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RESEARCH ARTICLE

Cross-transferability of SSR markers in *Osmanthus*

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Abstract Developing a molecular tool kit for hybrid breeding of *Osmanthus* species and related genera is an important step in creating a systematic breeding program for this species. To date, molecular resources have been aimed solely at *Osmanthus fragrans* with little work to develop markers for other species and cultivars. The objectives of this study were to (1) determine cross-transferability of *O. fragrans* and *Chionanthus retusus* derived SSRs in diverse *Osmanthus* taxa, (2) quantify the influence of locus-specific factors on cross-transferability, and (3) determine the genetic relationships between accessions. We tested 70 SSR markers derived from *O. fragrans* and *C. retusus* in 24 accessions of *Osmanthus*. Sixty-seven markers showed transfer to at least one other *Osmanthus* species with an overall transfer rate of 84% of loci across taxa. Genotyping with 42 microsatellite markers yielded a total of 367 loci. Number of alleles per locus ranged from 2 to 17 with a mean of 8.7 ± 4.8. Mean observed and expected heterozygosities were 0.560 ± 0.225 and 0.688 ± 0.230, respectively. Percent of polymorphic loci ranged from 40% in *Osmanthus delavayi* to 100% in *O. fragrans*. *Osmanthus fragrans* had the highest mean number of alleles per locus (4.2) while *O. delavayi* had the lowest (1.1). A reduced suite of eight-markers can distinguish between accessions with non-exclusion probabilities of identity from $3.91E-^{04}$ to $2.90E-^{07}$. The SSR markers described herein will be immediately useful to characterize germplasm, identify hybrids, and aid in understanding the level of genetic diversity and relationships within the cultivated germplasm.

Keywords Oleaceae · Microsatellites · Marker-assisted breeding · Ornamental horticulture

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Introduction

The genus *Osmanthus* Lour. (Family: Oleaceae Tribe: Oleinae) consists of about 30 species of evergreen trees and shrubs distributed primarily throughout temperate and tropical China. *Osmanthus fragrans* (Thunb.) Lour., commonly known as sweet osmanthus or fragrant tea-olive, is the most popular *Osmanthus* species with at least 166 named cultivars (Xiang and Liu 2008). *Osmanthus fragrans* is a common landscape commodity throughout its native range where it is prized for its fragrant flowers and leaf extracts used in traditional medicine (Shang et al. 2003). Four groups of *O. fragrans* (Albus, Asiaticus, Aurianticus, and Luteus, a.k.a Thunbergii) are defined by morphological and phenological traits including flower color, peduncle length, and flowering time (Xiang and Liu 2008). Other popular *Osmanthus* species include *Osmanthus heterophyllus* (Don.) Green (holly tea olive, false holly) and *Osmanthus* × *fortunei* Carr., an *O. fragrans* × *O. heterophyllus* hybrid, *Osmanthus armatus* Diels, and *Osmanthus delavayi* Franch. These species have cultivars with diverse plant architecture, leaf forms, and flowering times and are commonly found in landscape plantings throughout USDA Hardiness zones 7 and 8 in the United States.

Nursery growers desire *Osmanthus* cultivars with vibrant flower color, flower fragrance, increased growth rate, and tolerance to colder climates throughout both the US and China (Dong 2010). Germplasm evaluation and breeding are underway to find species and cultivars suitable for the US market and incorporate favorable traits into *Osmanthus* through hybrid breeding. Developing a molecular tool kit for hybrid breeding of *Osmanthus* species and related genera is an important step in improving this species. Initial molecular studies using chloroplast and nuclear intergenic regions to resolve *Osmanthus* phylogeny showed that *Osmanthus* is a polyphyletic genus with close genetic relationships to *Chionanthus* L. and other species (Arias et al. 2009; Yuan et al. 2010). Molecular work targeted at *O. fragrans* includes the development of SSRs from *O. fragrans* using repeat-enriched libraries (Zhang et al. 2011; Duan et al. 2013), sequencing of the *O. fragrans* transcriptome (Mu et al. 2014), and shot-gun sequencing of the *O. fragrans* genome (Yuan et al. 2015). Highly polymorphic DNA markers resulting from these efforts are ideal for breeding applications including assessing relationships between wild germplasm or cultivated material, genetic map construction, clonal identification, controlled cross certification, species and hybrid identification, and paternity determination (Grocer and Sharma 2016).

To date, Osmanthus molecular resource development has been aimed solely at *O. fragrans* with little work to develop markers for other species and cultivars. Because SSR markers are often transferable within families, markers developed for other species and genera within Oleaceae are a good starting point for developing an *Osmanthus* molecular toolkit (van Heusden and Arens 2010). The objectives of this study were to (1) determine cross-transferability of *O. fragrans* and *Chionanthus retusus* derived SSRs in diverse *Osmanthus* taxa, (2) quantify the influence of locus-specific factors on cross-transferability, and (3) determine the genetic relationships between accessions. The development of these SSR markers will aid in assessing relationships and diversity within the genus *Osmanthus*, prioritizing germplasm for breeding, and providing a tool for cultivar and hybrid identification.

Materials and methods

Twenty-four *Osmanthus* accessions representing six species were included in this study (Table 1). Plants from Nurseries Caroliniana (North Augusta, South Carolina, USA) were maintained in 3-gallon pots under full sun and micro-irrigated using spray stakes. Growing media consisted of pine bark amended with 6.6 kg m^{-3} 19 N-2.1P-7.4 K Osmocote Pro fertilizer (Scotts-Sierra Horticultural Products Co., Maryville, Ohio, USA), 0.6 kg m^{-3} Micromax (Scotts-Sierra Horticultural Products Co.), 0.6 kg m^{-3} iron sulfate, and 0.2 kg m^{-3} Epsom salts. Accessions from the US National Arboretum were received as semi-hardwood cuttings, dipped in liquid K-IBA (1000 ppm), and rooted in pine bark under mist. Growing media consisted of pine bark amended with 3.3 kg m^{-3} 19 N-2.1P-7.4 K Osmocote Pro fertilizer.

