**Development of polymorphic genic-SSR markers by cDNA library sequencing in boxwood, *Buxus* spp. (Buxaceae)**

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- **Premise of the study:** Genic microsatellites or simple sequence repeat (genic-SSR) markers were developed in boxwood (*Buxus* taxa) for genetic diversity analysis, identification of taxa, and to facilitate breeding.
- **Methods and Results:** cDNA libraries were developed from mRNA extracted from leaves of *Buxus sempervirens* ‘Vardar Valley’ and sequenced using the Illumina MiSeq system. Approximately 11.9 million base pairs of sequence data were examined and 845 genic-SSRs were identified, including 469 dinucleotide, 360 trinucleotide, seven tetranucleotide, one pentanucleotide, and eight hexanucleotide repeats. Primer pairs were designed for 71 selectively chosen genic-SSRs containing trinucleotide repeat motifs and were used to amplify the corresponding loci in 18 diverse boxwood accessions. Twenty-three primer pairs amplified polymorphic loci, with two to 10 alleles per locus.
- **Conclusions:** These novel polymorphic genic-SSR markers will aid in evaluating genetic diversity of boxwood germplasm and allow verification of hybrids and cultivars for breeding programs.

**Key words:** boxwood; Buxaceae; *Buxus sempervirens*; cDNA library sequencing; genetic diversity; genic simple sequence repeat (genic-SSR) markers; microsatellites; polymorphism.

Boxwood (*Buxus L. spp.*, Buxaceae) are popular woody landscape shrubs grown for their diverse forms and broad-leaved evergreen foliage (Batdorf, 2004). The genus contains approximately 90 species originating in Africa, Eurasia, the Caribbean, and Central America (Batdorf, 2004). Boxwood plants grown in temperate zones are increasingly threatened by a destructive new blight disease caused by the ascomycete fungus *Calonectria pseudonaviculata* Henricot (syn. *Cylindrocladium pseudo-naviculatum*, *Cylindrocladium buxicola*). First identified from the United Kingdom in 1994, the disease has spread throughout continental Europe, parts of western Asia, and into North America. (Ivors et al., 2012; Elmhirst et al., 2013; Gehesquière et al., 2013; Malapi-Wight et al., 2014). To date, all tested cultivated *Buxus* taxa are affected by boxwood blight, although some taxa appear to be more susceptible to the fungus than others (Henricot et al., 2008; Douglas, 2012; Lamondia, 2014). There is an urgent need to develop blight-tolerant boxwood cultivars because of the impact this disease has on landscapes and commercial growers.

The National Boxwood Collection at the U.S. National Arboretum (USNA) contains more than 700 *Buxus* accessions, making it one of the most complete collections in the world and a valuable genetic resource for developing blight-tolerant varieties. However, genetic relationships and diversity among these accessions have not been determined. Although morphological features can be useful in determining phylogenetic relationships in Buxaceae (Carlquist, 1982; Köhler and Brückner, 1990), molecular markers are needed to distinguish among closely related accessions and to assess diversity. Van Laere et al. (2011) used amplified fragment length polymorphism (AFLP) markers to characterize and differentiate between European and Asian boxwood. In the current study, we developed and characterized 23 polymorphic genic simple sequence repeat (genic-SSR) markers to facilitate genetic diversity analysis of boxwood taxa from the National Boxwood Collection and elsewhere. Compared to AFLP markers, SSRs are multiallelic, codominant, transferable between related species, and can be used to reproducibly fingerprint organisms in different laboratories. Our objective was to generate a suite of polymorphic genic-SSRs from coding regions of the *Buxus* genome, as these markers may also be useful in analyzing the functional diversity in germplasm collections.

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Total RNA was extracted from frozen leaf tissue of *B. sempervirens* L. ‘Vardar Valley’ (Appendix 1) using the QIAGEN RNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). RNA was quantified using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA), and quality was evaluated using the QIAxcel capillary electrophoresis system (QIAGEN). cDNA libraries were constructed using the TruSeq RNA Sample Preparation LS kit (Illumina, San Diego, California, USA) following the manufacturer’s protocol. Validated pooled cDNA libraries were prepared for sequencing following the Illumina protocol and sequenced using the MiSeq system on a 300-cycle MiSeq sequencing cartridge (Illumina). From a single sequencing run, 3,506,048 reads containing 0.5 Gbp of data with an average length of 140 bp per read were generated. Reads were trimmed of adapters and for quality, then assembled and mapped using the CLC Genomics Workbench version 6 software (CLC Bio).

