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RESEARCH ARTICLE

Genetic relationships of boxwood (*Buxus* L.) accessions based on genic simple sequence repeat markers

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Abstract Boxwoods (*Buxus* L., Buxaceae) are popular woody landscape shrubs grown for their diverse forms and broad-leaved evergreen foliage. We used genic simple sequence repeat (genic-SSR) markers to assess genetic diversity and relatedness of 275 accessions from the National Boxwood Collection at the U.S. National Arboretum. Flow cytometry was conducted to determine the relative ploidy of each accession. Genic-SSR loci were highly variable among the accessions, detecting an average of 6.7 alleles per locus based on 17 primer pairs. Data were analyzed with a distance matrix based on Jaccard's similarity index, followed by Unweighted Pair Group Method with Arithmetic Mean clustering. Two major clusters were identified, with four subclusters consisting of individual accessions from *B. balearica* Lam., *B. bodinieri* Lévl., *B. harlandii* Hance, *B. microphylla* Siebold et Zuccarini, *B. sempervirens* L., *B. sinica* (Rehd. et Wils.) M. Cheng, and their putative interspecific hybrids. The accessions generally clustered by cultivar, provenance, or species. Clustering within each group typically reflected breeding pedigrees, when known, and the clusters were supported by bootstrap results. This information will be used for breeding programs and collection management, and for identifying possible sources of disease tolerance for boxwood blight and other diseases and pests.

Keywords *Buxus* · *Calonectria pseudonaviculata* · Cultivar diversity · Flow cytometry · Germplasm · Microsatellite markers · Polymorphism

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Introduction

Boxwoods (*Buxus* L., Buxaceae) are popular landscape shrubs or small trees grown for their diverse forms and broad-leaved evergreen foliage. They are used in the landscape as specimen plants, hedges, mass plantings, or as topiary (Larson 1999; Batdorf 2004, 2005; Van Trier and Hermans 2005). Each year, more than 13 million boxwood plants are sold in

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the United States, with an annual value of over $100 million (USDA-NASS 2010). The genus *Buxus* grows in a range of ecological conditions from dry scrub to montane rain and cloud forests (Van Laere et al. 2015). The genus contains 95-100 species originating in Africa, Eurasia, the Caribbean, and Central America (Larson 1999). The most economically important species, *B. sempervirens* L., has approximately 400 named cultivars (Niemiera 2012). Boxwood plants grown in temperate zones are increasingly threatened by a destructive blight disease caused by the ascomycete fungus *Calonectria pseudonaviculata* Henricot (syn. *Cylindrocladium pseudonaviculatum, Cy. buxicola*). This disease causes necrotic stem and leaf lesions, defoliation, and usually plant death (Henricot and Culham 2002; Douglas 2012). First identified in 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). In the United States, the first confirmed reports of the disease were made from Connecticut and North Carolina in November 2011 (Ivors et al. 2012), followed by diagnosis in 12 additional states and three Canadian provinces (Elmhirst et al. 2013; Hagan and Conner 2013; Malapi-Wight et al. 2014; Williams-Woodward 2014). To date, all tested cultivated *Buxus* taxa are susceptible to boxwood blight, although some taxa seem to be more vulnerable to fungal infection than others (Henricot et al. 2008; Douglas 2012; LaMondia 2014, 2015). Published reports indicate that boxwood blight can be effectively managed with fungicides (Henricot et al. 2008; LaMondia 2015); however, developing blight-tolerant boxwood cultivars is a favored, long-term management strategy for this disease (Guo et al. 2015).

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, the genetic diversity and relatedness of these accessions has not been determined. Although morphological features can be useful in determining genetic relationships in Buxaceae (Carlquist 1982; Köhler and Brückner 1990), molecular markers are needed to differentiate among closely related accessions, identify plants, and help determine provenance, origins, and genetic divergence (Harris-Shultz et al. 2015). Van Laere et al. (2011) used amplified fragment length polymorphism (AFLP) markers to characterize and differentiate between European and Asian boxwood taxa, and von Balthazar et al. (2000) used internal transcribed spacer regions and plastid *ndhF* sequences to describe phylogenetic relationships. Our objective was to show the utility of previously developed genic SSR markers (Thammina et al. 2014) and ploidy analysis to characterize the diversity and, in some cases, the identity of *Buxus* accessions in the National Boxwood Collection at the USNA.

Materials and methods

Plant materials

Young leaves were collected in May/June from 275 *Buxus* accessions (Table 1 and Supplemental Table 1) maintained in the USNA National Boxwood Collection in Washington, DC and Beltsville, MD. These were selected from the entire boxwood collection, which includes approximately 700 individual plants, to include reference taxa, economically important cultivars, wild-collected species, and unknown or questionable accessions for verification and identification. Taxa included *B. balearica* Lam., *B. bodinieri* Lévl., *B. harlandii* Hance, *B. microphylla* Siebold et Zuccarini, *B. sinica* (Rehd. et Wils.) M. Cheng, *B. sempervirens* L., hybrids, cultivars, and unknown species. Leaf material was stored at −20 °C until use.