Primer selection

DNA was isolated from *Osmanthus* accessions using modified alkaline lysis (Alexander 2016). Primers

Table 1 *Osmanthus* germplasm used to assess cross-transferability of 70 SSR loci

Genus	Species	Cultivar[a]	Source[b]
Osmanthus	*armatus*	Jim Porter	Nurseries Caroliniana
Osmanthus	*armatus*	Longwood	Nurseries Caroliniana
Osmanthus	*armatus*		US National Arboretum
Osmanthus	× *burkwoodii*		US National Arboretum
Osmanthus	*delavayi*		US National Arboretum
Osmanthus	× *fortunei*	Fruitlandi	Nurseries Caroliniana
Osmanthus	× *fortunei*	San Jose	Nurseries Caroliniana
Osmanthus	× *fortunei*		US National Arboretum
Osmanthus	*fragrans*	Apricot Echo[c]	Nurseries Caroliniana
Osmanthus	*fragrans*	Asiatic[d]	US National Arboretum
Osmanthus	*fragrans*	Aurantiacus[c]	US National Arboretum
Osmanthus	*fragrans*	Beni Kin Mokusei[c]	Nurseries Caroliniana
Osmanthus	*fragrans*	Fodingzhu[d]	Nurseries Caroliniana
Osmanthus	*fragrans*	Thunbergii	Nurseries Caroliniana
Osmanthus	*heterophyllus*	Goshiki	Nurseries Caroliniana
Osmanthus	*heterophyllus*	Hariyama	Nurseries Caroliniana
Osmanthus	*heterophyllus*	Head-Lee Fastigate	Nurseries Caroliniana
Osmanthus	*heterophyllus*	Ilicifolius	US National Arboretum
Osmanthus	*heterophyllus*	Kembu	US National Arboretum
Osmanthus	*heterophyllus*	Ogon	Nurseries Caroliniana
Osmanthus	*heterophyllus*	Purpureus	US National Arboretum
Osmanthus	*heterophyllus*	Rotundifolius	Nurseries Caroliniana
Osmanthus	*heterophyllus*	Sasaba	US National Arboretum
Osmanthus	*heterophyllus*		US National Arboretum
Syringa	× *oblata*	Betsy Ross	US National Arboretum

[a] Missing cultivar name indicates an unimproved species

[b] Germplasm was received from Nurseries Caroliniana as plants in 1 or 3 gallon containers; germplasm from the US National Arboretum was received as cuttings. All plants were repotted or rooted in pine bark medium

[c] *O. fragrans* Aurantiacus group

[d] *O. fragrans* Asiaticus group

were selected from three sources: *Osmanthus* enriched-library (OEL) screening (Zhang et al. 2011), *Chionanthus* enriched-library (CEL) screening (Arias et al. 2009), and *Osmanthus* shotgun sequencing (OSS; Yuan et al. 2015). We used all primers developed from *O. fragrans* through OEL screening (n = 20). We chose primers developed from CEL screening that (1) had previously amplified in *Osmanthus*, (2) had a polymorphic information content (PIC) above 4, and (3) were located in an equal mix of genic and intergenic regions (n = 24). Repeat-containing sequences discovered during *Osmanthus* pyrosequencing (1471 sequences) were analyzed through the following method: each sequence was downloaded from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/nucleotide) and queried using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) to find similar nucleotide sequences. A sequence was considered a duplicate if it showed at least 99% identity to the query sequence and an Expect value, or *E*-value, of 0.0. A sequence was considered to be genic if there was an *E*-value of 10^{-10} or less to a gene or putative gene region in another species (Pearson 2013). Unique loci were analyzed via Tandem repeats finder with match, mismatch, and delta values set to 2, 7, and 7, respectively and a minimum period length of 15 (Benson 1999). Primers were chosen from sequences with suitable repeat regions using Primer3 v.0.4.0 set to default parameters (Untergasser et al. 2012).

PCR amplification

SSR loci were amplified using the PCR Core Kit (Qiagen, Carlsbad, California, USA) according to manufacturer's instructions with final reaction concentrations as follows: 10 μM of each primer, 10 mM

dNTPs, 15 mM $MgCl_2$, and 50 ng template DNA. PCR conditions consisted of: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55–60 °C for 45 s, and 72 °C for 45 s; followed by 8 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s; followed by a final extension at 72 °C for 10 min. All PCR reactions included a positive control (locus specific), an outgroup control (*Syringa* × *oblata* Lind. 'Betsy Ross'), and a no template control.

PCR products were electrophoresed on 2% agarose gel at 4.8 V/cm for 2 h and visualized under UV light. Cross-transferability was determined from presence or absence of bands on agarose gels of PCR products. Each sample was given a '1' if a band or bands was present and a '0' if no band appeared. Percent cross-transferability for each locus = (number of accessions with amplified locus)/(total number of accessions) × 100%. Cross-transferability of a marker locus to a given species was considered successful if 50% or more of accessions of that species showed amplification products of the expected size.

Genotyping

SSR loci with clear, single-locus amplification representing a mix of di- and trinucleotide repeats and a mix of locations (genic or intergenic) were sized via genotyping (n = 54). Products were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using 1 µL of PCR product, 10 µL of formamide (Applied Biosystems), and 0.2 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Allele sizes and number of alleles per locus were determined with GeneMarker version 2.6.3 (SoftGenetics, State College, PA, USA). Non-amplified loci were scored as missing data. Markers with irreconcilable stutter peaks were not included in downstream analysis. A total of 42 loci showed clear, unambiguous peaks in all species.

