

Relationship of *Buxus L.* Accessions from Longwood Gardens Based on Morphological and Molecular Characters

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Abstract Boxwoods (*Buxus L.*) are among the most widely cultivated ornamental shrubs. Longwood Gardens displays and trials many boxwood taxa from around the world. Cultivar identification and species delineation in boxwoods is problematic when based solely on morphological characteristics. To verify identity of 20 boxwood accessions from the trial beds of Longwood Gardens, their morphological features (leaf characters, growth habit, and landscape appearance) were recorded. These data were validated later with the analysis of AFLP markers. The AFLP markers were generated separately without any knowledge of the morphological characteristics. From the three-primer-pair combinations, a total of 212 useful markers between 75 and 500 bp fragment sizes were obtained. The average number of markers for each accession was 74 and the total markers for each accession varied from 50 to 108. The unique bands, which can be the potential diagnosis markers, were among zero to 15. Genetic distances ranged from 0.028 to 0.439. Only one UPGMA tree was generated using PAUP and the number of characters that changed unambiguously on each branch was recorded. From molecular data, the 20 accessions might derive from four or five different species and could be distinguishably classified into 12 different cultivars. The morphological data fully supported the results from molecular data. The combination of both morphological and molecular data is a powerful tool for determining genetic relations, cultivar discrimination, and targeted breeding.

Key words boxwoods (*Buxus L.*); germplasm identification; molecular characters; morphological characters; Longwood Gardens in USA

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从形态和分子特性来探讨美国长木植物园黄杨种质之间的关系

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摘要: 黄杨是栽植很广的庭园观赏灌木, 美国长木植物园从世界各地收集了许多优良品种。仅从形态上来区别这些黄杨品种是很困难的。为鉴定20个黄杨优良品种, 我们分别从形态特征(叶、习性和长势)和分子标记(AFLP标记)两方面着手, 综合分析它们的亲缘关系。三个AFLP引物结合在75和500 bp DNA片段间一共产生212个条带。每一个黄杨品种平均有74个条带, 最低者有50条,

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最高者有108条,通常可用于鉴定的特有条带在0到15条之间。品种间的遗传距离介于0.028和0.439之间。用PAUP软件,我们得到一个UPGMA树状图,并将每一步有显著差异的标在图上。从分子特性来讲,这20个黄杨优良品种可能起源于4到5个种,并可很清楚地分为12个栽培变种。形态特征也支持分子特性的结论。由此可见,结合形态特征和分子标记是确定遗传关系、鉴定栽培变种和分子辅助定性育种的一个很好的方法。

关键词: 黄杨; 种质鉴定; 形态特性; 分子特性; 美国长木植物园

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0 Introduction

Buxus L. is a member of boxwood family (Buxaceae). It consists of profusely branched evergreen shrubs and trees with opposite and entire leaves. The young branches are usually with angles and exstipulate. The genus was named by Carl Linnaeus in 1753 and *Buxus* is the Latin word of boxwood. The plants naturally distribute throughout temperate (Eurasia), subtropical (Asia and North America) and tropical regions (South Africa, Caribbean, and Central America). They are widely used as ornamentals and special wood production^[1].

Taxonomically, the phylogenetic relationships within *Buxus* and the number of its species are contradictory and confusing. Some researchers^[2,3] indicated that there were approximately 30 species, while others^[1,4,5] included more than 70 species. *Buxus sempervirens* L. (Common Box) and *B. microphylla* Sieb. Et Zucc. (Littleleaf Box or Boxwood) are the most important taxa in ornamentals. They are widely cultivated for hedges, screens, borders, and topiary and for specimen plantings of pendulous or prostrate form^[5]. However, both species are extremely variable which has given rise to many cultivars with overlapped morphological characteristics. "Cultivars are almost indistinguishable and once they are put to the shears no one can reliably separate them"^[6]. Obviously, the cultivar identification, the species delineation, and the genus circumscription, had created the problems. The differences among the taxonomists and horticulturists could be not resolved with limited morphological characteristics and it is important to clarify this contradictory information with modern molecular technologies.

