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Development of Simple Sequence Repeat Markers for *Chionanthus retusus* (Oleaceae) and Effective Discrimination of Closely Related Taxa

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***Abstract*. The genus *Chionanthus* (Oleaceae Hoffmans. & Link) includes deciduous or evergreen trees and shrubs distributed widely in tropical and sub-tropical areas, including a few temperate species. Although *Chionanthus* species are planted as ornamental garden plants and commercialized for natural products, genetic information for *Chionanthus* spp. is lacking. We created microsatellite-enriched libraries of *Chionanthus retusus* Lindl. & Paxton, assembled 1072 contigs, and detected 1010 repeats. The frequency of the repeats decreased with the increase in repeat length, and the most abundant motifs were: AG, AC, AAG, ACC, AT, and ACTC. We screened 384 markers on 12 accessions of four related taxa that included *C. retusus*, *Chionanthus virginicus* L., *Chionanthus pygmaeus* Small, and *Osmanthus americanus* (L.) Benth. & Hook. A total of 195 simple sequence repeat (SSR) markers amplified and discriminated six accessions of *C. retusus* and 57 SSR markers amplified and discriminated across the four Oleaceae species screened. To identify the best markers to use in future experiments, the "Unique Pattern Informative Combination" (UPIC) values were calculated for all the markers and the 100 markers that were most effective are reported here. The percentage of heterozygous loci across the 384 markers was lowest for *C. retusus* (29.3%) and highest for *O. americanus* (68.9%). The SSR markers developed here could assist in taxonomy and hybridization investigations for breeding programs and authentication of varieties used as medicinal plants.**

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Chionanthus L. is a member of the Oleaceae, a morphologically diverse family that includes economically important genera cultivated for food (*Olea europaea* L.), timber (*Fraxinus* L.), medicinal (*Forsythia* Vahl.), and ornamental (*Jasminum* L., *Ligustrum* L., *Osmanthus Lour*., and *Syringa* L.) purposes. The genus *Chionanthus* has ≈100 species (Chang et al., 1996; Wallander and Albert, 2000) distributed throughout tropical and subtropical areas worldwide but includes three temperate species (Green, 2004). Only the three temperate species are economically important as ornamentals and natural products. Temperate *Chionanthus* spp. (*C. retusus* from eastern Asia and *C. pygmaeus* and *C. virginicus* from eastern North America) are cultivated for their white flowers in feathery panicles and decorative blue fruits. With the exception of the pink-flowered *C. pubescens* Kunth from Ecuador, the evergreen tropical and subtropical *Chionanthus* spp. (syn *Linociera* Sw.) are usually not cultivated.

Very little is known about the genetics of *Chionanthus*. The base chromosome number in tribe *Oleeae* is $x = 23$ with published counts for *C. virginicus* and *C. retusus* of $2n = 46$ (Chang et al., 1996; Taylor, 1945; Wallander and Albert, 2000). A hypothetical phylogeny for the Oleaceae, including *C. retusus* and *C. virginicus*, was developed using sequences of two noncoding chloroplast regions, the *trnL-F* (intron-L/spacer-F) and *rps16* intron (Wallander and Albert, 2000); however, no other information at the molecular level is available that could assist in *Chionanthus* breeding programs or identification of species. In addition, understanding the level of genetic diversity as well as inter- and intrageneric relationships within the cultivated germplasm is important for planning a systematic breeding program for *Chionanthus*. Furthermore, the pollination and breeding systems of *Chionanthus* appear complex with individual species exhibiting varying levels of polygamodioecy (Dirr, 1998; Nicholson, 1990; Ueda, 1996) combined with occasional selfing that has implications for crossing and inheritance studies.

The development of molecular markers, specifically SSRs, will aid in assessing relationships, diversity, and parentage within the genus *Chionanthus*. In addition, because SSR marker transferability is usually feasible within plant families, i.e., Casuarinaceae (Yasodha et al., 2005), Meliaceae (White and Powell, 1997), Fagaceae (Barreneche et al., 2004), the markers we report here for *Chionanthus* could potentially transfer to species of *Olea*, *Fraxinus*, and *Syringa*.

Materials and Methods

Plant material. Source and accession data for all samples used in this study are shown in Table 1. *Chionanthus retusus*-derived SSR markers were tested on six accessions of *C. retusus*, three accessions of *C. virginicus*, two accessions of *C. pygmaeus*, and one accession of *Osmanthus americanus*, a species related to *Chionanthus* according to Wallander and Albert (2000). Total genomic DNA was extracted from leaf tissue using a Qiagen Plant Mini Kit (Qiagen, Valencia, CA).

Isolation of simple sequence repeats from Chionanthus retusus. For the construction of SSR-enriched libraries, we used the method of Techen et al. (2010), briefly described here. DNA from *C. retusus* was cut with restriction enzymes *Alu*I, *Hae*III, *Dra*I, *Rsa*I (New England Biolabs, Ipswich, MA) and combinations of pairs of these enzymes. The blunt-end DNA fragments were A-tailed with Taq-DNA polymerase and then ligated to an optimized

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Table 1. *Chionanthus* and *Osmanthus* samples tested with simple sequence repeats developed from *Chionanthus retusus*.[z]

Species	Accession no.	Source and provenance	Voucher no.	Sample
Chionanthus retusus	37681-HL	Kunming Inst. Bot.	6013	CR1
Chionanthus retusus	—	Cultivated, Greenbelt, MD	6021	CR2
Chionanthus retusus	37681-HH	Kunming Inst. Bot.	6012	CR3
Chionanthus retusus	45247-J	Coastal Japan 1978	6020	CR4
Chionanthus retusus	14089-L	Arnold Arboretum	6006	CR5
Chionanthus retusus	40121-H	Kyoto Univ. Forest Stat.	6016	CR6
Chionanthus virginicus	—	BARC-E, Beltsville, MD	6002	CV8
Chionanthus virginicus	—	BARC-E, Beltsville, MD	6003	CV9
Chionanthus virginicus	77196-H	Shemin Nurs.	6007	CV10
Chionanthus pygmaeus	76993–3	U.S. Botanic Garden	—	CP11
Chionanthus pygmaeus	76993–2	U.S. Botanic Garden	—	CP12
Osmanthus americanus	56806-H	Tom Dodd Nurs.	6008	OA7

[z]All voucher specimens are located in the herbarium of the U.S. National Arboretum, Washington, DC, and are collections of J. Kirkbride and R. Olsen. Living plants of *C. pygmaeus* are only listed by accession number because no voucher specimens have been collected.

linker (SSRLIBF3: 5′-CGGGAGAGCAAGG AAGGAGT-3′, SSRLIBR3: 5′-/5Phos/CTCC TTCCTTGCTCTCCCGAAAA'-3′). After 20 cycles of polymerase chain reaction (PCR), the amplified products were hybridized to four groups of biotinylated oligo repeats. Sequences containing repeats were captured using streptavidin-coated magnetic beads M-270 (Invitrogen, Carlsbad, CA) and the DNA was eluted. The eluate was PCR-amplified for 20 cycles; the PCR products were cloned in vector TOPO4 (Invitrogen) and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled in contigs using DNAStar Lasergene7 (DNASTAR, Inc., Madison, WI) and visually checked. Repeats were searched using SSRFinder (Sharopova et al., 2002) and Sputnik (Abajian, 1994). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) with stringent parameter conditions: Tm 63 optimum (60/65) minimum/maximum, length 24 optimum (20/28) minimum/maximum, and maximum overlap of repeat within the primer was 5 bp. Contig sequences containing microsatellites were screened against the NCBI Protein Database (BLASTx) (Altschul et al., 1990).