Data analysis

We used Cervus 3.0 (Field Genetics Ltd., London, England) to calculate allele frequencies, number of alleles per locus (k), observed heterozygosity (H_O), expected heterozygosity (H_E), PIC, and non-exclusion probability of identity (NE-PI) from multi-locus genotypes of 24 *Osmanthus* accessions (Kalinowski et al. 2007). A pairwise genetic distance (GD) matrix was calculated using R (R Core Team 2016) using a binary (Jaccard) distance; values for each locus were first converted into a presence-absence matrix. This allowed us to code the loci for the outgroup, *S.* × *oblata* 'Betsy Ross' as zeroes rather than missing for loci not possessed by this cultivar. Using the Jaccard distance measure, a dendrogram was created with the UPGMA clustering method, and bootstrapped probability values for each cluster were generated using the pvclust package (Suzuki and Shimodaira 2015), using 20,000 (re-)samples. Analysis of molecular variance (AMOVA) was performed using the presence-absence matrix (after removing the outgroup) to partition molecular variance among and within species. In order to determine whether there was significant additional variation attributable to species-to-species differences, we performed a Monte-Carlo randomization test based on 10,000 replicates. Additional statistical data analysis was performed using SAS® software, version 9.4 of the SAS System for Windows (SAS Institute, Cary, North Carolina, USA). Analysis of variance (ANOVA) general linear models were used to partition the variance in percent cross-transferability into sources attributable to marker source (OEL, CEL, or OSS), locus function (genic or intergenic), and repeat motif (di- or trinucleotide). An ANOVA general linear model was used to partition variance in marker statistics (k, H_O, H_E, and PIC) into sources attributable to marker source, locus function, and repeat motif. Means of percent cross-transferability and marker statistics for each source of variation were separated using Tukey's multiple range test. To assess the utility of a reduced panel of markers, loci that were polymorphic in all species were ranked by PIC. Eight markers with the greatest average PIC rank across species were selected for an additional analysis as a reduced panel.

Results

Primer selection

OEL- and CEL-derived primers were selected because of previous ability to transfer and/or high information content (Arias et al. 2009; Zhang et al. 2011). The 1471 repeat-containing contigs from *O. fragrans* sequencing (Yuan et al. 2015) represented 945 unique

loci. Of these unique loci, 152 of were in a putative gene and 793 were intergenic. Most loci (90%) were dinucleotide repeats, 7% were trinucleotide repeats, 1% were tetranucleotide, and 2% were more than 4 or a complicated mixture. We chose tri-nucleotide repeats (for ease of scoring, van Heusden and Arens 2010) that were mixed between genic and intergenic. Twenty-one loci fit these descriptions. A total of 70 loci were selected from three sources to test for cross-transferability (Table 2).

PCR amplification

PCR amplified an average of 84% of loci across taxa, with 68 out of 70 loci amplifying in at least one *Osmanthus* cultivar (Table 2). Mean percent amplification across species by marker source ranged from 98% (*O. fragrans* with OEL markers) to 71% (*Osmanthus* × *burkwoodii* with OSS markers).

OEL markers showed the highest mean amplification across taxa, followed by CEL and OSS. Markers containing dinucleotide repeats amplified 12.8% more accessions on average than those containing trinucleotide repeats. There was no difference in mean amplification percentage between genic and intergenic loci (Table 3).

Genotyping

Six *Osmanthus* species assessed using 42 microsatellite markers yielded a total of 367 loci. Number of alleles per locus (k) ranged from 2 to 17 with a mean of 8.7 ± 4.8. Mean observed and expected heterozygosities were 0.560 ± 0.225 and 0.688 ± 0.230, respectively. Observed heterozygosity (H_O) was less than expected heterozygosity (H_E) for most markers, however, that trend varied with marker source. OEL- and OSS-derived markers had 11 and 12% of markers that showed greater H_O than H_E, respectively, indicating fewer than expected heterozygotes. For CEL-derived markers, 56% showed an excess of heterozygotes relative to random mating. PIC of markers ranged from 0.074 to 0.92 with a mean of 0.64 ± 0.23 (Table S1).

Marker source, repeat size, and marker location all significantly influenced genetic diversity parameters (Table 3). OEL-derived markers had almost twice the k as CEL- and OSS-derived markers averaged across all species. The k for dinucleotide repeats was more than double that of trinucleotide repeats. Similarly, k for intergenic loci was double that of genic loci. PIC followed the same pattern, where OEL-derived markers, dinucleotide markers, and intergenic markers had the highest PICs (Table 3).

H_E was significantly higher for OEL-derived markers as compared to other sources, was higher for dinucleotide than trinucleotide repeats, and was higher for intergenic than genic loci. However, there was no difference in H_O for any of these parameters. H_E and H_O were equivalent for loci with trinucleotide repeats and intergenic loci, whereas H_O was reduced compared to H_E for dinucleotide repeats and intergenic loci (Table 3).

Percent of polymorphic loci ranged from 40% in *O. delavayi* to 100% in *O. fragrans* (Table 4). *Osmanthus fragrans* had the highest mean k (4.2) while *O. delavayi* had the lowest (1.1). There was little difference between expected and observed heterozygosities for all species tested. Mean polymorphic information content of markers low for *O. delavayi* (0.15) and moderate for the other species, ranging from 0.24 to 0.55. Using a reduced panel of eight marker increased mean expected heterozygosity and PIC for each species and resulted in more evenly distributed PICs and probabilities of identity across species (Table 4).

Genetic distance

AMOVA results indicated significant genetic structure; that is, the additional variance attributable to species was significant ($p < 0.001$). Most genetic variation (64.4%) was found within species. The most genetically similar accessions were the *O.* × *fortunei* cultivars 'San Jose' and 'Fruitlandi' ($\Phi = 0.29$). The most genetically distant pair of accessions (excluding the outgroup) were *O. heterophyllus* 'Kembu' and *O. fragrans* 'Fudingzhu' ($\Phi = 0.91$). The accessions of *O. fortunei* are most closely related to one another ($\Phi = 0.57 \pm 0.25$) while accessions of *O. fragrans* are the most differentiated ($\Phi = 0.71 \pm 0.079$; Fig. 1).