DNA extraction and PCR amplification

Genomic DNA was extracted from frozen leaf tissue using a DNeasy Plant Mini Kit in a QIAcube (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations, and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Twenty-three trinucleotide genic-SSR primer pairs (Supplemental Table 2) developed in our lab to amplify polymorphic loci in previous tests (Thammina et al. 2014) were used to assess genetic diversity. Polymerase chain reactions (PCR) and analysis of PCR products were carried out as previously published (Thammina et al. 2014). To avoid false negatives, primers that resulted in null

Table 1 Summary of the number of accessions of each *Buxus* taxon included in this study

Taxon	Number of accessions included in this study
Buxus balearica Lam.	5
Buxus bodinieri Lévl.	2
Buxus harlandii Hance	18
Buxus microphylla Siebold et Zucc.	26
Buxus microphylla var. *japonica* (Müll. Arg. ex Miq.) Rehder et E.H. Wilson	23
Buxus sempervirens L.	139
Buxus sinica (Rehder et E.H. Wilson) M. Cheng	8
Buxus sinica var. *aemulans* (Rehder et E.H. Wilson) M. Cheng	1
Buxus sinica var. *insularis* (Nakai) M. Cheng	31
Buxus sp.	7
Cultivars of undesignated species	15

Detailed accession information can be found in Supplemental Table 1

alleles in some of the samples were tested at least twice. Non-amplified loci were scored as missing data. Allele sizes and number of alleles per locus were determined with GeneMarker version 2.6.3 (SoftGenetics, State College, PA, USA).

Data analysis

The amplified allele data were converted to a binary matrix (presence/absence) for each allele by using R-polysat package, version 1.1 (Clark and Jasieniuk 2011). Because it was unknown whether our accessions were autopolyploids or allopolyploids, each genic-SSR allele was treated as independent in this study. A genetic distance matrix was generated based on Jaccard's similarity index implemented in the 'pvclust' package version 1.3-2 of R software (Suzuki and Shimodaira 2014). The Jaccard distance was chosen since it does not use the absence of an allele as a shared characteristic (Legendre and Legendre 1998). Accessions were then clustered by using the UPGMA algorithm implemented in 'pvclust.' The UPGMA algorithm was chosen because in a phenetic analysis, it makes no assumptions about evolutionary rate or phylogenetic relationships. The confidence level for the dendrogram was determined by bootstrapping analysis based on 20,000 replications. Only bootstrap values greater than 70 % are shown in the figures. A Bayesian method (STRUCTURE, Pritchard et al. 2000) was also tested to determine higher-level groupings. Following Evanno et al. (2005), STRUCTURE was run with a range of groups (two to seven) and results for the most parsimonious number of groups were examined in detail. Following the first split, this methodology was repeated on each subset, and the probabilities of individuals belonging to particular groups were examined.

Flow cytometry

Boxwood samples were processed and analyzed on a CyFlow Space flow cytometer (Partec, Munster, Germany) with ploidy determination based on the total DNA content per nucleus (linear fluorescence intensity). Nuclei were extracted with a Partec CyStain UV Precise P kit according to the manufacturer's instructions. Approximately 0.5 cm^2 fresh leaf tissue harvested in May/June was chopped with a razor blade in a 55-mm-diameter Petri dish containing 400 µL nucleus extraction buffer (Partec). The resulting slurry was incubated for 2 min at room temperature and filtered through a 50-µm CellTrics disposable filter (Partec) placed on top of a plastic 12 × 75-mm culture tube. 1.6 ml of staining buffer was added to each tube and incubated at room temperature for 60 s. Instrument parameters and peak sizes were standardized with accession 6395-H (*B. sempervirens* 'Vardar Valley'), a confirmed diploid ($2n = 2x = 28$) based on published chromosome counts (Van Laere et al. 2011). Samples were analyzed on the flow cytometer in the blue fluorescence channel. At least 3000 events were collected for each sample with a flow rate of 1 µL

sample per min and the mean FL1 recorded for peaks. Two samples of each accession were analyzed, and ploidy level was determined based on mean FL1 of sample peaks relative to the standard diploid control peak.

Results

SSR analysis

Six of the original 23 genic-SSR primer pairs tested (BSVV22, BSVV30, BSVV60, BSVV72, BSVV74, and BSVV99) amplified allele numbers that were inconsistent with our ploidy determinations in 37, 2, 1, 2, 1, and 5 accessions, respectively (data not shown). PCR amplification was repeated for these primer/sample combinations, and the allelic data still did not match the ploidy. Specifically, these primers amplified many fragments, even at high annealing temperatures, indicating that they were amplifying multiple regions in the genome. Therefore, the data from these primer pairs were not used in the analysis. The remaining 17 SSR loci were highly variable among the accessions, detecting an average of 6.7 alleles (amplicons) per locus ranging from 96 to 289 bp (Supplemental Table 2). These 17 genic-SSR primer pairs generated 114 scored fragments (alleles) across the 275 accessions. Locus BSVV56 was the most polymorphic (23 alleles), and loci BSVV41 and BSVV64 were the least polymorphic (2 alleles each) (Supplemental Table 2) (see Fig. 1).

Flow cytometry

Four different ploidy groups—diploid ($2n = 2x = 28$), triploid ($2n = 3x = 42$), tetraploid ($2n = 4x = 56$), and mixoploid ($2x/4x$)—were observed among the *Buxus* accessions analyzed in this study (Supplemental Table 1; Figs. 2, 3, 4). A total of 44 accessions were triploid, 20 were tetraploid, three were mixoploid, and the remainder were diploid. Ploidy level generally corresponded with species. For example, most of the *B. sinica* var. *insularis* (Nakai) M. Cheng, *B. balearica*, *B. sempervirens*, and *B. harlandii* were diploid. CV % values of the peaks were <6.0 %, indicating well-resolved peaks. Even with variation in diploid genome sizes among species (1.38–1.69 pg per 2C; Van Laere et al. 2011), distinctions among the different ploidy levels were unequivocal.