Fingerprinting. Forward primers were 5′ tailed with the sequence 5′-CAGTTTTCCC AGTCACGAC-3′ (Waldbieser et al., 2003) to permit product labeling, and reverse primers were tailed at the 5′ end with the sequence 5′-GTTT-3′ to promote non-template adenylation (Brownstein et al., 1996). Primer 5′-CA GTTTTCCCAGTCACGAC-3′ labeled with 6-carboxy-fluorescein (IDT-Technologies, Coralville, IA) was used for amplification of 10 ng DNA using Titanium Taq DNA Polymerase (Clontech, Mountain View, CA) in 5-μL reactions on an M&J thermal cycler (BioRad, Hercules, CA) at 95 °C for 1 min, 60 °C for 1 min (two cycles), 95 °C for 30 s, 60 °C for 30 s, 68 °C for 30 s (27 cycles), and a final extension at 68 °C for 4 min. Fluorescently labeled PCR fragments were analyzed on an ABI 3730XL DNA Analyzer and data-processed using GeneMapper Version 3.7 (both from Applied Biosystems). Presence of alleles was converted to a binary matrix. The accessions were clustered using the unweighted paired group method and arithmetic averages (UPGMA) algorithm implemented in the SAHN program of NTSYSpc Version 2.2 (Exeter Software, Setauket, NY). The confidence level for the dendrograms was assessed by bootstrap resampling (5000 replicates) (Efron et al., 1996; Felsenstein, 1985) using WINBOOT (Yap and Nelson, 1996).

Unique Pattern Informative Combination and heterozygous loci calculations. Markers that effectively discriminated the samples tested were identified using the UPIC software (Arias et al., 2009), and the number of unique patterns (UPIC values) identified by each marker was reported for the best 100 markers. Percentage of heterozygous loci was also calculated using the UPIC software (Arias et al., 2009) for each DNA sample across all 384 SSR markers tested.

Results

Repeats found. SSR-enriched libraries of *Chionanthus retusus* were made using four groups of biotinylated oligo repeats. A total of 2208 clones from those libraries was sequenced. Sequences were assembled to 1072 contigs in which 1010 repeats were detected by SSRFinder and Sputnik combined. We considered repeats only those that were non-mononucleotides, had a minimum repeat length of 8 bp, and a minimum 20-bp length of flanking region upstream and downstream of the repeat. Sequences of contigs containing repeats were submitted to GenBank with accession numbers (GQ117288 to GQ118148). We designed 394 primers on the flanking regions of the repeats and tested 384 of those primers on 12 DNA samples. Primer sequences and repeat motifs as appeared in the original sequences are provided in Table 2. DNA sequences corresponding to 28 of the markers had significant hits on BLASTx indicated in Table 2 in bold. To simplify the recording of the repeat motifs, those that were circular permutations and reverse complements of each other were grouped together as one type, i.e., AAC, ACA, CAA, GTT, TGT, and TTG were recorded as AAC. This resulted in 55 non-redundant repeat motifs isolated from the *C. retusus* SSR-enriched libraries. The first 11 most abundant motifs detected had frequencies ranging from 560 to six as shown in Figure 1. The remaining 44 non-redundant motifs, including one CG repeat, were found in a total of 57 repeats (Table 2) with frequencies lower than four and were not included in the plot (data not shown). Frequencies of the isolated repeats decreased as their length increased. The number of di-, tri, tetra-, penta-, and hexanucleotides are shown in Figure 1. Only 10 of 384 markers tested did not amplify any of the 12 DNA samples used in this study.

Markers that amplified four Oleaceae species (genera Chionanthus and Osmanthus). Based on the electropherograms in GeneMapper and presence across samples, we selected 57 markers that amplified across the four species tested (*C. pygmaeus, C. retusus*, *C. virginicus*, and *O. americanus*). Selected primers are listed in Table 3. From those, 43 amplified all 12 DNA samples and 14 amplified nine or more DNA samples. Only two of the 57 markers were monomorphic for the four species tested; the other 55 corresponded to polymorphic loci and amplified between two and 13 alleles. A total of 350 alleles was detected by these 57 markers. From these markers, 18 were polymorphic (detected up to five alleles) within the species *C. pygmaeus* allowing distinction of the two accessions tested, whereas 35 were polymorphic (detected up to five alleles) within the species *C. virginicus* allowing distinction of the three accessions tested.

Because the SSRs in this study were isolated from *C. retusus*, most markers amplified predominantly this species. Of 384 markers tested, based on their ease to score, we selected 195 that amplified the six lines of *C. retusus;* 33 of them were monomorphic, whereas the other 162 polymorphic markers detected between two and 15 alleles. A total of 837 alleles was detected in these 195 loci within the species *C. retusus*.

Unique pattern informative combinations of markers, Unique Pattern Informative Combination values. UPIC indicates the number of DNA samples that can be discriminated by each particular marker (Arias et al., 2009). The UPIC value is more informative for selecting subsets of SSRs than the use of its polymorphism information content. We calculated UPIC values for all the markers and report the UPIC values of the best 100 in Table 3. Twelve DNA samples (Table 1) representing four species of Oleaceae (*Chionanthus retusus*, *C. virginicus*, *C. pygmaeus*, and *Osmanthus americanus*) were used for the analysis. Combinations of these markers run on the 12 DNA samples described can detect as many unique patterns or alleles as to the sum of their UPIC values. In general, the markers allowed a clear distinction among *Chionanthus* taxa as well as within *C. retusus* accessions, which was shown by the high bootstrap resampling coefficients obtained (Fig. 2).

Heterozygosity (%). The percentage of heterozygous loci, based on 384 markers, for each of the four Oleaceae species tested was 29.9% to 30.7% (*C. pygmaeus*), 30.1% to 39.8% (*C. retusus*), 29.3% to 43.4% (*C. virginicus*), and 68.8% for *O. americanus*.

Cluster analysis. Genetic similarity coefficients based on UPGMA were calculated

Table 2. Markers that amplified all the Oleaceae accessions tested detected by Unique Pattern Informative Combination software (Arias et al., 2009).[z]