Discussion

Osmanthus appears to be a polyphyletic group, with accessions of *Chionanthus*, *Olea* L., and *Linociera*

Table 2 Cross-transferability to *Osmanthus* species of 70 SSR loci developed from *O. fragrans* or *Chionanthus retusus*

Locus[a]	Tm (°C)		Sequence	Repeat motif	Size (bp)[b]	Transferability[c]
OSM005	51.6	F	CCTGAACCCATACAAAGAGA	$(TG)_{13}(AG)_9$	150–208	†, ‡, *, **, ∓, β, γ
	53.9	R	CTAAGCAACAGTACCCAGGA			
OSM009	49.7	F	GCATCTTCATTTTACACACG	$(CT)_{17}$	130–178	†, ‡, *, **, ∓, β, γ
	48.0	R	GTTGAGATTCCTGAATATGG			
OSM010	54.7	F	GTA TTC CAG GCT GAT GGG TT	$(CA)_{11}(TA)_8$	148	†, ‡, *, **, ∓, β, γ
	53.0	R	AAA GCC CAA AGT ATG TTC CC			
OSM011	51.6	F	CCC ACT ACA AAC ACG GTA TA	$(CT)_{19}$	170–218	†, ‡, *, **, ∓, β, γ
	48.5	R	GAA CTA TCA CAA TAT GTG CC			
OSM013	52.4	F	CCC TCA AGG TCT CAA ACA AT	$(AC)_{23}$	205–231	†, ‡, *, **, ∓, β, γ
	50.2	R	CTT ATG TTT TGC CTT AGG GT			
OSM014	49.9	F	CCA TCA CAT ACC AAG TCA AT	$(AG)_8$…$(AG)_{18}$	128–162	†, ‡, *, **, ∓, β, γ
	52.6	R	GTC ACA ACA AGG AGA CCT AC			
OSM017	47.9	F	CCT TAG ACA ATT ATT CCG AC	$(AC)_{17}$	179–209	†,‡, *, **, ∓, β, γ
	48.8	R	CAG GTC AAA TAA GTT ATG CC			
OSM023	49.1	F	GCC TTC TCT CTT ATC ATC AA	$(CT)_{12}$	198–250	†, ‡, *, **, ∓, β, γ
	50.0	R	TGG AGA TGA AAG GGA TAG AA			
OSM025	49.3	F	GAC ACC ATA ACA GGG AAT AA	$(TG)_9(AG)_8$	244–290	†,‡, *, **, ∓, β, γ
	54.2	R	CTG GAG GAA CTG CTG TAC TT			
OSM034	54.6	F	CCA TTC CAA TAC GGC CAC TA	$(AC)_{13}$	175–205	†, ‡, *, **, ∓, β, γ
	54.3	R	AAT GCC AGA CCC TTC TTG AA			
OSM036	49.2	F	CGC TTT AGA ATG AAC AAG TC	$(AC)_8$	246–256	†, ‡, *, **, ∓, β, γ
	51.2	R	CTT CTC TAT TTC CAC GCA AC			
OSM037	51.4	F	CTT GAA TCA CCA TAC CTC CT	$(TTC)_{12}$	121–133	†, ‡, *, **, ∓, β, γ
	49.4	R	AGA GGA ATG AGA AAT AGC AC			
OSM038	53.6	F	AAG TAT TGG GAC CAT TGG GA	$(TC)_9(AC)_9$	184–220	†, ‡, *, **, ∓, β, γ
	50.2	R	TCC ACC ACT AGA AAG GTA TT			
OSM041	50.7	F	CTC GTC CAA TAA TGT GTA GC	$(TG)_{10}$	173–197	†, ‡, *, **, ∓, β, γ
	51.7	R	ATG TCC ATA GTG AAT GCC AA			
OSM042	51.5	F	GTT TCC AAC TAA TCC AAC GG	$(AG)_{20}$	216–260	†, ‡, *, **, ∓, β
	55.9	R	CCC TTT CTT CTG TGC TTC CC			
OSM048	58.2	F	CGA TTG CTG GTG AAG CCC TT	$(CCT)_5$…$(CTT)_{16}$	174–204	†, ‡, *, **, ∓, β, γ
	58.3	R	TCG TAG GGT GAG CCA TCG TT			
OSM052	53.1	F	GTG GTG CTG GGA AGA TTA TC	$(TC)_{11}$	105–128	†, ‡, *, **, ∓, β, γ
	51.8	R	CCA TTA GGT TTC TTT GTG CC			
OSM058	48.4	F	ATG TAG GAA AGG AAG AAA GT	$(AG)_{23}$	180	‡, *, **, ∓
	47.2	R	GTA AGA GTG ATA CAC AAC AA			
OSM061	58.6	F	AGA AGG TGG CGG TCA CAG AT	$(TG)_{10}(AG)_{12}$	250–320	†, ‡, *, **, ∓, β, γ
	56.2	R	CAC CAA CTC TCC TCA CTC CA			
OSM063	54.3	F	ACT CTC GGT CAG ATC GTA GA	$(AG)_{15}$	254–286	†, ‡, *, **, ∓, β
	48.7	R	TCC AGA CTT CCA TTT ATT GT			
StvChR_15_a	54.8	F	GGA AAA AGA AAG GGA GAA GGA GAA	$(AG)_{19}$	178–192	†, ‡, *, **, ∓, β, γ
	58.2	R	CTC TGT GAC CAT GAC TGT CTG TGA			
StvChR_35_a	57.7	F	GCC GCT ACT AAT GAG AGA GGA GAA	$(AG)_{19}$	102–161	†,‡, *, **, β
	57.7	R	ACA GTG TTG GTG CAA GTG ATT GTT			
StvChR_57_a	56.8	F	TTT GTC CTC TGT CAA CTC TTT CCC	$(AC)_{17}$	107–139	†, ‡, *, **, ∓, β, γ
	56.4	R	ATC ACC AGA ATG CCA CTT CCT TTA			
StvChR_76_a	59.1	F	TCA GTC TTC ACC ACT ACC ACC GTA	$(ATT)_4$	105–165	†, ‡, *, **, ∓, β, γ
	56.1	R	TGG GCT TTT AGA CGA GTA TTT GGA			
StvChR_94_a	54.6	F	CGG AGA CAA TTT AAA GCA CGA TTC	$(TTC)_2$	151–188	†, ‡, *, **, ∓, β, γ
	55.8	R	CGA CAA TAG TTC AAG CAT TGC GTA			