Deoxyribonucleic acid (DNA) fingerprinting techniques (DNA markers) are the preferred methods for identifying cultivars or genotypes and investigating the genetic variability within species because the DNA markers are not influenced by environmental or cultural factors, such as geographical location, microclimate, and nutrition^[7,8]. The rapid accurate information derived from DNA can be used to distinguish closely related plants, especially a morphologically homogenous group of plants^[8]. Currently, several DNA fingerprinting techniques are available. The widely used techniques are restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD). RFLPs have been used to investigate genetic diversity in cultivated plants^[9] and their wild relatives^[10,11]. Hubbard et al.^[12] successfully applied RFLP techniques to the identification of rose cultivars. The RAPD assay is less expensive and overcomes some technical limitations of RFLPs. It can be used for cultivar discrimination^[7], such as clonal identification of red maple (Krahl et al., 1993) and American elm^[13]. Michmore et al.^[14] reported that RAPDs were useful in the construction of genetic maps and could be used as linkage markers to downy mildew resistance in lettuce.

Amplified fragment length polymorphism (AFLP) is an ingenious combination of RFLP analysis and PCR that results in highly informative fingerprints. Compared with the RFLPs or RAPDs, AFLP is the most useful, reliable, and promising molecular marker technique for genotypic comparisons within species^[15-17]. Zhang^[18] reported 99.9% reproducibility among three replications of 27 bermudagrass genotypes. He also concluded that the AFLP technique not only had high reproducibility and revealed a high frequency of polymorphism, but also could be used for cultivar and hybrid identification and protection^[19,20]. Obviously,

the AFLP assay offers more reproducible information and increased opportunities to research molecular studies on ornamental taxa

Since no DNA work has been done for *Buxus*. This study will conduct the preliminary exploration of genetic diversity and clone discrimination using *Buxus* collection from Longwood Gardens. Further studies can lead to screening of all available clones and identifying the best clones for our gardens

1 Materials and Methods

1.1 Plant Materials

Fresh leaves of 20 *Buxus* accessions were collected from the trial beds of Longwood Gardens in Kennett Square, Pennsylvania, USA. The source information is listed in Table 1.

Table 1 Plant Sources for *Buxus* accessions from Longwood Gardens

No. Z	Plant name Y	Source X	Suggested Name W
1	<i>B. sempervirens</i> 'Albania'	Albania	<i>B.</i> × 'Albania'
2	<i>B. s</i> 'Belleville'	E. W. Seibert, 1956- 0368	<i>B. sp.</i> 'Belleville'
3	<i>B.</i> 'Glencoe'	Scarff's Nursery, OH; 1998- 0849	<i>B. s</i> 'Glencoe'
4	<i>B.</i> 'Green Beauty'	Saunders Brothers, VA; 1999- 0649	<i>B. microphylla</i> 'Green Beauty'
5	<i>B.</i> 'Jensen'	Saunders Brothers, VA; 1999- 0653	<i>B. s</i> 'Jensen'
6	<i>B.</i> 'Justin Brouwers'	Saunders Brothers, VA; 1999- 0652	<i>B. s</i> 'Justin Brouwers'
7	<i>B.</i> 'Longwood Cemetery'	Longwood Gardens, PA	<i>B. s</i> 'Suffruticosa'
8	<i>B.</i> 'Main Fountain Garden'	Longwood Gardens, PA	<i>B. s</i> 'Suffruticosa'
9	<i>B.</i> 'Martin'	Longwood Gardens, PA	<i>B. s</i> 'Suffruticosa'
10	<i>B.</i> 'MBG'	Missouri Botanical Garden, MO	<i>B. s</i> 'Myrtifolia'
11	<i>B.</i> 'Myrtifolia'	Missouri Botanical Garden, MO; 1961- 1294	<i>B. s</i> 'Myrtifolia'
12	<i>B.</i> 'Red Lion East'	Longwood Gardens, PA	<i>B. s</i> 'Red Lion East'
13	<i>B.</i> 'Rte50'	Woodlanders, SC, 1998- 0686	<i>B.</i> 'Rte50'
14	<i>B.</i> 'Suffruticosa'	Scarff's Nursery, OH; 1995- 0946	<i>B. s</i> 'Suffruticosa'
15	<i>B.</i> 'T45'	Longwood Gardens, PA	<i>B. s</i> 'Suffruticosa'
16	<i>B.</i> 'Vardar Valley'	Gulf Stream, 1985- 0964	<i>B. s</i> 'Vardar Valley'
17	<i>B.</i> 'Vardar Valley'	US Midwest, 1985- 0424	<i>B. s</i> 'Vardar Valley'
18	<i>B.</i> 'Vardar Valley'	Woodlanders, SC, 1992- 0076	<i>B. s</i> 'Vardar Valley'
19	<i>B.</i> 'Watkins'	Unknown	<i>B. s</i> 'Suffruticosa'
20	<i>B.</i> 'W intergreen'	Saunders Brothers, VA 1999- 0648	<i>B. microphylla</i> 'W intergreen'