Marker ID	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size range	No. all	Max all/S	Motif
StvChR_15_a	GGAAAAAGAAAGGGAGAAGGAGAA	CTCTGTGACCATGACTGTCTGTGA	114–212	7	3	AG
StvChR_94_a	CGGAGACAATTTAAAGCACGATTC	CGACAATAGTTCAAGCATTGCGTA	151–188	10	5	TTC
StvChR_76_a	TCAGTCTTCACCACTACCACCGTA	TGGGCTTTTAGACGAGTATTTGGA	105–165	10	2	ATT
StvChR_114_a	CCTTCCCCATTTAATCAATCACAA	TATGTTTGTTTACTTGTGCCCGTG	160–165	4	2	AC
StvChR_124_a	AATTCTCAGCCAATCACCTCATTC	ATGACGTGACCTTTTTGAGAGGAG	110–132	6	3	TC
StvChR_140_a	TTCTTCGCTCTGTAAAATTGGTCC	CCCTCCTTCTGTGAACTGATGACT	151–161	5	2	TG
StvChR_194_a	CAAGATCTGTTTTGGGTCATTCG	CTCCTTTGAAGCTTTCAACCCTTA	167–172	5	2	TC
StvChR_221_a	GGCTGAGGTTGATTACCTCTGATT	TTTCAGCCTGTAAAACCCTACTCTC	82–110	10	2	AG
StvChR_251_b	TTCTGTTTATTCATCTTCCACCGC	TAGACCAGGGTATTGGTCTTGCAT	132–191	9	4	TG
StvChR_284_a	AAAAATGTTGTGGTAGAAGTGCCG	GTTCCTCAACTCCCGAGTCCTAAT	69–225	9	4	GGC
StvChR_285_a	TTGACAGTGAAGGTGCAGAAGAAG	AGCCAGTCAGTGTTATTCTCCAGG	143–144	2	1	AAG
StvChR_291_a	TGCATTTCGTTACATTTTGTACGG	AGGATAATCACAGGAGGAAGGGAG	110–389	9	3	TC
StvChR_316_a	TCTCTTTCCGCTTCTTTTCCTTCT	GCTACCAACATCATTCGTTCCTCT	172–193	9	3	TTC
StvChR_337_a	AGAAGGGCTACGGAATTGTAAAGG	GCTAAGTAAGGGGGTCGGACTAAG	104–174	2	1	TC
StvChR_350_a	GCTTCACAATCCTCCAAAATGTCT	AGGCAACGTGATCTCAGTTCTTTT	121–196	9	2	TC
StvChR_353_a	CAAATCCATTTGAAAGCAAATCAAC	CCATTGATATGTAGTGCCGTAGCA	140–378	6	2	TC
StvChR_356_a	CGAACTGATGCATCTAGCCTTCTC	TCGAGAATTTAAATCAGTCGCTCC	145–216	4	2	TTC
StvChR_357_a	ATAAGGGAACAAATGTGCCAAGAA	GTATAGCAGGTCATTGATTTCCCG	175–373	8	3	AG
StvChR_360_a	AAAGTGAAGCAAGAGAGATGGTGC	CTCCTCTGTTTCTCTCCCATCTTTT	105–318	6	2	AG
StvChR_381_a	GCAGGAAGGATATGAGTCATGTTG	AAGACTAATACAGCCCTCAAATTGC	116–139	6	1	AG
StvChR_429_a	TTTTCAATCCATTTTCTTCTCCCC	AAGAGAGATGTCGAGAGAGGAGGG	104–153	6	3	TC
StvChR_430_b	TAGAAGAATCCATACATCACCGCC	TCACACTTATTTCACTGAGCCAACA	159–327	8	3	AAG
StvChR_438_a	AAAATTAAGAAACTCAACCATGCCC	TACATCCCAACTCACTCAGGGAA	103–138	2	2	AG
StvChR_454_a	AAATTGACACCCACAACCAATACC	GTGTTTTGCAATTGTTGATAGCGA	86–263	4	2	ACC
StvChR_480_a	AGAAGTGAAGATCTGAAGATGGCG	CGGAGATGTGTCGAAAGAAGAGA	103–338	11	6	AG
StvChR_488_a	GTCATGATAGCGCTAAGCGAGTTT	TCCCATGTGACAATACCGATAAGA	99–120	7	5	TG
StvChR_493_a	ATCTCCCTTCTCTAGCGACGTTTT	GATCTAACAAAATCCCACAGCGAC	98–309	7	4	TC
StvChR_534_a	GGAAATGAGTGATTGGAGAGCAAC	TCCTTTCCTGCTAGTGAGATTTGC	110–116	5	2	AT
StvChR_540_b	ACACCCAATCACAATCACAATCAC	AACATAAAGAAGGCGGAATTTGGT	141–171	9	4	AAG
StvChR_544_b	TCTTTCTTTTGGCTCAGGAAGACT	AATTTGTCATGCACAGGTCTCATC	100–172	8	4	AT
StvChR_563_b	TTTTGGGGGTTGCTCTTGATAATA	CTTTCATTGAATTTGGTTCAAGCC	161–162	7	4	AAG
StvChR_592_a	CCAAAATTGAGCAAAAATCTGGAG	ATGGATGAAGATAGGGATGGTTGA	126–136	7	3	AAG
StvChR_603_a	TGAAGATTATTCACGTTGGTTGGA	CCCCTATTAGCACAGCAAGAAGAA	132–183	12	4	TC
StvChR_632_a	CATGACCGAAGAAAACAACATGAG	CAGTTTCCAAATCCTCCATTCATC	147–170	2	1	AG
StvChR_632_b	CCACGAAAATGCACATATGTTTGA	TTGTTGATAATTGACCAGTGCAGG	106–114	4	2	AT
StvChR_656_a	AAAATGAGGAAAAATCAGGGGAAG	CATCCTTTTTCTTCAATCTCCTCAA	126–151	7	2	AGG
StvChR_680_a	CAAATAATTCGAGTTTCCACGACC	ATGTTTTCATTGGAGATGCGACTT	108–240	8	2	AG
StvChR_709_a	CATTGTTGTGGTTTTGCCTTCATA	CATTTCCGCCATTTGAATGTTTAT	174–183	4	2	CT
StvChR_711_a	GCAAAAACAGCCATGATTATTTCA	TAATGCCACCCATTCTTACCTCTC	156–168	5	2	AG
StvChR_732_a	CGTCTGCATACATCAAGCCAATTA	ATTCCTCGATCTCTCCGATCATTT	154–170	3	2	AG
StvChR_777_a	ACTCCAAGAAAAGTCGGGAAGTCT	ATCAGATCGCTGCCTACTAGTGCT	170	1	1	TA
StvChR_793_a	TGGAACCACATAAATAGATGCACG	GAGCCTGAAACCAAGCCTTAGAC	109–132	8	3	AG
StvChR_807_a	AAATGTCAGGGTGTAAATATTGTCAGT	ATGAAGTTGGATGTTGACGTTCTT	169–173	3	2	AG
StvChR_813_b	TACAAAATCATCCCGAGGAAGAAA	TCTGAATCTTCTAGGCTCTCTCGTTT	113–114	2	1	AG
StvChR_852_a	TCAGTGTGGTGTGCGTATGTATTG	CTCTCTCAAATCCACCTTGACTCG	107–322	5	2	AC
StvChR_879_a	GTCTGAGACCCGATCTGTCTGTTT	TAATTTTTCTTCCCTCAATCGCTG	165–170	4	2	TC
StvChR_881_b	AGGTGCTAAAAATGCTGAAAAACAA	CCCAGTAATATTGTGGAGGAGGG	112–198	13	4	AAG
StvChR_917_c	ATTTGGTGTGTCCAAAAACGAATC	AAATAGTGCGATCCAATGGCTAAA	159–160	2	1	TG
StvChR_925_a	AGTCTTGCAACTGCTCCTTTGTCT	TGTCCATTCTCCAGACTTGAATGA	98–121	9	3	AC
StvChR_926_a	GTTTGACGGTTACGATCAATCCAT	AATCCTGACATCTCATCATCGTCC	182	1	1	ATG
StvChR_945_a	CCAACTCTTACGCCAAACTTCAAT	TTCCGTCTTTATTTCTCTCGCTCT	108–174	9	2	AG
StvChR_961_b	AACCAGATCATCCAACCACCATAG	TGCAAATTGTGGTGGTGTTTTTAG	92–124	8	3	ACC
StvChR_967_a	TGTCATCCTATTTCATCATTCAGCA	AGGGAACGTGTTATGAAAAGCAGA	139–151	7	2	AG
StvChR_974_a	CGGTGGTTTTTGAGGTGGTAGTAG	CGGCACATCTCCTCTATCTTCATT	155–391	6	2	TGG
StvChR_989_a	AATCGGACAAGAACGAGATTGAG	AACCAGCAAATTAGTAAAAGACACAGAT	182–405	6	2	AG
StvChR_1003_c	TGACTAGTGGGTGAATGTGGATGT	TGACCAAACTCTCTCCAAGGAATC	112–297	2	2	AAG
StvChR_1032_a	CCTTATGACTCCATGGAAGATTGC	TCAGCAATCAAGTCCAAGTCTGAA	129–134	2	1	ACT
StvChR_7_a	CGATAGGTCAGTCGACTCTCTTGC	TCATCTCCTTCTCCTTTCACCTCA	130–152	6	3	GAA
StvChR_9_a	AAATCATAGAAATGGCGGTTGTTG	GATATTCATCTCCCCTCTTCTCCC	125–279	12	4	GA
StvChR_11_b	TCAATCCATATACAGAACGAAAATTGAA	CCTTCCCCAAATTCTCTCTTCATC	169–194	5	2	CT
StvChR_13_b	TTATTAGGATGCATCTCACATCAATTT	ATTCTCGATGATTTGTAGCGCAT	167–232	5	2	AG
StvChR_19_a	TCATCATAGAAACAACACTTGCAACA	ACTAAGACTGGCAAGGTTTTGACC	182–209	3	2	AG
StvChR_21_a	AAAATCACACCCATTTACTGCTCTTC	GGAATCAAATCTTCGACAATAAACACA	103–105	2	1	TA
StvChR_32_a	AACCGCCTCGGTATCAGACTAAAT	AAGTTGTTTGGAACATCAGCGTTT	149–162	4	1	GAT
StvChR_33_a	AGGCGATGCATAAACACACTTTTT	CAAGGAACGAAATCCCGAATACTA	182–183	2	1	AC
StvChR_35_a	GCCGCTACTAATGAGAGAGGAGAA	ACAGTGTTGGTGCAAGTGATTGTT	135–157	7	4	AG
StvChR_42_a	CTCTGTTGAGAGTGTTTGATTCGG	AAACGGTTTATCATTCAAGCAGGT	102–109	3	2	TC
StvChR_44_a	CCAAAACTTGCTCATCTCGCTTAT	TGTTGACTCGAAGAGGTATGCATTT	144–176	7	3	CA
StvChR_52_a	GCTTCTTCTGCAGGCGATTTACT	ATCATCCTCCCTTCCTTCTCTTTG	128–172	5	3	AG
StvChR_54_a	GGTGAAGAAATATCCCCCGATTAC	TTGATTCATCAAACAAAGAGGCAA	154	1	1	TC
StvChR_57_a	TTTGTCCTCTGTCAACTCTTTCCC	ATCACCAGAATGCCACTTCCTTTA	116–162	6	2	AC
StvChR_62_a	GAGGAATTCGAGTGCGAAAGAATA	TGTCAACAGACGCACTCTCTTTCT	161–167	4	3	AG
StvChR_66_a	TTGGCATTGCACCCTTAGTTATTT	TATATCTCTCCCCAACAACCCCTT	118–340	7	5	GA