Table 2 continued

Locus[a]	Tm (°C)		Sequence	Repeat motif	Size (bp)[b]	Transferability[c]
StvChR_114_a	53.6	F	CCT TCC CCA TTT AAT CAA TCA CAA	$(AC)_4$	160–165	†, *, **
	56.0	R	TAT GTT TGT TTA CTT GTG CCC GTG			
StvChR_124_a	55.4	F	AAT TCT CAG CCA ATC ACC TCA TTC	$(TC)_{17}$	105–109	†, ‡, *, **, β, γ
	56.8	R	ATG ACG TGA CCT TTT TGA GAG GAG			
StvChR_221_a	56.6	F	GGC TGA GGT TGA TTA CCT CTG ATT	$(AG)_{11}$	66–106	†, ‡, *, **, ∓, β, γ
	56.5	R	TTT CAG CCT GTA AAA CCC TAC TCT			
StvChR_251_b	55.5	F	TTC TGT TTA TTC ATC TTC CAC CGC	$(TG)_{10}$	108–114	†, ‡, *, **, ∓, β, γ
	57.7	R	TAG ACC AGG GTA TTG GTC TTG CAT			
StvChR_284_a	56.3	F	AAA AAT GTT TG GTA GAA GTG CCG	$(GGC)_4$	69–225	†,‡, *, **, ∓, β, γ
	58.0	R	GTT CCT CAA CTC CCG AGT CCT AAT			
StvChR_285_a	57.2	F	TTG ACA GTG AAG GT CAG AAG AAG	$(AAG)_3$	107–119	†, ‡, *, **, ∓, β, γ
	58.4	R	AGC CAG TCA GTG TTA TTC TCC AGG			
StvChR_316_a	56.2	F	TCT CTT TCC GCT TCT TTT CCT TCT	$(TTC)_8$	152–161	†, *, **, ∓, β, γ
	56.9	R	GCT ACC AAC ATC ATT CGT TCC TCT			
StvChR_350_a	55.8	F	GCT TCA CAA TCC TCC AAA ATG TCT	$(TC)_8$	150–174	†, ‡, *, **, ∓, β, γ
	56.9	R	AGG CAA CGT GAT CTC AGT TCT TTT			
StvChR_356_a	57.3	F	CGA ACT GAT GCA TCT AGC CTT CTC	$(TTC)_5$	137–148	†, ‡, *, **, ∓, β, γ
	55.0	R	TCG AGA ATT TAA ATC AGT CGC TCC			
StvChR_357_a	55.2	F	ATA AGG GAA CAA ATG TGC CAA GAA	$(AG)_7$	175–373	–
	55.6	R	GTA TAG CAG GTC ATT GAT TTC CCG			
StvChR_430_b	56.4	F	TAG AAG AAT CCA TAC ATC ACC GCC	$(AAG)_3$	159–327	–
	56.7	R	TCA CAC TTA TTT CAC TGA GCC AAC A			
StvChR_438_A	54.5	F	AAA ATT AAG AAA CTC AAC CAT GCC C	$(AG)_8$	103–138	†, ‡, *, **, ∓, β, γ
	57.4	R	TAC ATC CCA ACT CAC TCA GGG A			
StvChR_508_a	54.5	F	TGA TGA ACA ATC TCG TTT ATG TCC A	$(AAG)_5$	141–153	†, ‡, *, **, ∓, β, γ
	56.9	R	CTC ACC CTC ATC ATC ACA ACT CTC			
StvChR_563_b	54.6	F	TTT TGG GGG TTG CTC TTG ATA ATA	$(AAG)_3$	125–149	†, ‡, *, **, ∓, β, γ
	53.5	R	CTT TCA TTG AAT TTG GTT CAA GCC			
StvChR_656_a	53.7	F	AAA ATG AGG AAA AAT CAG GGG AAG	$(AGG)_5$	97–139	†,‡, *, **, ∓, β, γ
	53.4	R	CAT CCT TTT TCT TCA ATC TCC TCA A			
StvChR_709_a	54.5	F	CAT TGT TGT GGT TTT GCC TTC ATA	$(CT)_{14}$	174–183	†, ‡, *, **, ∓, β, γ
	52.8	R	CAT TTC CGC CAT TTG AAT GTT TAT			
StvChR_777_a	57.9	F	ACT CCA AGA AAA GTC GGG AAG TCT	$(TA)_4$	170	†, ‡, *, **, ∓, β, γ
	59.5	R	ATC AGA TCG CTG CCT ACT AGT GCT			
StvChR_925_a	58.8	F	AGT CTT GCA ACT GCT CCT TTG TCT	$(AC)_9$	98–121	†, ‡, *, **, ∓, β, γ
	55.9	R	TGT CCA TTC TCC AGA CTT GAA TGA			
StvChR_926_a	55.5	F	GTT TGA CGG TTA CGA TCA ATC CAT	$(ATG)_5$	153–159	†, ‡, *, **, ∓, β, γ
	56.4	R	AAT CCT GAC ATC TCA TCA TCG TCC			
StvChR_945_a	55.9	F	CCA ACT CTT ACG CCA AAC TTC AAT	$(AG)_5$	108–174	†, ‡, *, **, ∓
	55.8	R	TTC CGT CTT TAT TTC TCT CGC TCT			
StvChR_961_b	56.7	F	AAC CAG ATC ATC CAA CCA CCA TAG	$(ACC)_6$	100–181	†, *, **, ∓, β, γ
	54.8	R	TGC AAA TTG TGG TGG TGT TTT TAG			
StvChR_1032_a	55.6	F	CCT TAT GAC TCC ATG GAA GAT TGC	$(ACT)_4$	130–136	†, ‡, *, **, ∓, β
	56.5	R	TCA GCA ATC AAG TCC AAG TCT GAA			
Of_03	55.4	F	AAG GGG CCT AGG ATC GTT TA	$(GGA)_{14}$	261–290	†, *, **, β
	56.6	R	GTG AGA TGG CAC CGT AGG AT			
Of_06	53.6	F	TCC GCT AAA TAA CCG AGA AGA A	$(CTT)_{13}$	364	†, ‡, *, **, ∓, β, γ
	52.7	R	GCC AAC CAA ATG AAG AGA AAA G			
Of_07	52.1	F	GCA AAA ATG TGA AAC GAC GA	$(GT)_{14}$	312–342	†, ‡, *, **, ∓, β, γ
	54.0	R	TGG CAT GTA TTC CAG GTT CA			

