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ABSTRACT *Magnolia virginiana*, the type species of genus *Magnolia*, is a native American species belonging to section *Magnolia*. To better understand intraspecific taxonomy of *Magnolia virginiana*, we conducted molecular phylogenetic analysis based on sequences of cpDNA. Fresh leaves were collected from 28 populations (a total of 133 individuals) covering the entire distribution of the species, including the recently discovered Cuban population, and sequences of seven non-coding regions of the cpDNA were determined (ca. 5,000 bp). Based on nucleotide substitutions, ten haplotypes were recognized in *M. virginiana*. Phylogenetic analysis of the data matrix clearly indicated that populations of *M. virginiana* were divided into two major groups—one in the north and one across the south—which are essentially concordant with the morphological classification. Five nucleotide substitutions were found between them. Within the southern group, one common haplotype widely distributed, and populations of Texas (and adjacent areas) and western Tennessee showed a unique haplotype with an additional substitution(s), respectively. Less common haplotypes were found in Florida. The haplotype of the Cuban population was the same as the common haplotype of the southern group.

INTRODUCTION *Magnolia virginiana* L. is a native American species belonging to the section *Magnolia* of subgenus *Magnolia* (Figlar and Nooteboom 2004). Recently a small wild population of *M. virginiana* was discovered in western Cuba and has been taxonomically treated as subsp. *oviedoae* A. Palmarola, M.S. Romanov & A.V. Bobrov (Oviedo-Prieto et al. 2006, Palmarola-Bejerano et al. 2008). This discovery provided the initial motivation for this study—to determine whether this Cuban population is genetically different from populations in North America—and also rekindled our interest in the intraspecific genetic variation of the species within North America, which shows distinct morphological and ecological variation between the northern (var. *virginiana*) and southern (var. *australis* Sarg.) parts of its geographic range. The northern individuals are deciduous or partially deciduous, are mostly multi-trunked or shrub-like (to 9m tall), have glabrous twigs, and produce flowers that open in the mid-afternoon. In contrast, individuals from southern populations are mostly evergreen, are typically single-trunked (to 25 m tall), have densely pubescent twigs, and produce

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Table 1. Primer sequences used in this study

Region	Forward	Reverse	Ref.
trnG intron	GGTAAAAGTGTGATTCGTTC	GTTTCATTCGGCTCCTTTAT	(1)
trnT-trnL	CAAATGCGATGCTCTAACCT	CGTAGCGTCTACCGATTTCG	(2)
trnL intron-*trnF*	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	(3)
trnK5′-matK	GGGGTTGCTAACTCAACGG	GTTCGTAAAAAAATCGATCCA	(2), (4)
trnH-psbA	CGCATGGTGGATTCACAATC	AGACCTAGCTGCTATCGAAG	(4)
trnS-trnG	AGATAGGGATTCGAACCCTCGGT	TTTTACCACTAAACTATACCCGC	(5)
rpl32-trnL	CTGCTTCCTAAGAGCAGCGT	GGATCCCCTTTAGGTCGATA	(6), (2)

(1) Nishizawa and Watano (2000), (2) newly designed in this study, (3) Taberlet et al. (1991), (4) Azuma et al. (1999), (5) Shaw et al. (2005), (6) Shaw et al. (2007).

flowers that open near sundown (Meyer 1997, Weakley 2010). Moreover, intraspecific variation in the floral scent chemistry was also reported between northern (Maryland) and southern (Louisiana) individuals, which suggests different pollination syndromes (Azuma et al. 1997). Subsequently, Azuma et al. (1999, 2001) conducted a molecular phylogenetic analysis of Magnoliaceae including both northern and southern individuals of *M. virginiana*, and unexpectedly found some nucleotide substitutions between them.

The distribution of the two varieties overlaps geographically in South Carolina and adjacent areas, and thus, it is sometimes difficult to distinguish these varieties in herbarium collections as well as in the field. Therefore, if these two varieties (forms) are genetically distinct, we may be able to detect sequence divergence in the DNA between the two varieties. In this study we conducted sequencing of non-coding regions of cpDNA of *M. virginiana* to detect intraspecific sequence variation and geographic structure of the haplotypes if any.