**METHODS AND RESULTS**

**Table 1. Characteristics of 23 polymorphic genic-SSRs developed for *Buxus* spp.**

| Locus  | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | Tα (°C) | Hα | He | GenBank accession no. | BLAST top hit description (organism) |
|--------|--------------------------|--------------|------------------------|---------|----|----|-----------------------|-------------------------------------|
| BSVV14 | F: GATGAGGAAAGCCAGGTTGA | (GAT)6       | 174–180                | 3       | 58 | 0.30 | 0.265 | KM660701             | Predicted: protein SET *Vitis vinifera* |
|        | R: CGCAGCAACATGGAAGCTT   |              |                        |         |    |     | NM_171950             |                                     |
| BSVV15 | F: GTGCGGGAACCCTATAGTG   | (CTC)5       | 181–196                | 5       | 58 | 0.30 | 0.395 | KM660702             | Predicted: transcription factor TCP2-like *Vitis vinifera* |
|        | R: CAGAAGGCGTGAACTGTTAGA |              |                        |         |    |     | NM_171948             |                                     |
| BSVV22 | F: AGCCTTTAAAAGGAGAGAGG  | (TGA)3       | 130–145                | 6       | 58 | 1.00 | 0.775 | KM660703             | Hypothetical protein JCGZ 26794 [Jatropha curcas] |
|        | R: GCATTTCCTTTGCGAAACGGC |              |                        |         |    |     | NM_171949             |                                     |
| BSVV25 | F: TACGCTGATCCAACTCCG    | (AAG)5       | 199–223                | 5       | 60 | 0.40 | 0.485 | KM660704             | Predicted: probable nuclear protein 5-2-like *Vitis vinifera* |
|        | R: AGTGTCCTCAAGCTGAGCCT  |              |                        |         |    |     | NM_171951             |                                     |
| BSVV27 | F: TTCTCGTCATATCCCTCTCA  | (TTC)3       | 146–152                | 3       | 58 | 0.00 | 0.320 | KM660705             | Predicted: G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300 [Vitis vinifera] |
|        | R: AAGCTCTCGTTGGGAGACCT  |              |                        |         |    |     | NM_171952             |                                     |

**Note:** A = number of alleles per locus; Hα = expected heterozygosity; He = observed heterozygosity; Tα = annealing temperature.  
1The number of repeat units is the value for *Buxus* ‘Vardar Valley’, from which the original SSR markers were derived.  
2The allele size ranges for each locus and number of alleles per locus (A) were determined based on the data from 18 boxwood accessions.  
3The annealing temperature (Tα) was chosen based on experimental results using a gradient PCR machine to optimize PCR results. Optimization was necessary because each forward primer had an additional 18-bp sequence used for fluorescent labeling.  
4Observed heterozygosity (Hα) and expected heterozygosity (He) were determined by genotyping the 10 diploid boxwood accessions. Locus BSVV64 was monomorphic for these 10 diploid accessions tested.  
5Putative functions of homologous predicted proteins from a BLASTX search in the NCBI nonredundant database, with a threshold E-value of 1.0E-06.
The partial transcriptome assembly was mined for microsatellites using the PrimerPro Perl pipeline (http://webdocs.cs.ualberta.ca/~yifeng/primerpro/), which used the MISA algorithm (Coello Coello and Cortés, 2005) to detect tandem repeats of two to six nucleotides for at least five perfect repeat core motifs. A total of 845 SSR motifs were identified, including 469 dinucleotide, 360 trinucleotide, seven tetranucleotide, one pentanucleotide, and eight hexanucleotide repeats (sequences available from the authors). PCR primer pairs were designed using the Primer3 algorithm in the PrimerPro pipeline, with the following settings: primer length of 20 ± 2 nucleotides, GC content of 40–60%, and a PCR product size ranging from 100 to 300 bp. Trinucleotide motifs possessing unique PCR priming sites within the genome, as determined by BLASTN searches using PrimerPro, were evaluated visually for heterozygosity and mutation consistent with stepwise evolution. A total of 71 candidate markers were selected for testing from the trinucleotide SSR sites meeting these in silico criteria. PCR primers were manufactured by Integrated DNA Technologies (Coralville, Iowa, USA). The forward primers had an additional M13–(21) universal sequence (TGTAAAACGACGGCCAGT) attached to the 5′ end to allow indirect fluorescent labeling of PCR products using just one universal FAM (6-carboxy-fluoresceine)–labeled M13 primer (Schuelke, 2000). These 71 primer pairs were used to amplify SSR loci in 18 boxwood accesses representing diverse species and cultivars (Appendix 1). Twenty-three of these primer pairs proved to be polymorphic and resulted in expected amplification profiles (Table 1). In addition, eight primer pairs amplified monomorphic loci, 33 primer pairs amplified multiple regions or an unexpected size product, and seven primer pairs did not amplify a product at all (data not shown).