Table 2 continued

Locus[a]	Tm (°C)		Sequence	Repeat motif	Size (bp)[b]	Transferability[c]
Of_10	54.7	F	TCT GCA AGT CTA TTA TGG GTG C	$(GTGA)_9$	338–366	†, ‡, *, **, ∓, β, γ
	54.2	R	AGG TTC AAT TTT GGG TGA GAG A			
Of_12	54.0	F	AAT GTG TCA TTC AAG GGG GA	$(CA)_{15}$	258–334	†, ‡, *, **, ∓, γ
	54.9	R	ATG CCT TGT TTG GCT CTC AT			
Of_14	54.5	F	TGG TCC GCT AAA TAA CCG AG	$(TCT)_{14}$	315	†, ‡, *, **, ∓, β, γ
	55.8	R	TGG GAG CTC TCC TTC AAC AT			
Of_21	54.5	F	CAA AAC ACC ACC ACA ACA AAC T	$(AAC)_9$	216	**, ∓
	53.5	R	GTT CAA GCA ATA CCA TTC ACG A			
Of_22	56.5	F	AAA GAA GAG AGG GAG AGG GAG A	$(AAG)_6$	274	*, **, ∓, β, γ
	53.9	R	GAC ATT ACG ACT TTC ATC GCA G			
Of_34	53.7	F	CCA AAA GAA TCG TCT TCA CCT C	$(CAC)_{12}$	216	†, *, **, ∓, β, γ
	55.0	R	TAG TGC TAA TGG GCT GAT GTT G			
Of_42	56.8	F	CGC TAC AAG AGA GCC GTT TAC T	$(GGT)_{11}$	340	†, ‡, *, **, ∓, β, γ
	56.8	R	ATC CAC CTT CAC CTA CAC CAA C			
Of_50	54.9	F	ACC CCG TTT GTC TAC ATT CAT C	$(GTG)_9$	357	†, ‡, *, **, ∓, β, γ
	55.1	R	ACA CCA ACA CCA CCA ATA ACA A			
Of_51	54.4	F	GAA CCT AAA GAA CCT TAT CAC CCA	$(GTT)_{12}$	385	*, **
	54.5	R	TCA AGA TGT CTC AAC CAC CAT AC			
Of_69	54.8	F	CTA ATG AGT CGC ACA CAA AAG G	$(TCG)_6$	274	†, ‡, *, **, ∓
	56.4	R	ATT TCA GAG AAG GAA GAC GGC			
Of_70	54.7	F	AAT ATC CAA CAT GCC TAC CCT G	$(TCT)_{13}$	265	†, ‡, *, **, ∓, β, γ
	55.7	R	GTA ACC CAC GCG ATA CAA GAA G			
Of_90	53.1	F	GAT CGA ATC TTT TAT CTG CGC T	$(TTC)_{13}$	275	†,‡, *, **, ∓, β, γ
	53.6	R	ATC AAA CGG GCA TGT TCA TC			
Of_92	54.4	F	TTG TAG CTT GCC ACT TGT TTT G	$(TTG)_{13}$	200	†, ‡, *, **, γ
	55.6	R	TTG TCT CGT ACT CCT TGG TTC A			
Of_400	55.3	F	TGG TCT TGT TCA AAG CTC TTC C	$(TCT)_{15}$	352–386	†, ‡, *, **, ∓, β, γ
	55.0	R	CAG CCT ATT GTA TCC GGT CTT C			
Of_581	56.4	F	CCT CTA TTA CCT CTC AAC ACC CC	$(CAA)_{11}$	167–188	†, ‡, *, **, ∓, β, γ
	53.3	R	CAT TGT ATC TTG GCA CCT GAA A			
Of_832	55.2	F	CGA AGA GTA TCA GCA TCG ACA C	$(GAC)_6$	311–317	†, ‡, *, **, ∓
	55.0	R	CGA ATA TAG GCT CCA CCA AGA C			
Of_860	55.1	F	GCT GGATAT GAA GGC ACA TTC T	$(TTC)_7$	358–382	†, ‡, *, **, ∓, β, γ
	56.9	R	GCC ATA CGT CTA CAA GTC TCC C			
Of_1050	54.6	F	CAC CAC AAC CAC CAA CAA TAA	$(CCA)_6$	174	γ
	55.5	R	AAA ACC TAT GAT CCC CTC ACC T			
Of_1407	54.6	F	ATC CAT GTA AAG AAG GCT CTG G	$(GT)_{16}$	107	†, ‡, *, **, ∓, β, γ
	54.6	R	ATC TGC TAT TGC CCA AGT TCA T			
Of_1463	54.1	F	GAA GTC AAA GTC TGC AAA GAT CAG	$(GAG)_{13}$	353	†, ‡, *, **, ∓, β, γ
	55.3	R	TTC TTC TTC CTC CTC CTC CTC			

† = *O. armatus*, ‡ = *O. fortunei*, * = *O. fragrans* Aurantiacus group, ** = *O. fragrans* Asiaticus group, ∓ = *O. heterophyllus*, β = *O. burkwoodii*, γ = *O. delavayi*

[a] Loci in italics (n = 54) were assessed for size-based genotyping of 24 *Osmanthus* accessions

[b] Size ranges for loci not genotyped in this study were taken from Zhang et al. (2011) for *Osmanthus* enriched library loci, Arias et al. (2009) for *Chionanthus* enriched library loci, and Yuan et al. (2015) for *Osmanthus* sequence-derived loci

[c] Transferability—marker loci were considered transferable to a given species if gel electrophoresis revealed bands in the expected position for 50% or more of tested accessions of that species

Table 3 Mean genetic diversity parameters of SSR markers originating from a *Chionanthus* enriched library (CEL), an *Osmanthus* enriched library (OEL), and *Osmanthus* shotgun sequences (OSS)