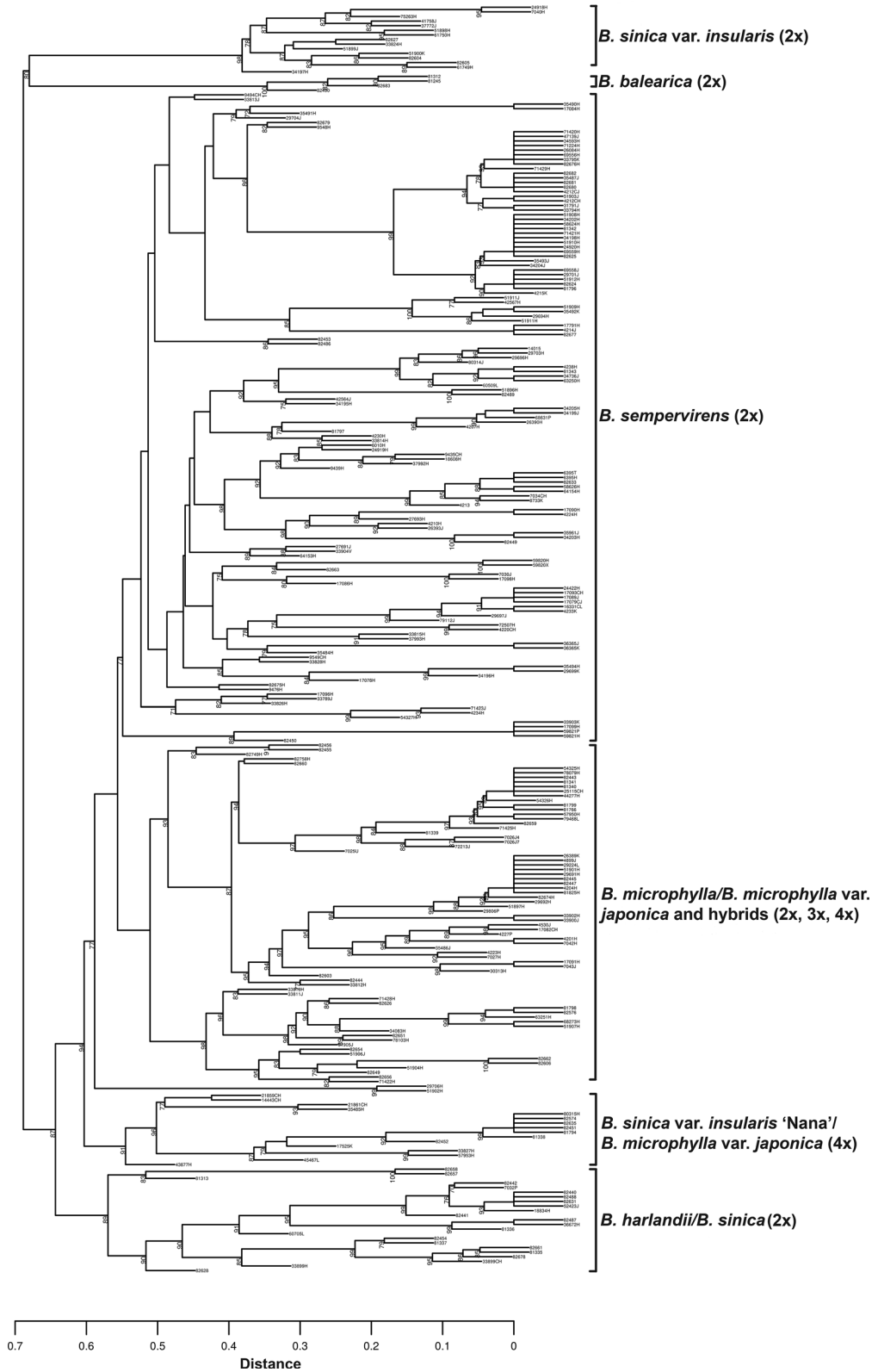
Fig. 1 Dendrogram of 275 boxwood accessions generated by ▶ UPGMA cluster analysis showing the overall clustering. Details of higher-order groups are shown in Figs. 2, 3, and 4. *Labels* indicate the predominant taxon or taxa and ploidy level in each cluster

Cluster analysis

UPGMA cluster analysis revealed two major clusters supported by high bootstrap values (>70 %), with further subclusters in each group. Accessions tended to cluster with like taxa (discussed in detail below) as indicated by the species labels to the right of the branches (Fig. 1). STRUCTURE analysis revealed an optimum population size (k) of two; however, these populations were separated by species (*B. sempervirens* and non-*sempervirens*), so they did not correspond with the UPGMA clustering. We followed the phenetic (UPGMA) approach rather than a Bayesian (STRUCTURE) one for several reasons. First, our accessions are not part of a population, but rather are clonally-propagated selections representing several species with varying ploidy levels. Alleles cannot be assumed to be in Hardy–Weinberg equilibrium, and there are very few known progenitor or wild species in our collection that can be used to determine origins. Finally, our interest was in learning about the relationships among our accessions for collection management purposes, and a predefined population structure could not be assumed. UPGMA clustering was therefore the more appropriate analytical method for our collection and our objectives.