Z: Plant materials were provided by Longwood Gardens, Kennett Square, PA 19348, USA.

Y: Plant names were not verified or identified by authors and were listed as they were labeled

X: Based on the living collection record of Longwood Gardens

Z: Suggested name after AFLP analysis with reference of morphological data

1.2 DNA Extraction

Total genomic DNA was isolated from fresh leaves following the procedures suggested by DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California, USA). The extra two steps were centrifuging at $5\,000\text{ r}\cdot\text{min}^{-1}$ for one minute after incubation at 65°C and at $12\,000\text{ r}\cdot\text{min}^{-1}$ for five minutes after ice incubation. Isolated DNA was visualized on 1% Agarose gel with 312 nm Variable Intensity Transilluminator (FBT IV-816, Fisher Scientific, Pittsburgh, Pennsylvania, USA) and the high yielded samples were used for the following AFLP experiment

Table 2 Oligonucleotide adaptors and primers for AFLP analysis of *Buxus* accessions

Adaptor or primer	Oligonucleotide (primer dye color)
MesI adaptors	GACGA TGA GTCCTGAG TACTCA GGACTCA T
EcoRI adaptors	CTCGTA GACTGCGTACC CATCTGACGCA TGGTTAA
AFLP Primers	
MesI+ 1	GATGA GTCCTGAG + TAA + C
EcoRI+ 1	AGACTGCGTACC + AATTC + A
MesI+ 3	GATGA GTCCTGAG + TAA + CTC
EcoRI+ 3	AGACTGCGTACC + AATTC + ACT (blue) AGACTGCGTACC + AATTC + ACG (green) AGACTGCGTACC + AATTC + AGC (yellow)

1.3 AFLP Procedure

Perkin Elmer (Foster City, California, USA) Large Plant Genome Kit was purchased to conduct this experiment. Amplified fragment length polymorphism reactions were conducted as the recommended by the AFLP Plant Mapping^[21]. Samples were electrophoresed (2 500 V) for 4 hours at 48 °C in 1× (TBE buffer) on an automatic DNA sequencer (model ABI377, Perkin-Elmer Applied Biosystems) equipped with GeneScan Analysis software (version 3.1, Perkin-Elmer Applied Biosystems). Fragment sizing were calculated automatically using the local Southern sizing algorithms^[22].

1.4 AFLP Data Analysis

Data files containing sizing data for all samples were created using Fragment Binner [Nancy Garnhart, personal comm.] for each primer combination. The threshold value for fragment detection was 50. All data files were reviewed by eyes, then exported into PAUP^[23]. Pairwise distance comparisons were generated and unweighted pair group method with arithmetic average (UPGMA) tree was derived using PAUP. To show the strength of each branch, we conducted 1 000 replicates of bootstrap analysis^[24] and listed the number of characters that changed unambiguously on each branch.