(*Continued on next page*)

Table 2. (*Continued*) Markers that amplified all the Oleaceae accessions tested detected by Unique Pattern Informative Combination software (Arias et al., 2009).[z]

Marker ID	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size range	No. all	Max all/S	Motif
StvChR_73_a	CTGGAGTGAGAGATGCAGTTCAAT	TCCCGAAACAACAGATTATTAAGGA	181–207	7	3	TC
StvChR_74_a	AGGTTTTCCTCTCTTTTCCTAGGGTT	CGGGAATTGAAAGGAGAAAACATT	134–144	3	1	GAA
StvChR_77_a	AAACAAAGTCACCACGACCACTACT	GCGAAAAGGCTTATTGTAGAAGCA	115–194	8	2	ACC
StvChR_80_a	TAAAACTTGGGGCTTAAGGGGTAA	GGCTCTGACCGTCAATTGGAT	154–164	3	1	TC
StvChR_89_d	TTGAACTACAGATCCAATTTCCAAGA	TTTTGATCCATCCCTGTATAGCGT	123–131	4	2	AC
StvChR_99_a	ACTGTGTTAGCGGTGCTGTAATCA	CTCGCCATTCTCTATCTCTTCTCG	112–154	8	3	AG
StvChR_112_a	AATTCTTGTTTTTATTTGACGGCG	TGCAGTTGATTAAGCATGTAGCAAT	192–260	7	2	AAG
StvChR_116_a	AGGAGCGCATAGAGAGGTAGGAAT	ATCAATTCAAAATTCAAGGGCAAA	162–166	3	1	AG
StvChR_120_a	CCGAACCCTATTTTCTTCCATCTT	AGAGAAGGATGCGTTCACTGTTG	107–136	8	5	TC
StvChR_121_a	AGTGGACCATCGTCTAACTCTTCG	TTGCAGACTTTTCTTTGTTTCAACTG	109–110	2	1	ATG
StvChR_131_a	AACCAAGTCTAGATCCATTGCAGG	TCCAACGAAATGTGATGAAATGAG	148–168	7	3	TC
StvChR_141_a	CTTGAGTGGTTGCCCTACTTGACT	CACAGATTCAAGACACGCAGATTC	173–184	4	2	TC
StvChR_142_a	TGACCATTCCAACACCAAAATATG	GATCATTGCAAAAATCAGGGTTTC	132–146	4	2	AG
StvChR_146_a	TCCACAGATTAATGGCATTGAAAA	GAAGAAGGAGAAGGAAAAGGAGAGA	128–131	3	1	TC
StvChR_150_a	TAATTAATGATTGAGCGGGATTGG	AAAGTTGAGGCAATTAATGATGCTG	269–324	7	2	TTC
StvChR_159_a	TCATTTACTTTTGGTCTTCCATCAC	CCTTGTTCTTGCTGCTTGATAAAT	165–184	6	3	AG
StvChR_169_a	TTTGGATGTATATGGATGTGAGCG	AAGAGAGAAACAGTCCAAATCCCC	103–283	4	2	AG
StvChR_184_b	AAGAAGGGAAAATGTAGCGTCGTT	GTATGGGATATGTGTCGGATCCTC	173–187	5	2	AAG
StvChR_185_b	TCTGAAACCCTAACCCCTCTATCC	CGCCATCAATGTTTCCGTTATATT	122–145	5	2	TC
StvChR_195_a	CCCTTCTATTTAGTGGGTTTGGGT	GATGAAAATGACTTCGACGATTCC	155–169	5	3	AG
StvChR_196_a	GCCAAACAGGGTTTTACTTTCCTT	TAAAAATGGTGAGGCAAATAACGG	162–164	2	2	TC
StvChR_198_a	TGCAATAGAACAGAAACAAGGCAA	TGTAGGACAGCAGTGAGTAGTCCG	134–161	10	4	TC
StvChR_209_b	AGAGAGAAACAAACACAAGGGCAC	GAGAAATCACTGAATAGACAGCGGA	189–212	8	3	TC
StvChR_227_b	CAGCCGTTGAACTTAAAGCTTCTC	CGATCCTCGCGTTTTCTATCTCTA	155–159	2	1	TC
StvChR_246_a	CTGTCCTGAATGCTGAATGTATGC	CCAAACCACAACAACCAACAATAC	168–172	4	2	AT
StvChR_253_a	GGAAGCAAGAAATTATTTGGGTGA	AATTATGGAGCCCAAAAAGGAAAA	165–174	3	2	TG
StvChR_254_a	CCCTAAATAACCAATAATGGATTGAAA	GTCAACCAAAACCTAGTCGTCAGA	165–219	5	2	AAG
StvChR_267_a	GGCAAATCTAATTAAATGAAAGGGTT	AACCTAAATATAACTAACGTGCACACAC	149–468	4	2	ATAC
StvChR_301_a	TGGCAATCTGAGACTTTGAGTGAG	CTACTAACCCCATCCATGCGTAAA	125–133	7	3	AG
StvChR_304_a	AAGATTGGACGATTCAATTTTTGTG	GAACATCGGTGACGTGGTAACTC	104–289	6	2	AG
StvChR_312_a	TCATTAGAGGTTGTCCAAGCCATT	TTCGAGAGGCTCGTTATTTTAGACA	172–221	7	2	AG
StvChR_314_a	GTAGCAAGTGAATCCAAGGAACAA	TGAAATTTTGGAACTAACTAAATCGTCT	135–160	7	3	AC
StvChR_318_a	GTTGTCGACGAACGTCGCTATTA	CATTTTGTCCAGCTCTACCTCCAT	119–458	4	2	AG
StvChR_321_b	TGATGGGGTACGAATTTTTGGTAT	ATTTTTGGAGATTTTTGGGGTGAG	248–485	7	4	TG