Discussion

Although the UPGMA dendrogram was complex and contained some clusters that were not supported by strong bootstrap values, it was quite effective in addressing collection management issues, such as identifying accessions, determining relatedness, and detecting labeling problems for specific accessions. Accessions grouped into two major clusters (Fig. 1). The smaller of these clusters contained what we labeled as the *B. sinica* var. *insularis* and *B. balearica* groups (Fig. 2A). The *B. sinica* var. *insularis* group


B. sinica var. insularis (2x)
B. balearica (2x)
B. sempervirens (2x)
B. microphylla/B. microphylla var. japonica and hybrids (2x, 3x, 4x)
B. sinica var. insularis 'Nana'/ B. microphylla var. japonica (4x)
B. harlandii/B. sinica (2x)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Distance

includes the historic accession NA 82627, which was originally collected by E. H. Wilson in Korea in 1918 and was the first Korean boxwood germplasm distributed in the U.S. (Batdorf 2004). Korean boxwoods are popular in the U.S. nursery and landscape industry for their horticultural appeal and exceptional cold-hardiness (Batdorf 2004). This cluster includes other known Korean wild-collected or seed-derived forms of *B. sinica* var. *insularis* (NA 61749-H, NA 61750-H, and NA 82605, NA 51899-J, NA 37772-J, and NA 41758-J). From this list, accessions NA 37772-J and NA 41758-J are both named 'Wintergreen', yet are distinct. Historical records indicate that 'Wintergreen', introduced by Scarff's Nursery in 1960, was sold as a mixture of up to 25 clones, all under the same name (Batdorf 2004). While most accessions labeled as *B. sinica* var. *insularis* are included in this group, several accessions clustered in the *B. microphylla* or hybrid groups. This could be due to one or more factors, including variability in this species caused by a diverse provenance base followed by selection, previously unidentified hybridization within this species or its cultivated progeny, or simply misidentification.

The *B. balearica* group (Fig. 2A) includes accession NA 82683, which was grown from wild-collected seed from the Balearic Islands (Spain), along with two other *B. balearica* accessions. An accession received as *B. sempervirens* (NA 82490) also grouped in this cluster, but its grouping as well as morphology indicates that it may be a misidentified accession of *B. balearica*. In contrast, two additional accessions labeled as *B. balearica* (NA 82453 and NA82486) grouped far from these wild-collected specimens in the *B. sempervirens* group, suggesting that they are misidentified. The *B. harlandii/sinica* group (Fig. 2B), supported by a 89 % bootstrap value, includes accessions related to *B. harlandii*, *B. sinica*, and *B. bodinieri*. *Buxus harlandii* is native to central and southern China (Batdorf 2004). The USNA accessions include historical accession NA 82442, the first U.S. introduction of *B. harlandii* (PI 23012), collected by agricultural explorer Frank Meyer in Hangchow, Chehkiang, China in 1907. Our study also reveals that an additional redistribution of this material by the USDA (NA 82441) in 1936 is distinct from NA 82442 and, in fact, is more similar to *B. harlandii* NA 7032 which originated from a collection in England (Hilliers). This indicates that several provenances of *B. harlandii* are currently available in the U.S. and include a number of cultivated forms collected in China as either *B. harlandii* (NA 36672-H) or *B.*

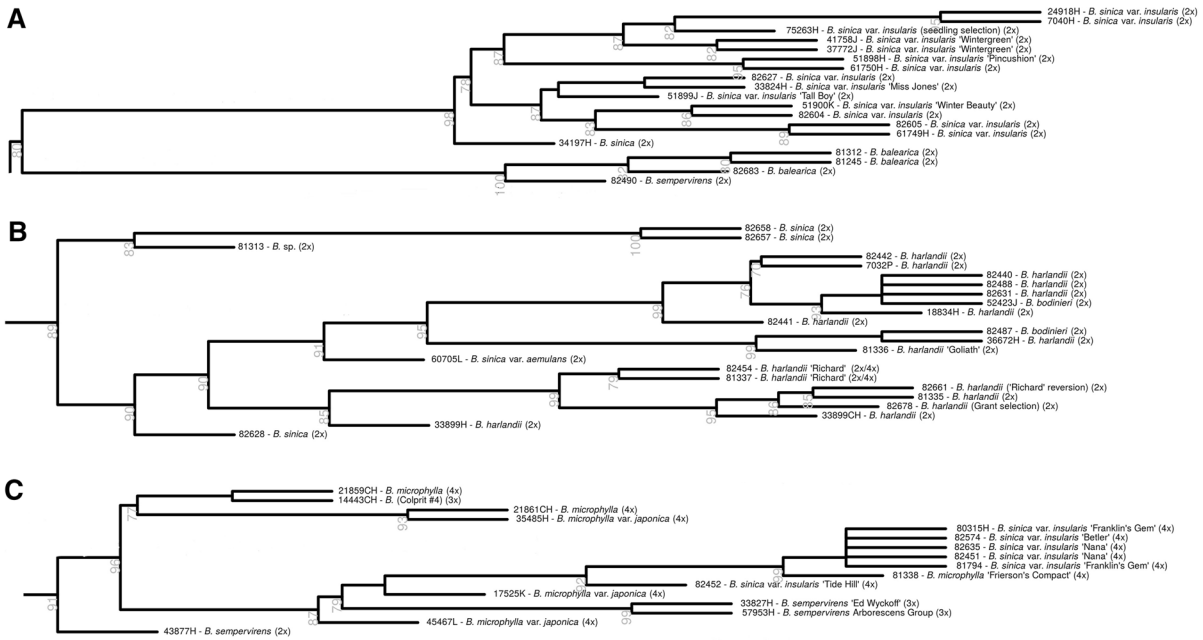


Fig. 2 Detail of clusters from Fig. 1 including those labeled **A** *B. sinica* var. *insularis* and *B. balearica* (from the top of Fig. 1); **B** *B. harlandii/B. sinica* (from the *bottom* of Fig. 1); and **C** *B.microphylla* var. *japonica/B. sinica* var. *insularis* 'Nana'