2 Results and Discussion

From the three-primer-pair combinations of 20 *Buxus* accessions collected from Longwood Gardens trial beds, a total of 212 useful bands (markers) between 75 and 500 bps fragment sizes were generated. Primer combinations labeled with blue, green, and yellow dye colors produced 90, 69, and 53 bands, respectively. The average number of markers for each accession was 74 and the total markers for each accession varied from 50 to 108 (Table 3). The unique bands, which can be the potential diagnosis markers, were among zero to 15. Obviously, the genetic diversity among these 20 accessions were very little, except the accession # 1 (8 unique bands), # 2 (14), # 3 (7), # 13 (15), and # 20 (8) [Table 1, 3]. It is important to indicate that the accession # 8, # 10, # 11, # 15, # 16, # 17, and # 19 did not have any unique band [Table 1, 3], which implied that the genetic information of these accessions was very similar to one or more taxa (included the above 7 accessions) in this study. It is not necessary to keep some of these taxa for further evaluation.

Based on all useful AFLP markers, genetic distances (pairwise distance comparisons) and total character (band) differences between *Buxus* accessions were generated by PAUP^[23] (Table 4). Genetic distances ranged from 0.028 to 0.439. Accession # 16, # 17, # 18 showed minimal genetic differences, while accession # 2 and # 20 had the highest one. Generally, high genetic distances among the different species and low genetic distances among the clones were expected. The values of genetic distances among the varieties and cultivars were intermediate to values among the species and clones. The total character (band)

Table 3 AFLP markers generated from six primer pairs of *Buxus* accessions

Taxon No. Z	Primer Dye Color			Total Bands	Unique Bands
	Blue	Green	Yellow		
1	38	23	21	82	8
2	56	29	23	108	14
3	37	11	25	73	7
4	36	13	16	65	2
5	36	12	21	69	3
6	36	12	20	68	2
7	38	8	23	69	1
8	38	12	23	73	0
9	38	10	22	70	1
10	37	17	19	73	0
11	35	14	20	69	0
12	25	4	21	50	1
13	44	40	18	102	15
14	33	12	28	73	1
15	36	9	22	67	0
16	35	14	21	70	0
17	38	17	21	76	0
18	41	21	21	83	1
19	39	12	23	74	0
20	34	15	14	63	8
Mean	38	16	21	74	3
Useful Bands	90	9	53	1 477	64

Z: The taxon number corresponds with the number in the first column of Table 1.

differences showed the same trend as the genetic distance (Table 4).

Table 4 Genetic distances and total character (band) differences between *Buxus* accessions (above diagonal: total character differences; below diagonal: genetic distances)

No.	Z	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	-	80	71	63	65	60	65	63	64	61	59	64	80	65	65	58	56	57	58	67	
2	0.378	-	67	69	75	60	73	67	68	39	41	74	58	71	69	74	74	75	58	93	
3	0.335	0.316	-	44	54	41	60	54	59	40	42	43	69	58	52	51	57	64	55	62	
4	0.297	0.326	0.208	-	48	43	60	56	55	40	40	43	71	56	50	49	53	60	55	40	
5	0.307	0.354	0.255	0.226	-	47	34	30	33	54	50	39	75	36	28	47	51	58	33	70	
6	0.283	0.283	0.193	0.203	0.222	-	45	43	44	27	29	40	56	45	41	42	46	53	44	59	
7	0.307	0.344	0.283	0.283	0.160	0.212	-	14	17	54	52	43	75	22	18	47	49	52	15	72	
8	0.297	0.316	0.255	0.264	0.142	0.203	0.066	-	9	52	48	41	67	12	8	47	49	52	9	68	
9	0.302	0.321	0.278	0.259	0.156	0.208	0.080	0.043	-	49	49	40	74	17	7	50	54	57	14	71	
10	0.288	0.184	0.189	0.189	0.255	0.127	0.255	0.245	0.231	-	8	37	41	48	48	43	47	52	47	62	
11	0.278	0.193	0.198	0.189	0.236	0.137	0.245	0.226	0.231	0.038	-	35	39	44	46	41	43	50	45	58	
12	0.302	0.349	0.203	0.203	0.184	0.189	0.203	0.193	0.189	0.175	0.165	-	70	37	35	46	52	59	42	53	
13	0.377	0.274	0.326	0.335	0.354	0.264	0.354	0.316	0.349	0.193	0.184	0.330	-	71	67	66	70	69	70	89	
14	0.307	0.335	0.274	0.264	0.170	0.212	0.104	0.057	0.080	0.226	0.208	0.174	0.335	-	14	51	51	54	15	68	
15	0.307	0.326	0.245	0.236	0.132	0.193	0.085	0.038	0.033	0.226	0.217	0.165	0.316	0.066	-	45	49	54	15	66	
16	0.274	0.349	0.241	0.231	0.222	0.198	0.222	0.222	0.236	0.203	0.193	0.217	0.311	0.241	0.212	-	6	13	50	63	
17	0.264	0.349	0.269	0.250	0.241	0.217	0.231	0.231	0.255	0.222	0.203	0.245	0.330	0.241	0.231	0.028	-	7	50	63	
18	0.269	0.354	0.302	0.283	0.274	0.250	0.245	0.245	0.269	0.245	0.236	0.278	0.326	0.255	0.255	0.061	0.033	-	51	70	
19	0.274	0.274	0.259	0.259	0.156	0.208	0.071	0.043	0.066	0.222	0.212	0.198	0.330	0.071	0.071	0.236	0.236	0.241	-	65	
20	0.316	0.439	0.293	0.189	0.330	0.278	0.340	0.321	0.335	0.293	0.274	0.250	0.420	0.321	0.311	0.297	0.297	0.330	0.307	-	