StvChR_322_a	TTTCATCGATTACAATTACCCAAATACA	TAAGATATGTGTGGGTGTTGGTGG	111–214	7	4	ACC
StvChR_326_b	TGACTCGTATGGAAGCAGGAAAAT	ACCGTATTCAACCACCTCAATCAT	168–188	4	1	TG
StvChR_327_a	GTTAGATGGGGTGAGTGAGCAAAT	TTTCATCTCCATTGGTTCCTTTCT	159–345	11	3	TC
StvChR_341_a	GACTTGGACTCTTGGTTGGAAAGA	GACCTCACCTTTTTGCCTCTTTTT	123–181	7	2	AT
StvChR_343_a	TTCTCTCTAATCTCGCCCCTTTCT	AGAGATGGAGGTCTGCTTCTTCAA	157–576	5	3	TC
StvChR_352_a	TTCGAGATATTTGAAATCTTCCTTTGA	AATGCATCAATCTCAAAGACATGC	87–187	6	2	TC
StvChR_371_a	GTTGACAGTGAGCGGTGTTCTCT	TTTTCTCCTATTTTCTTACCCCCG	132–171	8	3	AG
StvChR_379_a	AACATGCACGTCTTTACCTGTTCT	ATTTTCTTCTCCCTTCTGGGTTTC	111–141	5	2	TC
StvChR_411_a	GAAACTTTTCTTTGTCAGGGGTGA	TGGAAAATTTGAATTGGTAGGTGTT	156–191	4	1	TC
StvChR_423_a	TCTGCATAAAGCAACAATGAATCC	TGTGAGGACACTGAAAATTTGCTT	175–184	5	2	AG
StvChR_427_a	AGTCTTCTCTTGTCGTTCCATTCG	CTTCCACAGCACGAAAATCAGTTA	149–153	2	1	TC
StvChR_433_b	CCACCATCTCAATCCCAAATACA	TATCAACATCTTCAATTGCATCGG	175	1	1	TC
StvChR_437_a	TATAAATCGCCATTGAGGGATAGG	TCACTTTGCATAGATCAGGCGTAA	172–176	5	2	AG
StvChR_440_a	CACCATAATTATTGCGTTTGACCA	GGGTCACCCTCCTTTCATACTTTT	147–187	7	2	AC
StvChR_447_a	ATGGTCGAGATCACACAGTTGAGA	CAATTAGTCAGTAGCAGCCCCATC	178–206	6	2	ATG
StvChR_461_a	CCTCCATCCCTTCTTTTGTTCTTT	TAGAGCTGTGATTGGTGAAATGGA	151–165	6	2	TC
StvChR_468_a	TCATAATTGACTCCATTTTCACCG	CGAGAAATGATTGAAAGTGGCTC	102–118	6	2	TC
StvChR_481_a	AAGATGAATTCACTCATGGGTTGC	AAGAGATTCTTTAAACCCCCACCC	159–175	5	2	AT
StvChR_482_a	TGAGTCATTGACAGAAGTTGAGTTTG	AGATTGAACCAAATCAAGCGATTC	135–149	4	2	ATC
StvChR_487_a	TGACCATTTGATTTAGGTGAGTGAA	TTTTGAAACCTCTACCCGATTTTT	166–182	4	1	ATTT
StvChR_490_a	CTTGTTAGGCACAAGCATTCGAT	TGTTATTACATCTTCAAACCACCATTTT	218–224	5	3	AG
StvChR_494_b	CTTGATTGAATTTGGTAGGATCGC	CCATAATTTCTGCACACTTGCTCA	102–127	9	3	ATGT
StvChR_496_a	TTCCTATGCCTCTCTCCCTCTCTT	AGGGCGAGTTGGTGTAGACTGTAG	152–362	3	2	TCC
StvChR_504_b	ATCAATCCCACACAATTTCAAACC	AAATGAGTAGGTCGATAGGGGGAG	96–112	8	4	TC
StvChR_507_a	ATACTCCACCACCTGGAAATGATG	TCTCTATAGGAGGAGGAGATGGGG	149–184	9	4	ATG
StvChR_508_a	TGATGAACAATCTCGTTTATGTCCA	CTCACCCTCATCATCACAACTCTC	165	1	1	AAG
StvChR_510_a	GCAAGCGTATATTTTCCAACAAAA	CATGTTGAAGAATTATTTTACCCTCTTC	99–131	3	1	AG
StvChR_515_a	TGGCTATGGTTATGGTGTTGTGTC	ACCACGCAATCTCTCCCTACATC	143–185	3	2	TTG
StvChR_525_a	GGAGAGTCTGGGGTTTATGACAGA	TTCATACCGTTTTTAACTCGTCGC	120	1	1	TG
StvChR_543_a	GCGGAAGATGAAGCTATGAATGTT	TGTGTGGCTCTCATTTCACACTTT	157–165	2	2	AT
StvChR_546_a	TGGCTGGGTTTTCTTGTTACCTTA	CACACACCCATAAATTTTTCGTCA	149–207	7	2	AC
StvChR_566_a	TTTGAGATCTTGGTGTGGACTGTT	GATCCAAACCAGTTCTTGGAGAAA	180–193	5	2	ATG
StvChR_577_a	CATAATGCATGCACAAAACAGTCA	TTCCTGCTCCACACTACTAACATCC	171–177	3	2	AG
StvChR_580_a	CCCCACTTAGCATATTACATTCCA	TCGGACCTCAACATTACTCTTCAA	120–142	7	2	AC
StvChR_583_a	AGCAATGAGTGCTTGTAGTGTGGT	TCCAACGTTAATTATTGTCATTATCCTC	182	1	1	AG
StvChR_587_a	CTGCAGCAGAACGGTATACACACT	AACCGTTCCCATCCTAAACTTTGT	129	1	1	AG
StvChR_598_a	TGATTTTTGCATTTCTTGACATCTG	AAAGAAAGCGAAAGTTCAAGGACC	123–180	3	2	TTC
StvChR_604_a	CTTCTTGCTGGGACATTTTTCTTC	TGCTCTTGATTTCATTCCCAAAAT	148–165	4	3	TTC