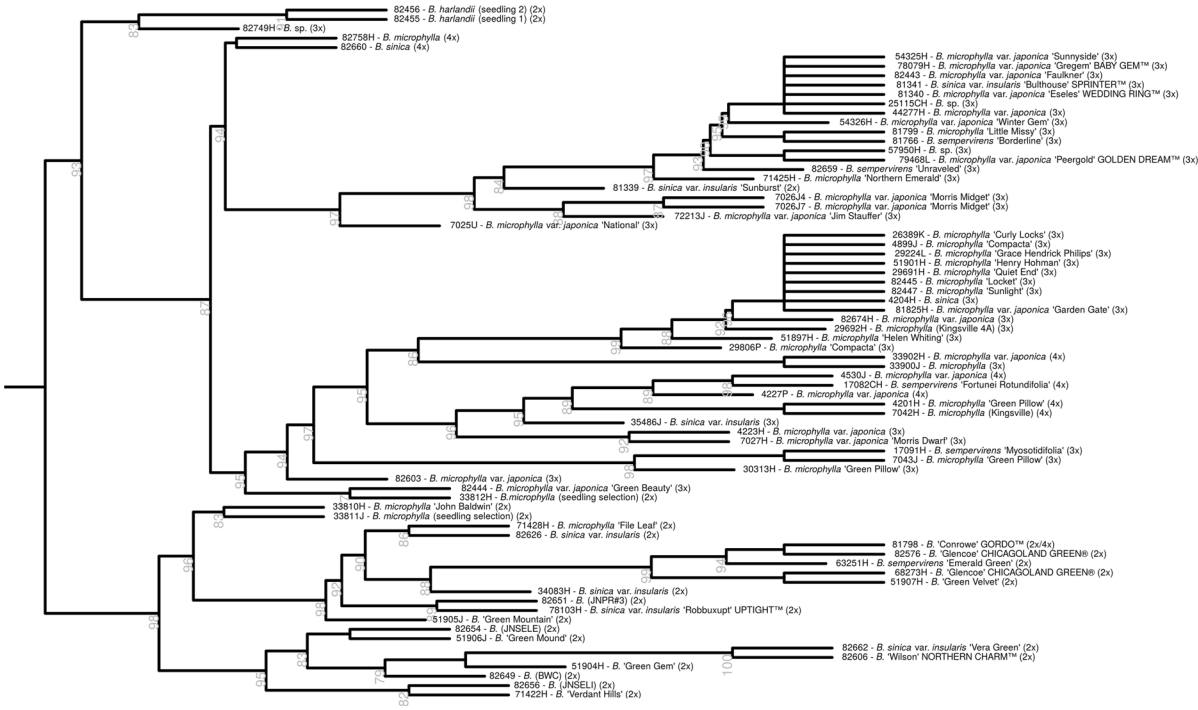


Fig. 3 Detail of the cluster from Fig. 1 containing *B. microphylla/B. microphylla* var. *japonica* and hybrids

bodinieri (NA 52423-J). According to Batdorf (2004), with support from Van Laere et al. (2011), *B. bodinieri* should be considered synonymous with *B. harlandii* and represents natural variation within the latter species. Additional accessions of *B. harlandii* (NA 18834H, NA 81336, NA 81337, and NA 82454) may be derived from the earlier species introductions, but their exact origins are unknown.

Buxus sinica is a morphologically variable, widespread species in China and as such is taxonomically and nomenclaturally confusing. In the *Flora of China*, six poorly understood intraspecific taxa are recognized, including *B. sinica* var. *aemulans* (Rehd. et Wils.) M. Cheng (Brückner and Ming 2008). Accession NA 60705-L was wild-collected as seed in Huangshan, China in 1988 and clusters with *B. sinica* and *B. harlandii* (Fig. 2B). The only three wild-collected *B. sinica* in our study are also found in this cluster (NA 82628 from Lianshan, Sichuan; and NA 82657 and NA 82658 from Gansu Province). Accession NA 81313, originally received as *B. harlandii* from a commercial nursery with no provenance data, does not match the species description, and may be a variety or hybrid of *B. sinica.* Additional wild-collected accessions of *B. sinica* and appropriate phylogenetic markers would shed much needed light on this species-complex.

The identification and nomenclature of the *B. microphylla* group is confounded by a long history of cultivation in Japan. *Buxus microphylla* was first described from a cultivated plant and is not known in the wild (Batdorf 2004). The small cluster labeled "*B. sinica* var. *insularis* 'Nana'/*B. microphylla* var. *japonica*" (Fig. 2C) is supported by a 91 % bootstrap value and contains wild-collected tetraploid *B. microphylla* and related accessions and tetraploid accessions associated with *B. sinica* var. *insularis* 'Nana'. *Buxus microphylla* var. *japonica* (Müll. Arg. ex Miq.) Rehd. et E.H. Wilson, represents the wild form, and thus should be applied to wild-collected or wild-type cultivated accessions NA 21861-H and NA 45467-L from Kyushu, Japan; NA 21859-CH from Hirado, Japan; and NA 17525-K from Nikko, Japan (Supplemental Table 1). Tetraploid *B. sinica* var. *insularis* 'Nana' and related accessions were also present in this group and not in the more distant *B. sinica* var. *insularis* group, where wild-collected Korean material is grouped. *Buxus sinica* var. *insularis* 'Nana' may