Z: The taxon number corresponds with the number in the first column of Table 1

Only one UPGMA (unweighted pair group method with arithmetic average) tree was generated using PAUP^[23] (Fig 1). Based on 1 000 replicates of bootstrap analysis, the number of characters that changed unambiguously on each branch, and the genetic distances (Table 4), the 20 accessions might derive from five different species or grex. Accession # 1, # 2 and # 13 should be originated from three different species (maybe grex), respectively. Morphologically, these three accessions have rounded or lanceolate leaves, which can be distinguished from the rest of plants. Accession # 4 and # 20 derived from the fourth distinguished species, *Buxus microphylla*. The rest of them were from the fifth species, *Buxus sempervirens*. Morphological data support these two species.

The taxa subordinate to species, especially cultivars and clones, are very important to the horticultural industries, plant breeding, and natural conservation. Normally, a group of individuals with one or more unique

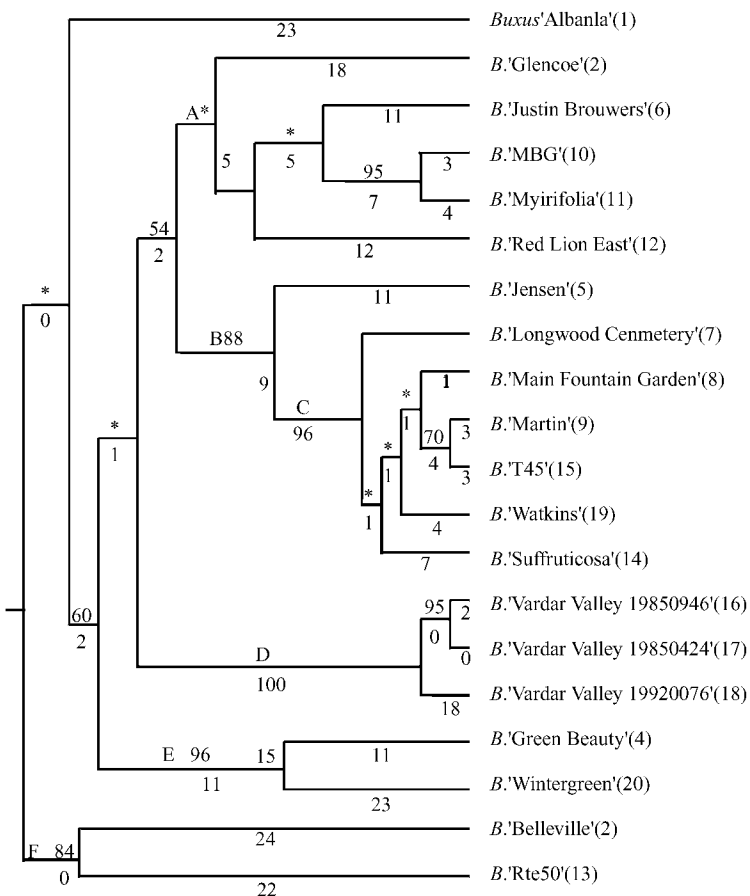


Fig 1 UPGMA tree of *Buxus* accessions from the Longwood Gardens based on AFLP data. Numbers above and below the branches are bootstrap percentages