MATERIALS AND METHODS A total 133 leaf samples (individuals) were collected from 28 wild populations of *Magnolia virginiana* covering most of the width and breadth of the distribution of the species in North America and Cuba. Voucher specimens are deposited in the herbaria of the Arnold Arboretum of Harvard University and the Department of Biogeography, Faculty of Geography, M. V. Lomonosov Moscow State University. Total DNAs were extracted from the silica-gel dried leaves by a modified method of Doyle and Doyle (1987). Seven intergenic or intron regions of chloroplast DNA were amplified and sequenced (ca. 5,000 bp). The regions sequenced are as follows; $trnG^{UCC}$ intron, $trnT^{UGU}$-$trnL^{UAA}$, $trnL^{UAA}$ intron-$trnF^{GAA}$, $trnK^{UUU}$*5′-matK*, $trnH^{GUG}$-*psbA*, $trnS^{GCU}$-$trnG^{UCC}$ and *rpl32*-$trnL^{UAG}$. The primer sequences are shown in Table 1. Because the *rpl32-trnL* intergenic spacer region was about 1,300 base pairs (Shaw et al. 2007), we amplified a half of the region (ca. 690 bp). The PCR mixture (20 μL) contained 1 μL of template DNA, 200 μmol/L of each dNTP, 1 μmol/L each primer, 2.5 mmol/L $MgCl_2$, Taq buffer, 1 U of Taq polymerase (TaKaRa ExTaq, Takara Bio Inc., Japan). The PCR was performed with a GeneAmp PCR System 2700 (Applied Biosystems Japan Ltd., Japan) starting at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (30 sec), annealing at 50°C (30 sec), and extension at 72°C (30 sec), and a final extension at 72°C (7 min). After checking a single band by electrophoresis on 1% agarose gel, the PCR products were purified with the QIAquick PCR Purification kit (Qiagen K. K., Tokyo, Japan). Direct sequencing of both strands was conducted on an ABI 3100 Genetic Analyzer (Applied Biosystems Japan Ltd., Japan) using a BigDye Terminator version 3.1 Cyclic Sequencing Ready Reaction kit (Applied Biosystems Japan Ltd., Japan) following the manufacturer's protocol. Alignment of sequence data was manually carried out. Phylogenetic analysis (maximum parsimony) was conducted using PAUP* 4.0b10 (Swofford 2002).

RESULTS Numbers of nucleotide substitutions, indels, polymorphic single-nucleotide track, and length of the aligned sequences are shown in Table 2. The DDBJ/EMBL/GenBank accession numbers of sequences determined in this study are as follows; AB553835–AB553838 (*trnG* intron), AB553839–AB553844 (*trnT-trnL*), AB553845–AB553849 (*trnL* intron-*trnF*),

Table 2. Sequence variation of seven non-coding regions of plastid DNA in *Magnolia virginiana*

	trnG intron	*trnT-trnL*	*trnL* intron-*trnF*	*trnK5′-matK*	*trnH-psbA*	*trnS-trnG*	*rpl32-trnL*
Nucleotide substitution	1	3	3	1	2	2	2
Indels*	0	1	0	0	0	1	0
Polymorphic single-nucleotide track	1	1	1	1	0	0	0
Aligned length	673	827	883	824	430	755	690

*Repeats of short sequence (18 bp and 25 bp).

AB553850–AB553852 (*trnK5′-matK*), AB553853–AB553855 (*trnH-psbA*), AB553856–AB553858 (*trnS-trnG*), AB553859–AB553861 (*rpl32-trnL*).

Each region showed one to three nucleotide substitutions among populations and individuals, which seem to be enough to resolve inter-haplotype relationships within *M. virginiana* (number of variable sites = 14, parsimony-informative sites = 11, consistency index = 1.00, retention index = 1.00). Therefore, we used only the nucleotide substitutions in the phylogenetic analysis (indels and polymorphic single-nucleotide track were ignored for determination of haplotype in our analysis).