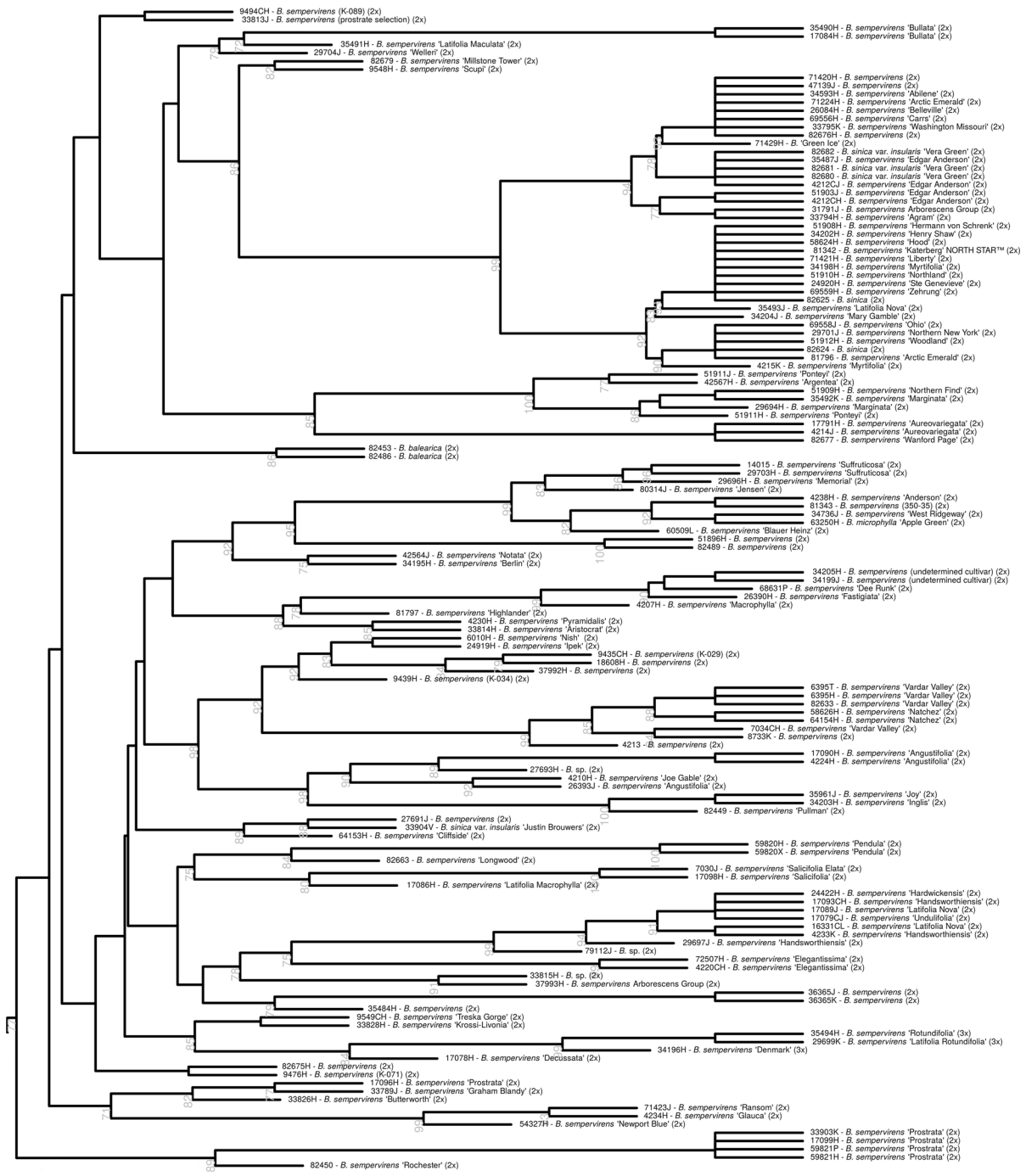


Fig. 4 Detail of the cluster from Fig. 1 containing *B. sempervirens*

have originated as a selection made by boxwood specialist Henry Hohman, Kingsville Nurseries, Maryland in the 1930s (Batdorf 2004). 'Tide Hill' and 'Frierson's Compact' are closely-related to 'Nana', while 'Franklin's Gem' and 'Betler' are indistinguishable from 'Nana' based on our markers. All of the above cultivars share a similar extreme dwarf habit and small leaves. Based on our study, and

the wide dispersion of these accessions across clusters, the name *B. sinica* var. *insularis* may cover a variable and unrelated group of taxa that may have diverged with natural or human selection or previously unknown hybridizations.

Most accessions of *B. microphylla*, *B. microphylla* var. *japonica*, and many hybrids grouped in a poorly supported cluster, which could be resolved into two subclusters (Fig. 3). The bottom, smaller cluster (98 % bootstrap support) contains accessions previously thought to be hybrids between *B. sempervirens* and *B. sinica* var. *insularis,* or derived from these purported hybrids, as well as cultivars not previously associated with these hybrids. For example, the first hybrids of *B. sempervirens* 'Suffruticosa' × *B. sinica* var. *insularis,* introduced by Sheridan Nurseries of Ontario, Canada, are found in this cluster. These include the popular cultivars 'Verdant Hills' (NA 71422-H), 'Green Gem' (NA 51904-H), 'Green Mound' (NA 51906-J), 'Green Mountain' (NA 51905-J), and 'Green Velvet' (NA 51907-H) (Batdorf 2004; Van Trier and Hermans 2005). Several open-pollinated seedlings of 'Green Velvet' (NA 82649, NA 82651, NA 82654, and NA 82656) also are included in this cluster, as are accessions NA 82662 (*B. sinica* var *insularis* 'Vera Green') and NA 82606 ('Wilson' NORTHERN CHARM™). In another subgroup, NA 33810-H (*B. microphylla* 'John Baldwin') and NA 33811-J (*B. microphylla*-seedling selection from 'John Baldwin') clustered together. 'John Baldwin', synonymous with 'J.T. Baldwin', is commercially available in the U.S. and used extensively in northern Europe for hedges (Batdorf 2004). Other notable accessions in this group include 'File Leaf' (NA 71428-H), 'Emerald Green', 'Green Velvet', and 'Conrowe' GORDO™ and 'Glencoe' CHICAGOLAND GREEN® (both sports of 'Green Velvet'). Our data support the report of Van Trier and Hermans (2005) indicating that most of the boxwood hybrids in cultivation were developed from interspecific crosses between *B. sempervirens* and *B. microphylla*. Clustering of the hybrids with *B. microphylla* accessions indicate they are more closely related to *B. microphylla* than to *B. sempervirens,* which is consistent with findings from AFLP analyses (Van Laere et al. 2011).