(* the bootstrap percentage was less than 50% and the branch clasped on the bootstrap tree) and unambiguous character changes. Letters A through F mark clades discussed in the text. The number in parenthesis is the taxon number, which corresponds with the number in the first column of Table 1.

From Fig 1, Clad A had the low support of bootstrap values, except the accessions # 10 and # 11. The accession # 10 and # 11 were very similar with only 0.038 genetic variations. Other three accessions in this clad had relatively higher genetic distances (0.189, 0.193, and 0.203). The AFLP data supported that there were four distinguished cultivars in this clad, the accession # 6 was closer to accessions # 10 and # 11 than other two accessions. Morphologically, their habits were variable, but leaves were similar, especially Accession # 10 and Accession # 11, which both should be called *Buxus sempervirens* 'Myrtifolia'.

Clad B included Clad C and Accession # 5 with high bootstrap value at 88%. The genetic distances between Accession # 5 and each accession of Clad C were relatively high to recognize the Accession # 5 as a distinguished cultivar. Within Clad C, Accessions # 9 and # 15 had very low genetic distance (at 0.08). Other accessions in this clad were relatively close to these two. The AFLP data supported these six accessions in Clad C should be derived from a cultivar. Morphologically, leaf shapes among these accessions significantly overlapped and could not be distinguished if mixing them. Both molecular and morphological data support that these six accessions originated from a cultivar and could be called *Buxus sempervirens* 'Suffruticosa'.

Clad D included Accessions # 16, # 17, and # 18. These three accessions formed a very strong clad with 100% bootstrap support. The genetic distances and the number of characters that changed unambiguously on each branch were very low. Obviously, these three accessions were from a cultivar and the accessions # 16 and # 17 were almost identical from AFLP data. All three accessions had elliptic to rounded, dark green foliage and we should call them *Buxus sempervirens* 'Vardar Valley'. Clad E only had Accessions # 4 and # 20. These two accessions were grouped with high genetic distance (0.189). However, the 96% bootstrap value indicated that they were much closer than any other accession. The AFLP data concluded that these two accessions were two distinguished cultivars even if they might derive from the similar gene pool long time ago.

Clad F consisted of Accessions # 2 and # 13 with 84% bootstrap support. Genetically, they were not close to each other with 0.274 genetic distance. Accession # 2 had 24 unambiguous character changes while Accession # 13 had 22. These data indicated they were two different cultivars, which could be derived from different species or grex or hybridizations. However, they were relatively close to each other compared with all other accessions. Further studies should be conducted to clarify their relationships.

Accession # 1 was far away from all other accessions. The genetic distances with other accessions ranged from 0.264 to 0.378 (average 0.303). The band pattern (total band differences, Table 4) was very different than all other accessions. Morphologically, its leaves had high variation on sizes, shapes and apices. Both AFLP data and morphological descriptions supported that Accession # 1 should be a distinguished cultivar derived from *Buxus* grex or species.

3 Conclusion

Both molecular and morphological traits supported that 20 *Buxus* accessions in the trails of Longwood Gardens should be derived from five species or grex. Among them, 12 distinguished cultivars could be further evaluated for their landscape performance. Other eight accessions should be removed to reduce expenses. For the future evaluation and conservation studies, AFLP data, together with morphological characteristics, could be used for screening for closer related clones. AFLP. Besides the application for cultivar determination, we could also apply AFLP technique to screen the specific gene(s), such as "genes" which control cold hardiness or disease-resistance.

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