Genomic DNA was extracted from frozen leaf tissue of 18 boxwood accesses using the Qiagen DNeasy Plant Kit and quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). PCR was carried out in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, California, USA). The 20-μL PCR reaction mixture contained 10 ng of template genomic DNA, 0.25 μM of each reverse and universal FAM-labeled M13–(21) primer, and 0.0625 μM of the forward primer with 1× Bio-line MangoMix and 2.5 mM Bioline MgCl2 (Bioline, Taunton, Massachusetts, USA). PCR profiles consisted of initial denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, annealing temperature of each primer pair (Table 1) for 45 s, and 72°C for 45 s; followed by eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. Products were analyzed on an ABI 373xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) using 1 μL of PCR product, 10 μL of formamide (Applied Biosystems), and 0.3 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Allele sizes and number of alleles per locus were determined using GeneMarker version 2.6.3 (SoftGenetics, State College, Pennsylvania, USA). Number of alleles per locus ranged from two to 10 with a mean of 4.34 (Table 1). The boxwood population we used included 10 diploids, four triploids, two tetraploids, and two mixoploids, as determined by flow cytometry in our laboratory (Appendix 1). Thus, we cannot report expected heterozygosities (Hs) for this population unless segregation analysis was performed to confirm dosage patterns of alleles for each locus (Dufrèse et al., 2014). Instead, we treated the 10 diploids as one population and calculated the observed heterozygosity (Ho) and Hs using GenAIEx software (version 6.5; PeaKall and Smouse, 2012) (Table 1). Excluding the monomorphic locus BSVV64, Hs and Hs ranged from 0.000 to 1.000 and 0.185 to 0.840 with means of 0.377 and 0.495, respectively. The contig sequences of the 23 SSR loci were subjected to a BLAST search against the National Center for Biotechnology Information (NCBI) nonredundant protein database using the BLASTX program to identify putative functions. With a threshold E-value of 1.0E-06, all 23 SSR sequences shared homology to protein sequences from dicots from diverse families (Table 1).
APPENDIX 1.  Boxwood accessions (*Buxus* spp.) evaluated in this study, from the National Boxwood Collection at the U.S. National Arboretum.

| Taxa* | Accession no. | Ploidy* | Source |
|-------|---------------|---------|--------|
| *Buxus balearica* Lam. | 81245 | Diploid (2x) | Cultivated; received from Dawes Arboretum, collected from garden in Yalta, Ukraine |
| *B. balearica* | 81312 | Diploid (2x) | Cultivated; received from Woodlanders Nursery, originally from College of William & Mary |
| *B. bodinieri* H. Lév. | 960187 | Diploid (2x) | Cultivated; received from J. C. Raulston Arboretum, originally from Heronswood Nursery |
| *B. harlandii* Hance | 36672-L | Diploid (2x) | Cultivated; collected from garden in Xi’an, Shaanxi Province, China |
| *B. harlandii* ‘Richard’ | 81337 | Mixoploid (2x/4x) | Cultivated; received from Nurseries Caroliniana |
| *B. microphylla* Siebold & Zucc. var. *japonica* (Müll. Arg.) Rehder & E. H. Wilson | 17525-CJ | Tetraploid (4x) | Collected directly from the wild, Nikko, Tochigi Prefecture, Japan |
| *B. microphylla* var. *japonica* ‘National’ | 7025-V | Triploid (3x) | Cultivated; received from Morris Arboretum |
| *B. microphylla* var. *japonica* ‘Sprinter’ | 81341 | Triploid (3x) | Cultivated; received from Spring Meadow Nursery |
| *B. sempervirens* L. | 36365-K | Diploid (2x) | Collected directly from the wild, Bzyb River, Georgia (as *B. colchica*) |
| *B. sempervirens* ‘Borderline’ | 81766 | Triploid (3x) | Cultivated; received from North Carolina State University |
| *B. sempervirens* ‘Highlander’ | — | Diploid (2x) | Cultivated; received from Conard-Pyle |
| *B. sempervirens* ‘Katerberg’ | 81342 | Diploid (2x) | Cultivated; received from Conard-Pyle |
| *B. sempervirens* ‘Vardar Valley’ | 6395 | Diploid (2x) | Collected directly from the wild, Vardar River, Macedonia |
| *B. sinica* (Rehder & E. H. Wilson) var. *aemulans* (Rehder & E. H. Wilson) P. Brückner & T. L. Ming | 60705-J | Diploid (2x) | Collected directly from the wild, Huangshan, Anhui Province, China |
| *B. sinica* var. *insularis* (Nakai) M. Cheng | 35486-J | Triploid (3x) | Cultivated; received from the Washington Park Arboretum |
| *B. sinica* var. *insularis* ‘Franklin’s Gem’ | — | Tetraploid (4x) | Cultivated; received from Conard-Pyle |
| *B. ‘Conroe’* | — | Mixoploid (2x/4x) | Cultivated; received from Conard-Pyle |
| *B. ‘Green Gem’* | 51904-H | Diploid (2x) | Cultivated; received from O. E. White Arboretum |

*Taxa include species, varieties, and cultivars. Hybrids of unknown parentage are listed by their cultivar names.

*National Arboretum accession numbers are given when available, with the exception of *B. bodinieri*, which is listed using its source number.

*Ploidy was determined using flow cytometry in our laboratory.