In the combined aligned sequence data matrix, we recognized ten haplotypes (A–J) in *M. virginiana* based on the nucleotide substitutions (Table 3). Phylogenetic relationships among these haplotypes and a distribution map of the haplotypes are shown in Figure 1. The phylogenetic relationships among haplotypes clearly indicated two major groups in *M. virginiana*, one in the north (haplotypes A–B) and one across the south (haplotypes C–J) (Figure 1). There are five nucleotide substitutions between the northern and southern groups. Parsimoniously haplotype B and C seem to be ancestral within each group. Within the northern group, a derived haplotype A tends to be found in a higher latitude than haplotype B. Within the southern group, an ancestral haplotype C was commonly and widely distributed. Populations in Texas (and adjacent areas) (J) and western Tennessee (D) showed unique haplotypes which had an additional substitution(s), respectively. The other minor derived haplotypes were found in Florida and Louisiana with or without the common haplotype C. The haplotype of the Cuban population was the same as the common haplotype (C) of the southern group.

DISCUSSION Molecular phylogenetic analysis based on nucleotide substitutions found in non-coding regions of cpDNA clearly indicated that there were two phylogenetic and geographical groups within *Magnolia virginiana* (northern and southern groups) which are essentially concordant with morphological classification and their distribution (var. *virginiana* and var. *australis*). There are five nucleotide substitutions between the northern and southern groups (Figure 1). This value is almost equivalent to what would be expected between two closely related species. For example, we tentatively analyzed the same regions of cpDNA of two closely related species pairs of *Magnolia* and found three and five nucleotide substitutions between *M. grandiflora* L. and *M. tamaulipana* A.Vázquez and between *M. kobus* DC. and *M. stellata* Maxim., respectively (Azuma et al. 1999, unpublished data). Thus, if one were to use this molecular data alone, northern and southern populations of *M. virginiana* could just as easily have been treated as different species instead of different varieties. In addition, this study could not support the treatment of Cuban population as a separate subspecies of *M. virginiana*. More detailed morphological study linked with haplotype analysis will be helpful to further increasing our understanding of the taxonomy of this species.

The relatively large genetic distance between the northern and southern groups (five nucleotide substitution) implies that they had been geographically or biologically isolated for a proportionately long period. Southeastern North America has been considered a refugia for evergreen plant species during the Tertiary (Graham 1999, Azuma et al. 2001). Indeed, minor haplotypes of *M. virginiana* have been restricted to Florida and adjacent areas, which is consistent with that idea (center of diversity). Originally the northern group (haplotypes A and B) may have been derived from the southern group at a much earlier time. Thus, it may be possible to say that the northern group, a mostly deciduous

Table 3. Observed nucleotide substitutions and haplotype in *Magnolia virginiana*

	ID	*n*	*trnG* intron	*trnT-trnL*			*trnL* intron-*trnF*			*trnK5′-matK*	*trnH-psbA*		*trnS-trnG*		*rpl32-trnL*		Haplotype
Northern Group																	
Massachusetts	V01	5	G	T	C	G	A	G	T	C	G	A	G	G	G	C	A
New Jersey	V02	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
New Jersey	V03	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
Virginia	V18	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
Virginia	V17	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
Virginia	V17	4	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
North Carolina	V04	5	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
North Carolina	V19	5	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
North Carolina	V20	5	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
North Carolina	V24	5	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
North Carolina	V25	5	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
South Carolina	V26	1	.	.	.	.	C	.	.	.	.	.	.	.	.	.	B
Southern Group																	
South Carolina	V05	5	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Tennessee	V06	5	A	.	T	.	C	.	.	.	.	C	A	T	.	T	D
Tennessee	V07	5	A	.	T	.	C	.	.	.	.	C	A	T	.	T	D
Georgia	V08	5	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Florida	V09	5	A	.	T	T	C	.	.	.	.	C	A	T	.	.	E
Florida	V10	5	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Florida	V21	4	A	.	T	.	C	.	C	.	.	C	A	T	.	.	F
Florida	V21	1	A	G	T	.	C	.	.	.	.	C	A	T	.	.	G
Florida	V22	3	A	.	T	.	C	.	.	.	A	C	A	T	.	.	H
Florida	V22	2	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Florida	V23	2	A	.	T	T	C	.	.	.	.	C	A	T	.	.	E
Florida	V23	3	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Florida	Fl	1	A	.	T	.	C	T	.	.	.	C	A	T	.	.	I
Florida	Fl	1	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Mississippi	V11	5	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Louisiana	V12	1	A	.	T	.	C	.	.	.	A	C	A	T	.	.	H
Louisiana	V12	4	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C
Louisiana	V16	5	A	.	T	.	C	.	.	T	.	C	A	T	T	.	J
Texas	V13	5	A	.	T	.	C	.	.	T	.	C	A	T	T	.	J
Texas	V14	5	A	.	T	.	C	.	.	T	.	C	A	T	T	.	J
Texas	V15	5	A	.	T	.	C	.	.	T	.	C	A	T	T	.	J
Cuba	Cu	6	A	.	T	.	C	.	.	.	.	C	A	T	.	.	C