(*Continued on next page*)

Table 2. (*Continued*) Markers that amplified all the Oleaceae accessions tested detected by Unique Pattern Informative Combination software (Arias et al., 2009).[z]

Marker ID	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size range	No. all	Max all/S	Motif
StvChR_605_a	AATTTGAGTGACACCACACAATCG	TTCATCCTTCTTCCTTCTCTCCCT	127–541	13	5	TGG
StvChR_613_a	AATGATCTCACGTAGATTCGTCCC	ATTTGGTCGGACAATGATGAGAAT	143	1	1	AT
StvChR_617_a	TACCACCGCCTGGTAAATTCTC	TAAAAGTGTTGTTCGATTCGCCTT	139–143	3	1	ACC
StvChR_622_a	TCCAAAGCTAGAAACTCTCACAGGA	GTGGACTCAACACCACCCTTATTC	175–194	4	2	AG
StvChR_627_a	GGGGTGAGGGTGTTATTACTGTTG	GCCACTTCCAAATCCCACTATACA	167–182	4	1	AG
StvChR_645_c	TCAACTCAACAGGACCAAACACTG	CACAGGTGACATTTTGCCCTCTTA	149	1	1	AT
StvChR_647_a	AAGCCGAAAAAGAAGAGGAAAAGA	TTAAGAATGAAGAAATGGAGCCTCA	117–125	3	2	AAGG
StvChR_655_b	ACGTAGCAGGATGAGATTCAGACA	GATTGGGTTCTGGAGTTTTGAATG	152	1	1	AC
StvChR_659_a	TTTTATACTTGGAGGAGTGCTAATGC	GACACTATCAATCAGGGTCAAGAAAA	149–283	4	2	AT
StvChR_662_a	ACTTCAGATGTGTCATCTCTCCGA	CTACCTCTCCCACTCCACCTCAT	178–181	4	1	AG
StvChR_671_a	GAATGAAAACCTGCAAGGTCAAGT	ACCTGCAGAAAAAGCATGAGAAAA	163	1	1	TC
StvChR_681_a	AATAATTTGTGATCACCACCCCAC	TGATGATCATGGAAGTGGTTATGG	152	1	1	ACC
StvChR_686_b	GAAAGTCCGAAAGCCAGGTAATTT	GCGTATCACTGTCACCACTTCAAC	116–126	5	2	TC
StvChR_689_a	TGATTGATACTTTTTGATGGGGCT	TTTTCATTTTGCACTCTCCTTTCC	175–201	6	2	AAG
StvChR_697_a	CACCACCACATCCACAATGAATA	ACGATCGTGCGAATCTATGAATTT	102–103	2	1	ACC
StvChR_704_c	ATTTTCGAAATGTACCGGAACTGA	GTGGAGGAAGACCTCCTAGGGATA	140–240	2	2	TC
StvChR_730_a	TGCCTGTGATTTTGTGTATATCGTG	CCAGTGACTAGTTGAAACTTGAATTGC	131–158	6	2	TC
StvChR_733_a	GATCGGCTCCATTGAAAGAGAGTA	ATGCCGAGGATGATGCTGTAAT	100–104	3	2	AG
StvChR_735_a	CACTCTAACCCTAACCCCTTTGCT	GTCGAGCAAGGAACTCTACCAAAA	121–139	7	3	TC
StvChR_740_a	GTTCCTGTGATGTTCGGTTTTCTT	AGGGTGACATAATCTCCACAAGGA	103–111	4	3	TG
StvChR_752_a	GACTTTTAGAAGATTATGAGTGCCCC	TTTATTCCCATTGTTACTTTTCACAAA	172–199	7	3	TC
StvChR_753_a	TCTCGCTTCATCTTATCCTGATCC	TCAAATGTGTTATACAGGGAGGGG	124–125	2	1	TC
StvChR_759_b	TAACACGGACACATCCTAAGCAAC	TGTGTCTCTGAAATGGTTGGTTGT	126–143	5	2	ACC
StvChR_786_a	GATTAACAAAATCATTCCTCCCCC	CATGCAAACATGTTCTATGTGCAG	108–118	6	2	TC
StvChR_795_a	TCATCTACCTCTGAACATAATTCCCA	GGCTGTATTGGCTTAGTGAAATGG	140–176	7	3	AACC
StvChR_808_a	GAGCAAAGGTAAACAAGAAGCGAA	GTCGATTTCTTCCTCGATGTCTGT	163	1	1	AG
StvChR_825_a	TTGGATTAATTTTGGTTTGTTTATTGTT	TGGAAGAGAGTTTGTGCAAGGTAG	172–192	5	2	TC
StvChR_832_b	TGTTTTATTGTGCAAAAATCCGGT	GGTGGAGAATGAGGAGAGAAGTGA	158–185	7	3	TC
StvChR_836_c	CACCAGATCAACAAATCCCATACC	GTGTGATAGATGTGGTGGTTGCAG	100–218	14	5	ACCATC
StvChR_858_a	TTTACCGTGAGAGATAATGGGAGC	TTAGTTGAGTGACTTGACCACCGA	165–203	6	2	AG
StvChR_862_a	GCTTTTAAGAAACTACGTAAGATGGGG	GCATTTGATTTCTTAAGGTCACTCG	159–179	3	2	AG
StvChR_865_a	GTCTCTTCAGGTTTACTCGACCCC	TTGACTGTGAAGTCGTGGGCT	136	1	1	ATC
StvChR_894_a	TGAACATTCACTAATCAGCCTCCTC	AGAGAAGAAGACGGACCGGTAGAC	178–358	5	3	TC
StvChR_896_a	GTCCGGATTCACTTTCTTCCTTTC	ATGATAATGGCGATGGTCTCGTAA	98–119	4	2	TTC
StvChR_918_c	AAAATCCTCCACATTCAACCAGAA	CGACGTTCACTGTTCGTTCACT	173–184	3	2	AC
StvChR_927_a	TAAAATCCGACTTTTCCCTCACAA	CACCAACTTTGGCTAAGAAAAATGA	172	1	1	TC
StvChR_935_a	TAAGCCTTGAAGTAGTAGCTGCCC	ATCGAACTTAAAGTGCTGCCAATC	83–189	5	2	TC
StvChR_943_a	TTACTCCAATCGCTTTCCTAAACG	CAGCCTACATCCAAACTTTGTTCC	123–146	2	2	AG
StvChR_950_a	TATTACCCTATTCCCTGACGAGCA	GGGAAGGAGAAAAACAAAGAGGAA	155–181	5	2	TG
StvChR_976_a	TACGTAGGGAGGAATGTTCAAACG	GCAAGGAACATCAAAAGTCCATCT	179	1	1	AAG
StvChR_996_a	ATTTGAGGGGAATAGGGAAATCGT	AAGTCGAAGACTCGAAGTCCCAC	99–134	5	3	TG
StvChR_1006_a	TGCTGACCACGTTGTTATCTGTTT	ACTGACGTGGAACCAAGAAAGAAG	97–113	3	3	TC
StvChR_1016_a	GCTTTCCTTTTTGTTTCCCTCTTC	TTCATCCAAAGATTTCGAGTGTAAAA	155–183	5	2	TTC
StvChR_1017_a	GACCAACCAAAACAATCCAACTC	CTCCGGTGGTAAATTGAAAGAGAG	92–127	15	5	TC
StvChR_1025_a	AGCCCATTTTTGCATTTTGAGTAT	ACAATGATTTTGTGTTTTGATTTGTCT	157–182	4	1	AC
StvChR_1031_b	TTTGAATCCATCCATTTGAGAACA	TGGGAGAAAAATGAGACTTGGTTT	139–146	8	4	AC
StvChR_1061_a	CACAATTGCACCCAATTCCTACAT	CCATCCTCCTAAACCCTCATTTTC	150	1	1	AT
StvChR_1072_a	TACCAGAGATGGGAACAACACAGA	TTTTAGGAGGGGTGAAATTGATGA	146–161	5	4	AAG
StvChR_217_sk/a	ACTTGAAGATTTATCGGCACATCC	GATGGACCTTTCCATGTTCTTGAC	147–169	5	3	TC
StvChR_351_sk/a	AGTTTTCTCTCTTTACTCACGCCACT	AATGAAGGCGACAATTCCACTAAA	154–184	6	3	ACTC
StvChR_432_sk/a	GTCTCCGACGATGAACCAAATACT	TTTCAAACCTTTGTTCTCGTCTCC	165–176	2	2	AG
StvChR_568_sk/a	TCCCAAAATACCCAAAGAAAACAA	TTGGAATCCTTTTCTCCTATTCCC	149–150	2	1	AAGAT
StvChR_710_sk/a	GTAGAAGAAGGGGAAATGGGAAGA	CTATCCTGGGTGGGTATGAAATTG	174–191	5	2	AAGAG
StvChR_745_sk/a	CACACTCCATCGCAACAATAGAAG	TGTGTGGTTGATTAGTTCGTGGTT	99–157	8	3	AACC
StvChR_955_sk/a	ATTTCCCGCCCATACACATACAC	CGACCGAGTCCTCCATAGAGATTT	172–217	7	2	AC

