223, 229, 240	104	138	118, 120, 122, 128	199, 209, 211, 225

Table 4. Ploidy estimation by flow cytometry for *M. hupehensis* accessions

Accession(s)	Sample size	Source	DNA/2C nucleus (pg)	Ploidy estimate ^z
PI 590052	1	Grown from seed sampled in the Shennongjia Forest District	1.2–1.5	2x
PI 590053	1			
PI 590057	1			
PI 588760	1	USDA, ARS Plant Genetic Resource Unit, Geneva, N.Y. Received from Oregon State Univ.	2.07	3x
PI 589522	1	USDA, ARS Plant Genetic Resource Unit, Geneva, N.Y. Received from Agriculture Canada Saanichton Plant Quarantine Station	2.16	3x
Cornell Arboretum <i>M. hupehensis</i>	1	Cornell purchased this tree as a sapling from Schmidt and Son Nursery Co., Boring, Ore.	2.42	3x
Half-sib families GMAL 4423-4439	35	USDA, ARS Plant Genetic Resource Unit, Geneva, N.Y. grown from seed sampled in the Yubei District	2.19–2.68	3x
R3T3-SF	1	Grown from seed gathered from AA 17474-1	3.46	4x

^zRanges of DNA content (pg/2C nucleus) were 1.2–1.85 for diploids, 2.07–2.73 for triploids, and 2.8–3.75 for tetraploids.

Table 5. Traits of *M. theifera* Rehd. and *M. hupehensis* (Pamp.) Rehder described by Rehder (1916, 1949) compared with those of the ‘Yichang’ clone accessions in the NPGS

Trait	Description from Rehder (1916)	GMAL 4423 - 4439	PI 588760 PI 589522 ^z
Leaf shape	Ovate, ovate-oblong, to elliptic-ovate	Ovate to elliptic ovate	Ovate standard
Leaf apex	Acuminate	Acuminate	Acuminate
Leaf base	Rounded to broadly cuneate	Rounded, rounded irregular, to broadly cuneate	Rounded to broadly cuneate
Leaf margin	Sharply serrulate	Serrulate	Serrulate
Leaf length	6–8 cm	5.5–7 cm long	---
Leaf pubescence	Pubescent on the veins beneath and finally glabrous	Pubescent, esp. on abaxial veins, becoming glabrous	Pubescent, esp. on abaxial veins, becoming glabrous
Young leaf color	Purple at unfolding, changing to green	Purple at unfolding, changing to green	Young lf. color rated brick-red, older lvs. green
Styles	3 or rarely 4	Juvenile	3 ^x
Blossom color	White, tinged pink	Juvenile	White with pink tinge
Calyx	Deciduous	Juvenile	Deciduous ^w
Fruit size	≈ 1 cm across		1.05–1.2 cm
Fruit shape	Globose	Juvenile	Globose, flat-globose
Fruit color	Greenish yellow with red cheek	Juvenile	Green-yellow with red-brown cheek

^zData from Morphological Characterization 1996 of the *Malus* Core Collection (MORPHOLOGIC 96) posted at www.ars-grin.gov

^yData not collected.

^xSample size of 50 blossoms. Magnification of blossoms at $\times 10$ showed styles connate at the base.

^wObservations by the author (LLB) in 1997 and 1998.

Fig. 2. Fruit characters of PI 589522, an 'Yichang' clone of *M. hupehensis*. SSR fragment length analysis by Hokanson et al. (2001) determined that this accession is a clone of PI 588760. Photo by Steven King posted at www.ars-grin.gov

Fig. 3. Comparison of *M. hupehensis* trees at the USDA, ARS Research Center, Beltsville, Md. (A) 'Yichang' clone R6T1-SF, 15 Apr. 1988; (B) 'Long Ashton' tree, 15 Apr. 1988; (C) 'Yichang' clone R6T1-SF, 8 Oct. 1985; (D) 'Long Ashton' tree, 8 Oct. 1985. Photos by Richard Zimmerman.

crop conservation committees frequently tally the number of accessions per species to identify gaps in the collection for further acquisition efforts. The discovery of at least 36 accessions of a single clone shows the danger of accepting accession counts as a measure of diversity. Over a period of 13 years, the U.S. NPGS has received the 'Yichang' clone from two ex situ sources and has germinated an additional 235 seeds collected in the Yubei District. No morphological variation was observed in the seedling population in the first year of growth.

The effort required to collect, propagate, maintain, and characterize a clone already present in the U.S. NPGS collection serves as a reminder that most plant collecting is done with little or no prior knowledge of the useful alleles in the target populations (Holden et al., 1993). Sampling strategies identified for populations of outcrossing, sexually reproducing congeners may be documented (Lamboy et al., 1996), but they are not readily applicable to apomictic species.

Accessions 9988(1), 9988(2), 9988(3), and 'Normal Triploid' *M. hupehensis* displayed a second set of identical phenotypes (Table 1). Although there is no clear provenance linking these accessions to each other or to their original collector, the prevalence of apomixis in triploid *Malus* and the custom of arboreta to exchange germplasm contributes to the possibility of a second clone with different accession identifiers.

R3T3-SF possessed all of the 'Yichang' clone SSR alleles plus additional alleles at seven of the eight loci tested (Table 3). The R3T3-SF DNA content/2C nucleus places this accession in the tetraploid range, a result consistent with the product of normal (1n) pollen fertilizing an unreduced (3n) aposporic megagametophyte (Dermen, 1936). Although R3T3-SF appeared to be similar to its half-siblings during juvenile growth, its fruit are yellow, and slightly larger than the 'Yichang' type. Ploidy estimates, SSRs, and morphological traits for R3T3-SF are indicative of sexual origin, documenting the facultative nature of apomixis in the 'Yichang' clone. Although additional morphological and ploidy data are not available for *M. hupehensis* 'Rosea', the presence of all the SSR fragments found in the 'Yichang' clone plus two additional alleles show it may be sexually derived (Table 3). Even with strict apomictic reproduction, somatic mutations can occur and accumulate over time. While SSRs provide excellent genetic resolution of changes from sexual recombination, they are limited in their ability to detect somatic mutations (Nybohm, 1990). Single gene somatic mutations at loci affecting fruit color or tree habit may be horticulturally important, and visual inspection remains the most effective method to detect such changes.

Description of an agamospecies. The morphological traits of *M. hupehensis* Pamp. Rehd. [*M. theifera* Rehd.] described by Rehder (1916, 1949) bear a remarkable resemblance to those

traits observed in the 'Yichang' clone (Table 5). Wilson Collection No. 451 is listed among several type specimens given for *M. theifera*, but Rehder (1916) viewed the new species as highly variable and widely distributed. The original species description is silent on the topics of fruit traits, ploidy level, mode of reproduction, or the extent of the species range (Pampanini, 1910). Botanists have augmented the original species description in different ways, but often repeat that *M. hupehensis* is a triploid, apomictic species with fruit characters repeated from descriptions of *M. theifera* (Chun, 1921; Dermen, 1936; Liberty Hyde Bailey Hortorium, 1976; Zhou et al., 1995). Whether *M. hupehensis* should be identified as the 'Yichang' clone, a collection of agamospecies, or a broader grouping including diploids, triploids, and tetraploids is subject to debate. Given the degree of ambiguity in *Malus* taxonomy and nomenclature, investigators should include accession numbers, germplasm sources, and basic descriptive information when reporting experimental results concerning *M. hupehensis* or other *Malus* species material.

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