Marker variable	n	k[a]		H_O^b		H_E^c		PIC[d]		% Amplification		n[e]
Source												
CEL	16	6.1 ± 4.3	b[f]	0.53 ± 0.23	a	0.55 ± 0.25	b	0.50 ± 0.25	b	83.3 ± 27.3	a	27
OEL	18	11.8 ± 3.8	a	0.62 ± 0.19	a	0.82 ± 0.12	a	0.78 ± 0.13	a	92.0 ± 10.9	a	20
OSS	9	7.3 ± 3.9	ab	0.50 ± 0.27	a	0.65 ± 0.20	ab	0.63 ± 0.20	ab	75.2 ± 26.4	a	24
Repeat size												
2	25	11.2 ± 4.2	a	0.59 ± 0.17	a	0.80 ± 0.15	a	0.75 ± 0.16	a	88.1 ± 19.1	a	35
3	16	5 ± 3.1	b	0.51 ± 0.30	a	0.52 ± 0.23	b	0.46 ± 0.22	b	78.5 ± 28.5	a	31
Location												
Genic	19	4.3 ± 2.1	b	0.45 ± 0.27	a	0.47 ± 0.23	a	0.43 ± 0.21	b	80.5 ± 29.8	a	23
Intergenic	23	8.3 ± 4.7	a	0.57 ± 0.22	a	0.67 ± 0.22	b	0.62 ± 0.23	a	79.3 ± 24.3	a	25

Means are followed by standard deviations

[a] Number of alleles per locus

[b] Observed heterozygosity

[c] Expected heterozygosity

[d] Polymorphic information content

[e] Number of marker loci for % amplification analysis

[f] Means followed by different letters within columns indicate a significant difference at $p < 0.05$ using Tukey's range test

(Oliver) Stearn intermingled with *Osmanthus* species in several independent phylogenies (Wallander and Albert 2000; Arias et al. 2009; Yuan et al. 2010; Guo et al. 2011). The close relationship between *Chionanthus* and *Osmanthus* may be exploited for hybrid breeding to increase resistance of native ornamental species to pests. For example, the North American native *Chionanthus virginicus* L. (white fringetree) is susceptible to emerald ash borer while *Osmanthus americanus* and *C. retusus* Lindley et Paxton were moderately and strongly resistant, respectively (Cipollini and Rigsby 2015). Evaluation of more *Osmanthus* and *Chionanthus* accessions and subsequent hybridizations will be necessary to introgress favorable genes and generate the variation necessary to expand the genus into new markets.

The SSR markers described herein will be immediately useful to characterize germplasm, identify hybrids, and aid in understanding the level of genetic diversity and relationships within the cultivated germplasm. The high genetic variability within species seen in the present study (64.4%) indicates that cultivars within a species likely have different origins; that is, they are relatively unrelated except for a few pairs. The inclusion of known hybrids (as in the case of *O. fortunei*) may have inflated the within-species differences; however, species such as *O. fragrans* where cultivars are largely unimproved also showed high within-species differentiation. Knowledge of genetic relatedness within *Osmanthus* will be immediately useful to breeders desiring to generate variability within the cultivated germplasm without the risk of inbreeding.

There were no significant differences in percent amplification across species due to high standard deviations. However, OSS-derived markers were clearly more successful in amplifying *O. fragrans* and *O. fragrans* hybrids than other species, while OEL- and CEL-derived markers amplified more evenly across species. New microsatellites being developed from enriched libraries (e.g., Duan et al. 2013) should serve as excellent sources for markers across many genera within the Oleaceae. SSRs from sequence data such as the *Osmanthus* shotgun sequence (Yuan et al. 2015) and transcriptome sequence (i.e., EST-SSRS, Mu et al. 2014) need an analysis pipeline and screening before use. We tested 23 of 945 unique OSS-derived markers. Eight loci (~35%) returned clear, unambiguous peaks in at least half of the accessions of each species. A 35% success

Table 4 Comparison of mean genetic diversity parameters for six species of *Osmanthus* assessed using panels of 42 and 8 microsatellite loci

	n*	Full set (n = 42 loci)							Reduced set (n = 8 loci)					
		Polymorphic loci (%)	*k*** range	*k*	$H_E^{\dagger}$	$H_O^{\ddagger}$	PIC$^{\Psi}$	NE-P(I)$^{\gamma}$	*k* range	*k*	H_E	H_O	PIC	NE-P(I)
O. burkwoodii	1	64	(1–2)	1.6	0.63	0.63	0.24	$3.2E^{-12}$	2	2	1	1	0.38	$3.91E^{-04}$
O. delavayi	1	40	(1–2)	1.1	0.40	0.40	0.15	$6.0E^{-08}$	2	2	1	1	0.38	$3.91E^{-04}$
O. armatus	3	83	(1–5)	2.6	0.62	0.71	0.43	$8.9E^{-24}$	(2–4)	2.5	0.62	0.63	0.44	$5.53E^{-05}$
O. fortunei	3	81	(1–5)	2.2	0.49	0.48	0.33	$5.7E^{-18}$	(2–4)	2.9	0.65	0.75	0.47	$2.10E^{-05}$
O. fragrans	6	100	(2–10)	4.2	0.67	0.62	0.55	$3.5E^{-33}$	(2–7)	4.3	0.72	0.71	0.60	$2.90E^{-07}$
O. heterophyllus	10	90	(1–11)	4.0	0.52	0.44	0.44	$1.0E^{-26}$	(2–9)	4.3	0.59	0.49	0.51	$2.32E^{-06}$

* Number of individuals genotyped

** Number of alleles

† Expected heterozygosity

‡ Observed heterozygosity

Ψ Polymorphic information content

γ Combined non-exclusion probability (identity)

rate in the overall set of 945 unique markers would yield roughly 330 SSRs for *Osmanthus* genetic data analysis. These results fall between results of similar studies with success rates of 20% (Yuan et al. 2015) and 39% (394 primers from 1010 repeat-containing contigs; Arias et al. 2009). Thus, roughly 30–40% of repeat-containing contigs may be expected to produce useful primers for plant breeding applications, with higher percentages attainable when markers are pre-screened for uniqueness.