[z]Markers that amplified all the Oleaceae (StvChR_15_a to StvChR_1032_a) and *Chionanthus retusus* only (StvChR_7_a to StvChR_955_sk/a). DNA sequences shown in bold had significant hits on BLASTx. In the columns, size range is: observed on the samples, includes the 23 bp of primer tailing. "No. all" = total number of alleles detected. "Max all/S" = maximum number of alleles detected on an individual sample. Sequences corresponding to the contigs were submitted to GenBank with accession numbers GQ117288 to GQ118.

for four species of Oleaceae (*C. pygmaeus*, *C. retusus*, *C. virginicus*, and *O. americanus*) using 57 markers as shown in Figure 2A. We also calculated genetic similarity coefficients for *C. retusus* using 195 markers as shown in Figure 2B. We observed a high level of polymorphism within the species *C. retusus* using these 195 markers. These markers allowed a clear distinction among *C. retusus* varieties as is shown by the high bootstrap resampling coefficients obtained. Bootstrap confidence values are indicated on the dendrograms. Clusters formed groups by species with the exception of *C. virginicus* (6007), which associated with *C. pygmaeus* based on the 57 markers analyzed as shown in Figure 2A.

Discussion

Chionanthus SSR-enriched libraries were generated using 24 oligo repeats; however, we detected twice as many repeat motifs, probably as a result of non-specific hybridization during the enrichment process. Although we detected a large number of repeats in the assembled contigs, only 394 pairs of primers were designed under the stringent conditions used. In *C. retusus*, the frequency of repeats decreased with the increase in length of the repeat motifs (Fig. 1). This trend has also been observed in the distribution of SSRs of other eukaryotic genomes (Katti et al., 2001). We also found that in *C. retusus,* the most abundant di-, tri, and tetranucleotide repeat motives were AG/AC, AAG/ACC, and ACTC/ATAC, respectively. In *Arabidopsis thaliana* (L.) Heynh., the same di- and trinucleotide motives were the most abundant across the entire genome;

however, the predominant tetranucleotide motives were of the type "AAA/(CTG)" (Anwar and Khan, 2005). Curiously, we found at least one $(CG)_n$ repeat in *C. retusus*, a particular repeat motif that has not been found in the entire *A. thaliana* genome (Anwar and Khan, 2005) and that is very rare in other eukaryotic genomes (Katti et al., 2001).

Ninety-six percent of 57 SSRs that amplified across the four species of Oleaceae (*C. pygmaeus, C. retusus*, *C. virginicus*, and *O. americanus*) were polymorphic. Within the species *C. retusus*, 61% of 195 SSRs we developed were polymorphic. The high level of polymorphism is not unexpected in this study, because we chose representative samples of *C. retusus* that covered two extremes of its range in Asia with *C. retusus* CR1 and CR3 originating from the Yunnan province in China and CR4 and CR6 from Japan. *Chionanthus retusus* CR2 represents the typical form in cultivation in the United States and is likely derived from the same parental stock as CR5, a plant we originally received as *C. retusus* var. *serrulatus* (Hayata) Koidz. from the Arnold Arboretum, but is of uncertain provenance. The varietal epithet *serrulatus* was first applied to a new species of *Chionanthus* from Taiwan, which was later reduced to a variety of *C. retusus* (Fogg, 1960). A larger survey of North American nurseries is planned to determine the level of genetic diversity of *C. retusus* in cultivation. Only a handful of *C. virginicus* and *C. pygmaeus* accessions were available for sampling and used to test cross-amplification of *C. retusus*-derived SSRs. A broader sampling, encompassing a greater range of *C. virginicus* in eastern North America and larger sampling in Florida, where it is in close proximity to *C. pygmaeus,* is needed to determine population structure and similarities between these species.

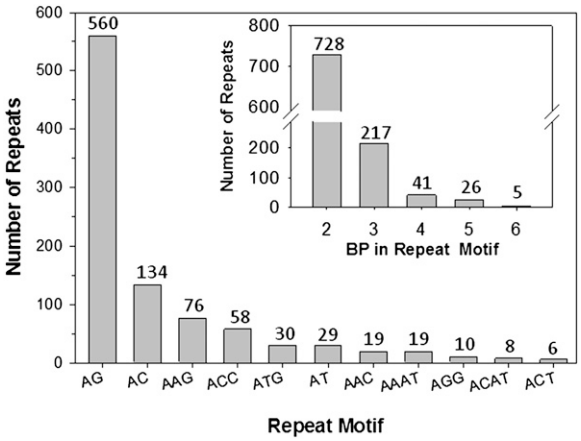

Fig. 1. Motifs and frequency of repeats detected in *Chionanthus retusus* simple sequence repeat (SSR)-enriched libraries. Frequency of motif repeats detected in an SSR-enriched library after screening 1072 contigs. Another 43 repeat motifs with frequencies lower than four were not included in the plot. Embedded graph shows the number of repeats detected in *C. retusus* arranged by repeat motif length (BP).

The percentage of heterozygous loci found in *O. americanus* (68.8%) was twofold higher than in *C. retusus* (30.1% to 39.8%). In addition, *O. americanus* shared 29% of detected alleles with *C. retusus*, which had a genetic similarity of 0.54. Very few alleles were shared between *O. americanus* and the species *C. virginicus* and *C. pygmaeus*. The amplification and sharing of alleles between *Chionanthus* sp. and *O. americanus* is indicative of a recent divergence and supports the conclusions of Wallander and Albert (2000) who reported *O. americanus* to be closely related to *Chionanthus*. The greater number of polymorphic loci in *O. americanus* may also be attributed to genome size. Whereas *Chionanthus* species used in this study are presumed diploid with $2n = 46$, *Osmanthus americanus* is a hexaploid with $2n = 138$ (Taylor, 1945).