The top, larger cluster in Fig. 3, supported by a 93 % bootstrap value, contains most of our accessions of *B. microphylla* var. *japonica* with primarily triploid and tetraploid accessions from cultivated sources. Notable accessions in the lower (95 % bootstrap support) subgroup include *B. microphylla* 'Compacta' (NA 29806-P) and sports or seedlings derived from it (NA 26389-K, NA 4899-J, NA 29224-L, NA 51901-H, NA 29691-H, NA 82445, NA 82447, NA 81825-H, NA 82674-H, NA 29692-H, and NA 51897-H). Several previously mis- or unidentified accessions in our collection also fell into this group, namely NA 4204-H (*B. sinica*), NA 81825-H 'Garden Gate', NA 33902-H (tetraploid), and NA 33900-J (triploid). The placement of accessions NA 4530-J (*B. microphylla* var. *japonica*) and NA 17082-CH (*B. sempervirens* 'Fortunei Rotundifolia') in this group sheds light on the origins of this round-leaved phenotype as well. Historically, the name 'Rotundifolia' has been used to describe various plant taxa with rounded leaves. Hence, accessions with this name would be expected to fall in different clusters, as they did in our study. Accessions NA 17082CH (*B. sempervirens* 'Fortunei Rotundifolia') fell in the *B. microphylla* cluster (Fig. 3) and NA 29699 K (*B. sempervirens* 'Latifolia Rotundifolia') and NA 35494H (*B. sempervirens* 'Rotundifolia') grouped closely in the large *B. sempervirens* cluster (Fig. 4).

The other subgroup in the *B. microphylla* var. *japonica* cluster (94 % bootstrap support) contains cultivated material, predominantly triploid *B. microphylla* var. *japonica*, as well as several accessions that our study revealed to be misidentifications, including, NA 82758-H, NA 82660, NA 81341, NA 25115-CH, NA 44277-H, and NA 57950-H. Based on this grouping, supported by high bootstrap values and shared ploidy levels and morphological traits, we conclude that these accessions are all types of *B. microphylla* var. *japonica*.

Buxus sempervirens is the most important species to commercial boxwood growers in the U.S. (Batdorf, 2005). Half of the samples included in this study were accessions of *B. sempervirens*, and most of these samples clustered in one large group in the dendrogram (Fig. 4). which fell into smaller subgroups based on phenotype, horticultural use, or origin. While this *B. sempervirens* cluster is too large to examine in detail for every accession included therein, the placement of several cultivars, as well as certain historical or horticulturally interesting groups, is worth noting. Starting at the bottom of the cluster in Fig. 4, *B. sempervirens* 'Prostrata' accessions (NA 33903-K,

NA 17099-H, and NA 59821-P/H) clustered together in one sub-group. 'Prostrata' is a cultivar name that has been applied to multiple boxwood genotypes that have a wide, low-growing phenotype (Batdorf 2004). The original 'Prostrata' was derived from an open-pollinated seedling of *B. sempervirens* in Europe (Batdorf 2004). However, NA 17096-H labeled as 'Prostrata' did not group with these accessions, suggesting that the prostrate phenotype has arisen multiple times under cultivation. Similarly to 'Prostrata', the "Arborescens Group" is another name that has been applied based on a distinctive phenotype, in this case, to any tall, tree-like boxwood (Batdorf 2004). Hence, the diverse placement of accessions with this name within the large cluster should not be surprising.

A historically significant group with strong bootstrap support is the "Handsworth Group," which contains NA 17093-CH ('Handsworthiensis'), and related accessions (NA 79112-J, NA 29697-J, NA 4233-K, NA 17079-CJ, NA 16331-CL, and NA 17089-J). According to Batdorf (2004), accession 17093-CH originated at Handsworth Nursery in England before 1872. Based on the grouping, and USNA records, the accessions in this group are selections from Handsworth Nursery or renamings of its introductions.

Another popular accession, *B. sinica* var. *insularis* 'Justin Brouwers', grouped clearly within the *B. sempervirens* cluster. Van Laere et al. (2011) reported similar findings, implying that 'Justin Brouwers' may actually be a cultivar of *B. sempervirens* and not of *B. sinica* var. *insularis*.

The English boxwood, *B. sempervirens* 'Suffruticosa', is one of the most recognized iconic boxwood forms in the landscape, made famous in the U.S. for its use in early Colonial gardens, and used extensively in modern U.S. landscapes (Batdorf 2004). In our study, this cultivar grouped in a cluster with 99 % bootstrap support with several other accessions [NA 29696H, NA 80314J, NA 4238H, NA 81343, NA 34736J, and NA 63250-H (*B. microphylla* 'Apple Green')], which sheds light on previously unnoted, possible shared origins of these accessions.

Another horticulturally significant group that tended to cluster based on phenotype is the *B. sempervirens* accessions with variegated foliage, including silvery-white margined leaves ('Argentea') and yellow or green striped/splashed leaves ('Aureovariegata'). This cluster (with 85 % bootstrap support) included NA 42567-H (the original 'Argentea' cultivar), and accessions with occasionally variegated leaves, including NA 29694-H, NA 35492-K ('Marginata'), NA 51911-H/J ('Ponteyi'), and NA 51909-H ('Northern Find'), as well as NA 4214-J, NA 17791-H ('Aureovariegata'), and the dwarf variegated 'Wanford Page' (NA 82677).