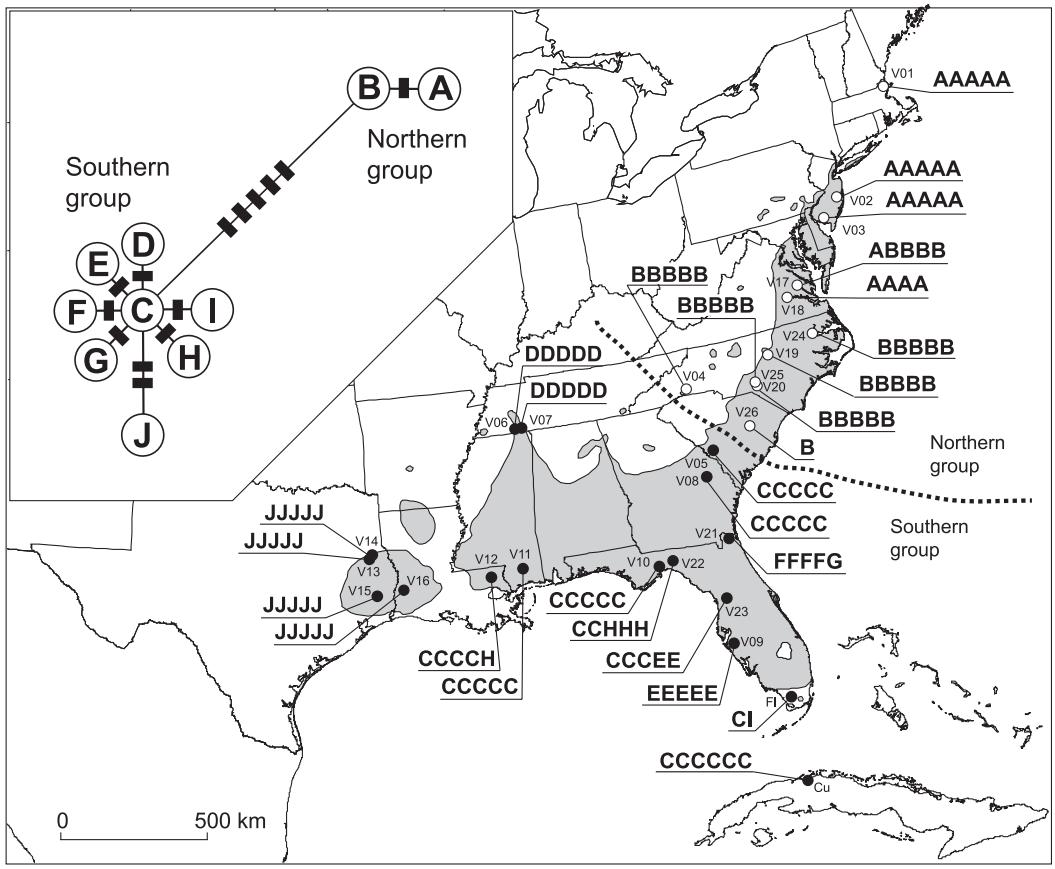

Figure 1. Unrooted phylogenetic tree and sample locations of ten haplotypes of *Magnolia virginiana*. Bar at branch in the unrooted tree indicates nucleotide substitution. Gray areas indicate the distribution of *M. virginiana* (from Little 1971, 1978). Each character (A–J) indicates haplotype of each individual (sample).

lineage which would be better adapted to cold climates, separately survived during the glacial periods beginning in the late Miocene at higher latitudes outside the refugia of the evergreen plant species including the southern group of *M. virginiana*, resulting in a separation of the two groups for a long period of time. It would seem to suggest that the two populations should be treated as separate species, but further work on the physiological ecology and reproductive biology of both northern and southern populations and population genetics at boundary area is needed to determine whether their degree of biological isolation warrants this level of taxonomic separation.

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