BLASTx screening of DNA sequences containing microsatellites showed significant hits on some interesting genes. For example, the sequence that originated marker StvCHR_508a had similarity to SORBIDRAFT of *Sorghum bicolor* that is related to the development of inflorescences (Polegri et al., 2010). The DNA sequence corresponding to marker StvCHR_680a had similarity to proteasome subunit alpha; both 26S proteasome and the *REV* (*REVOLUTA*) genes are required for the maintenance of root apical meristem and shoot apical meristem (Zhang et al., 2010), and it has been shown that plants with double mutations on both isoforms of the 26S proteasome are often non-viable (Gallois et al., 2009). Several DNA sequences had similarity to chromatin remodeling, DNA binding, and control of transcription such as those from markers StvCHR_356a, StvCHR_430b, StvCHR_777a, and StvCHR_943a. The DNA sequences associated to marker StvCHR_709a had similarity to the brahma-related gene (BRG1), which is implicated in tumor suppressor function (Hendricks et al., 2004). Further research taking in

Table 3. Markers that amplified DNA samples from four species of Oleaceae (*Chionanthus retusus, C. virginicus, C. pygmaeus,* and *Osmanthus americanus*) selected by their performance in terms of peak quality and distribution across species.

Marker	UPIC	Marker	UPIC	Marker	UPIC	Marker	UPIC	Marker	UPIC
StvChR_945a	12	StvChR_209b	6	StvChR_793a	5	StvChR_656a	4	StvChR_447a	4
StvChR_350a	10	StvChR_120a	6	StvChR_343a	5	StvChR_740a	4	StvChR_430b	4
StvChR_57a	10	StvChR_504b	6	StvChR_321b	5	StvChR_832b	4	StvChR_124a	4
StvChR_35a	10	StvChR_494b	6	StvChR_185b	5	StvChR_896a	4	StvChR_195a	4
StvChR_9a	8	StvChR_881b	6	StvChR_689a	5	StvChR_351sk	4	StvChR_1017a	4
StvChR_540b	8	StvChR_301a	6	StvChR_76a	5	StvChR_141a	4	StvChR_437a	4
StvChR_925a	8	StvChR_131a	6	StvChR_352a	5	StvChR_99a	4	StvChR_312a	4
StvChR_112a	8	StvChR_221a	6	StvChR_546a	4	StvChR_786a	4	StvChR_44a	4
StvChR_11b	8	StvChR_955sk	6	StvChR_440a	4	StvChR_825a	4	StvChR_989a	3
StvChR_66a	8	StvChR_198a	6	StvChR_184b	4	StvChR_314a	4	StvChR_304a	3
StvChR_62a	8	StvChR_490a	6	StvChR_468a	4	StvChR_745sk	4	StvChR_411a	3
StvChR_251b	7	StvChR_935a	6	StvChR_961b	4	StvChR_894a	4	StvChR_480a	3
StvChR_603a	7	StvChR_150a	6	StvChR_73a	4	StvChR_680a	4	StvChR_563b	3
StvChR_566a	7	StvChR_461a	6	StvChR_284a	4	StvChR_254a	4	StvChR_1025a	3
StvChR_316a	7	StvChR_580a	6	StvChR_94a	4	StvChR_1006a	4	StvChR_33b	3
StvChR_19a	7	StvChR_605a	6	StvChR_1016a	4	StvChR_481a	4	StvChR_598a	3
StvChR_795a	6	StvChR_13b	6	StvChR_967a	4	StvChR_327a	4	StvChR_711a	3
StvChR_836c	6	StvChR_371a	6	StvChR_507a	4	StvChR_159a	4	StvChR_77a	3
StvChR_710sk	6	StvChR_169a	5	StvChR_752a	4	StvChR_15a	4	StvChR_544b	3
StvChR_341a	6	StvChR_357a	5	StvChR_730a	4	StvChR_950a	4	StvChR_326b	3

UPIC values correspond to the number of DNA samples that were discriminated by the marker out of the 12 lines tested from Table 1. Combinations of these markers can detect a number of unique patterns or alleles equal to the sum of their UPIC values.
UPIC = Unique Pattern Informative Combination.

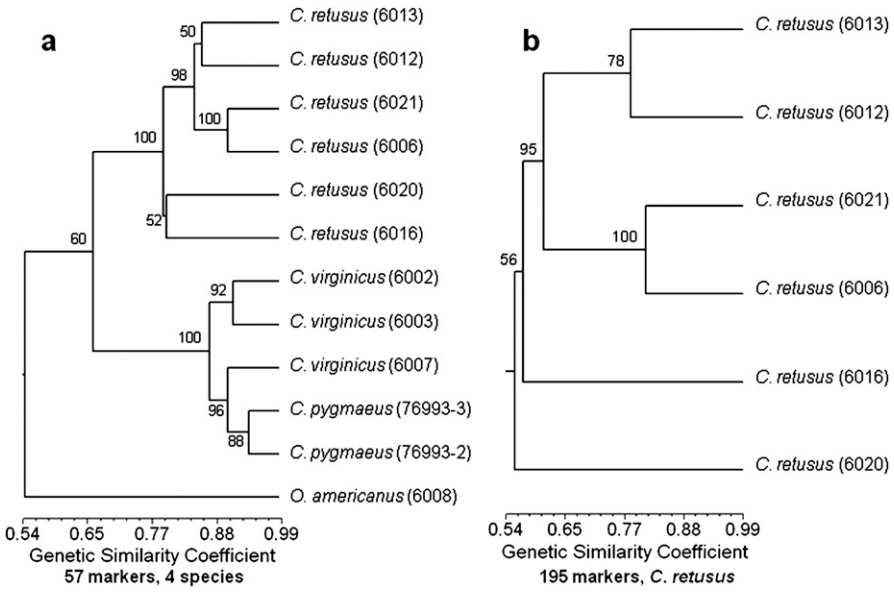

Fig. 2. Clusters calculated using the unweighted paired group method using arithmetic averages (UPGMA) of NTSYSpc 2.2. Confidence levels from bootstrap analysis (5000 replicates) are indicated at the nodes. (**A**) Cluster analysis of 12 DNA samples of *Chionanthus* and *Osmanthus* using 57 simple sequence repeat (SSR) markers that amplified across species; (**B**) cluster analysis of 6 DNA samples of *C. retusus* using 195 SSR markers that amplified this species.

consideration the gene functions potentially affected by the polymorphism of the markers presented here would provide useful insight for plant breeding programs.

Considering that transferability of molecular markers is feasible within plant families (Barreneche et al., 2004; White and Powell, 1997; Yasodha et al., 2005), the markers we developed for *Chionanthus* could be used to examine other economically important members of the Oleaceae such as *Fraxinus* and *Olea*. Although a number of microsatellites are available for *Olea* (Cipriani et al., 2002; Omrani-Sabbaghi et al., 2007; Stambuk et al., 2007), between 200 and 500 additional markers are necessary for 80% to 95% of the *Olea* genome to lie within 10 cM of a marker (Wu et al., 2004).

In the present study, we have characterized a large number of SSR markers for *Chionanthus*-related species: 55 that discriminate among the four species of Oleaceae tested, 162 that discriminate within the species *C. retusus*, 35 polymorphic ones for *C. virginicus,* and 18 polymorphic markers for *C. pygmaeus*. These markers could aid in identifying genetic diversity of *Chionanthus* germplasm and allow verification of hybrids, pedigrees, and cultivars for botanical characterization and ornamental tree breeding programs.

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