Selection of a commonly accepted set of microsatellites with wide transferability is an important step for strengthening the molecular discrimination of *Osmanthus* species and cultivars. Dinucleotide repeats are common in plant genomes, but difficult to score because separation of neighboring alleles requires reliable and precise protocols (Baldoni et al. 2009). In one study, an estimated 83% of discrepancies among laboratories in scoring dinucleotide microsatellites were due to erroneous length during the binning or "allele calling" process (Weeks et al. 2002). We attempted to increase the trinucleotide repeat microsatellites for use in *Osmanthus* by over-representing them in primers designed from repeat-containing contigs generated from *O. fragrans* shotgun sequencing. However, there was a clear trade-off between ease of scoring and genetic diversity, where loci with trinucleotide repeats had fewer alleles per locus, lower expected heterozygosity, and lower PIC as compared to markers from the same source that contained dinucleotide repeats. For example, only two markers in our reduced 8-locus suite contained a trinucleotide or tetranucleotide repeat. More testing of trinucleotide repeats is warranted for an international effort to improve *Osmanthus*, keeping in mind a larger suite markers will be needed to provide the same discrimination as a smaller suite of dinucleotide markers.

In cultivated olive, 7 of 11 markers showed an excess of heterozygotes that was attributed to the accumulation of allelic-mutations during asexual propagation (Baldoni et al. 2009). In our study, OEL- and OSS-derived markers showed a decrease in heterozygosity as compared to random mating. Because *Osmanthus* has been selected from wild germplasm over multiple generations, it is reasonable that heterozygosity has been reduced. For example, *O. fragrans* and *O. heterophyllus* showed the most reduced heterozygosity of any species, which is expected as it has undergone the longest and most extensive cultivation. However, CEL-derived markers show an excess of heterozygosity in the same individuals, giving a caveat to interpreting past mating and selection history of ornamental plants from molecular data.

Building a reduced panel of markers was a trade-off between depth and evenness. Choosing a panel by average PIC rank alone led to a panel of markers that

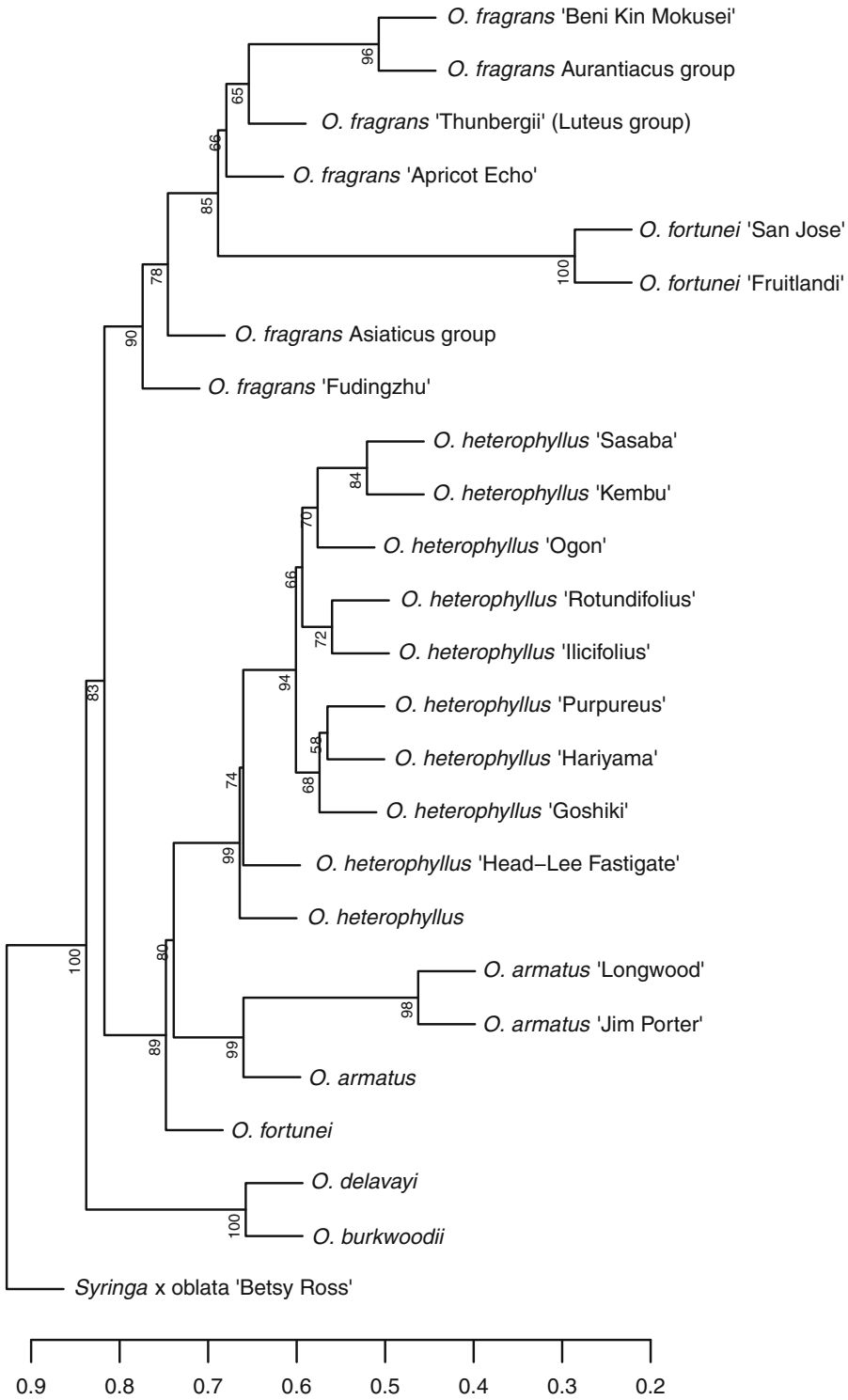

Fig. 1 Tree of 24 *Osmanthus* accessions representing six species and the outgroup, *Syringa* × *oblata* 'Betsy Ross', based on Jaccard distances calculated using 42 microsatellite markers and UPGMA clustering. Nodes are ordered by increasing genetic distance. Scale bar shows genetic distance

maximized H_E, PIC, and probability of identity for species with high allelic diversity. Species with low diversity suffered by having monomorphic markers included in the panel. Restricting the panel to markers that were polymorphic in all species removed the highest overall scoring markers, but led to PICs and probabilities of identity being more uniform across all species. These metrics will become even more favorable as more markers are tested in species relatively differentiated from the marker sources and replace less informative ones on the reduced panel.

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Compliance with ethical standards

Conflict of interest The authors report no financial interest or potential conflicts of interest in conjunction with the planning, execution, and publication of this research.

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