One of the most lasting impacts on boxwood cultivar development in the U.S. has been made by the plants collected by Edgar Anderson from the Balkans in 1934 for the Arnold Arboretum (Batdorf 2004). In our study, these accessions form several groups within the large *B. sempervirens* cluster (Fig. 4). One cluster (with 92 % bootstrap support) contains *B. sempervirens* 'Nish', 'Ipek', and other cultivars, including most significantly 'Vardar Valley', the most widely-grown cultivar in the U.S (Batdorf, 2004). Other accessions from Anderson's Balkan collections cluster near the top of the phenogram, along with the so-called "American boxwood" accessions, recognized by their characteristic large, often spreading, forms: NA 33794-H ('Agram'), NA 69556-H ('Carrs'), NA 26084-H ('Belleville'), NA 31791-J (Arborescens Group), and NA 34593-H ('Abilene').

Two accessions labeled as *B. sempervirens*, NA 51902-H 'Decussata' and NA 29706-H 'Varifolia', clustered distantly from the other *B. sempervirens* accessions and, in fact, formed a separate cluster from other species (Fig. 1). This unexpected clustering suggests that more research is needed to determine the correct origin or provenance of these cultivars, as they could be misidentified or unrecognized interspecific hybrids.

Accessions tended to cluster together by ploidy (Fig. 1). *Buxus sinica* var. *insularis* accessions and *B. balearica* accessions were all diploids (Fig. 2A; Supplemental Table 1), in agreement with the published ploidy data of Darlington and Wylie (1955) and Van Laere et al. (2011). Other diploids included many of the *B. sinica*, *B. bodinieri,* and *B. harlandii* accessions (Fig. 2B). The varied ploidy levels observed in *B. microphylla* in our study could be related to this species' adaptation to varied ecological ranges or its advanced genetic evolution as explained by Van Laere et al. (2011) and observed in other *Buxus* species (Gutierrez 2014). By superimposing the ploidy data on the phenogram, the probable hybrid origin of triploid taxa becomes evident (Fig. 1). Triploid plants

presumably arose from hybridizations between diploid and tetraploid taxa or possibly from hybrids between diploids involving unreduced gametes, although this phenomenon has not been reported in *Buxus*. The placement of triploids in the *B. microphylla* var. *japonica* and 'Nana' cluster (Fig. 2C), and in the hybrid cluster with and between tetraploids and diploids (Fig. 3), is consistent with their presumed hybrid origin.

We have completed a comprehensive study of the boxwood accessions in the USNA-National Boxwood Collection that included ploidy analysis and assessment of accessions based on historical, provenance, and SSR genotypes. Our results were generally consistent with studies conducted with AFLPs (Van Laere et al. 2011). AFLP markers predominantly reflect differences in non-coding regions of the genome (Meudt and Clarke 2007), whereas our genic SSRs are derived from transcribed genes. Hence, differences in patterns of genetic relatedness between these two studies could be due in part to the marker system used.

The 275 accessions that we tested included 24 cultivars that were represented by two or more accessions from different sources, which served as both an internal control and a measure of clonal drift that may be present in cultivated taxa. Of the 24 replicated cultivars, approximately half were indistinguishable from each other using our markers. The remaining plants clustered closely, usually differing by only one allele. The fact that these presumably identical plants showed minor genetic differences could indicate that the original cultivar was derived from a group of closely related plants or that, over time, through repeated vegetative propagation, selectively neutral mutations may have arisen in this material. Such genetic plasticity has been observed in other vegetatively propagated plants as well (Klekowski 1988; Walbot 1996). In addition, as discussed in the text, the grouping of a few cultivars with identical names in different clusters indicates misidentification.

Genic SSRs are likely not the best choice of marker for DNA fingerprinting or cultivar identification because they are more conserved and therefore have less variability than many other marker systems, such as genomic SSRs, AFLPs, or SNPs. But genic-SSR markers are still quite useful in collections management, because their repeatability allows new accessions to be analyzed as they are acquired, thereby confirming their identity and likely value to the collection. The genic-SSR markers used in this study were useful in characterizing genetic diversity and relatedness of boxwood germplasm, and, of significance for the National Boxwood Collection, they enabled the identification of unknown or duplicate accessions and recognition of accessions that may be misidentified. In particular, the unexpected placement of several accessions in the phenogram led to a re-examination of plant records and the correction of several mislabeled or misidentified accessions. While many accessions from known groups clustered as expected based on phenotype, horticultural use, and/or origin, this study also showed that plant names often lead to assumptions of relatedness where none may exist. This may be due to misidentified plants, but it could also reflect a much wider or more complex genetic structure among taxa. Such complexities could be a result of different ploidy levels and ease of interspecific hybridization, augmented by human selection, ploidy isolation, and asexual propagation, followed by rapid international movement of popular genotypes.

Conclusions

This study is of direct interest to boxwood growers, breeders, and ornamental horticulture professionals; the information will be used to drive decisions in breeding programs, especially as more information is amassed on sources of resistance to boxwood blight and associations between resistant germplasm and particular clusters or genotypes can be identified. However, a detailed analysis such as the one presented here has implications for collection management and germplasm conservation, particularly in woody horticultural crops, which are generally clonally propagated. Maintenance of large collections of woody plants requires a long-term investment of resources, so this type of analysis can be beneficial in identifying duplicates and misidentified accessions to maximize genetic diversity while managing resources efficiently.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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