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Discrimination and Genetic Diversity of *Cephalotaxus* Accessions Using AFLP Markers

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ABSTRACT. ***Cephalotaxus*** **Sieb. and Zucc. (plum yew) species and cultivars have become popular because of their sun and shade tolerance, resistance to deer browsing, disease and insect tolerance, and cold and heat adaptability. Unfortunately, the nomenclature and classification in the literature and nursery trade are confusing due to their extreme similarity in morphology. In this study, amplified fragment-length polymorphism (AFLP) markers were used to discriminate taxa and evaluate genetic differences among 90** ***Cephalotaxus*** **accessions. A total of 403 useful markers between 75 and 500 base pairs (bps) was generated from three primer-pair combinations. Cluster analysis showed that the 90 accessions can be classified as four species,** ***C. oliveri*** **Mast.,** ***C. fortunei*** **Hooker,** ***C. harringtonia*** **(Forbes) Koch., and** ***C.*** **×*****sinensis*** **(a hybrid species); four varieties,** ***C. fortunei*** **var.** ***alpina*** **Li,** ***C. harringtonia*** **var.** ***koreana*** **(Nakai) Rehd.,** ***C. harringtonia*** **var.** ***nana*** **(Nakai) Hornibr., and** ***C. harringtonia*** **var.** ***wilsoniana*** **(Hayata) Kitamura; and eight cultivars. Suggested names are provided for mislabeled or misidentified taxa. The** ***Cephalotaxus*** **AFLP data serve as a guide to researchers and growers for identification and genetic differences of a taxon, and a model to establish a cultivar library against which later introductions or problematic collections can be cross-referenced.**

Cephalotaxus (plum yew) taxa are native to the southern Asia and the Himalayas (China, Japan, and Korea) and were introduced to Europe, America, and Australia in the early 1800s and cultivated as landscape plants. Morphologically, plum yews are normally dioecious and infrequently monoecious. Throughout cultivated history, branch sports (chimeras) or seedling selections have originated throughout the world. Taxonomically, *Cephalotaxus* is a small genus with four to nine species, depending on the authority (Dallimore and Jackson, 1967; Fu, 1984; Krüssmann, 1985; Mabberley, 1993; Rushforth, 1987; Silba, 1984). Fu (1984) recognized five species as endemic to China, three species with distribution in China and adjacent countries, and one species, *C. harringtonia*, as native to Japan and Korea. *Cephalotaxus harringtonia* (Forbes) Koch was introduced to England in 1829 and to the United States in 1830. In the 1990s, the plants became popular because of their sun and shade tolerance [can substitute for junipers (*Juniperus* L. sp.)], resistance to deer browsing, tolerance to diseases and insects, and cold and heat adaptability (Dirr, 1990, 1992; Hillier Nurseries, 1995; Tripp, 1994).

Cephalotaxus harringtonia 'Prostrata', a low growing form, was awarded the Gold Medal by the Georgia Green Industry Association in 1994 (Harlass, 1994). Unfortunately, the nomenclature and classification in the literature and nursery trade are very confusing due to morphological similarities among species and the complicated history of *Cephalotaxus* taxonomy. During their 170-year period of cultivation in the western hemisphere, many new cultivars were introduced by growers around the world (Tripp, 1994). *Cephalotaxus* taxa with the same names are often not morphologically similar and morphologically similar plants do not necessarily bear the same name. Furthermore, some new cultivars are introduced into the trade without descriptions and origination. It has been difficult to classify the various taxa by morphological characters (Dirr, 1990), but modern molecular fingerprinting techniques could aid classification.

Deoxyribonucleic acid (DNA) fingerprinting techniques are preferred methods for identifying cultivars or genotypes and investigating the genetic variability within species because DNA markers are not influenced by environmental or cultural factors, such as geographical location, microclimate, and nutrition (Nybom, 1994; Staub and Meglic, 1993). The rapid, accurate information derived from DNA can be used to distinguish closely related plants, especially a morphologically homogenous group of plants (Nybom, 1994). 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 (Paul et al., 1997) and their wild relatives (Miller and Tanksley, 1990; Wang et al., 1992). Hubbard et al. (1992) successfully applied RFLP techniques for identification of rose (*Rosa* L. sp.) cultivars. However, the RFLP assay is more expensive because it requires large amounts of high quality DNA, frequently employs radioactive isotopes for probe labeling, and uses X-ray film for marker detection (Lin et al., 1996; Staub and Meglic, 1993). On the other hand, the RAPD assay is less expensive and overcomes some technical limitations of RFLPs. It has been used for cultivar discrimination (Staub and Meglic, 1993), such as clonal identification in red maple (*Acer rubrum* L.) (Krahl et al., 1993) and American elm (*Ulmus americana* L.) (Kamalay and Carey, 1995). Michelmore et al. (1991) reported that RAPD markers were useful in construction of genetic maps and could be used as linkage markers for downy mildew (*Bremia lactucae* Regel) resistance in lettuce (*Lactuca sativa* L.). How-

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Table 1. Plant sources for *Cephalotaxus* accessions and suggested name after AFLP data analysis and morphological comparison (➚ = male, ✚ = female, ? = unknown).

Accession no.	Plant name[z] and source	Suggested name[y]
1	✚ *C. drupacea*; F. Pokorny's Garden, Athens, Ga.	*C. harringtonia*
2	? *C. drupacea* (seedling); F.W. Schumacher Inc., Sandwich, Mass.	*C. harringtonia*
3	? *C. drupacea*; J.C. Raulston Arb., Raleigh, N.C.	*C. harringtonia* 'Duke Gardens'
4	? *C. drupacea*; Georgia State Arb., Braselton, Ga.	*C. harringtonia*
5	➚ *C. fortunei*; Arnold Arb., Jamaica Plain, Mass.	*C. fortunei*
6	✚ *C. fortunei*; R. Ellis' Garden, Aiken, S.C.	*C. harringtonia*
7	? *C. fortunei* (seedling); R. Ellis' Garden, Aiken, S.C.	*C. harringtonia*
8	? *C. fortunei* (361046); Edinburgh Bot. Garden, Scotland	*C. fortunei*
9	? *C. fortunei* (687276); Edinburgh Bot. Garden, Scotland	*C. harringtonia*
10	? *C. fortunei* (seedling); F.W. Schumacher, Inc., Sandwich, Mass.	*C. harringtonia*
11	✚ *C. fortunei* (69.16245); Kew Gardens, England	*C. harringtonia* 'Prostrata'
12	? *C. fortunei*; Nurseries Caroliniana, N.Augusta, S.C.	*C. fortunei*
13	? *C. fortunei*; Yucca Do Nursery, Waller, Texas	*C. fortunei*
14	✚ *C. fortunei* 'Grandis'; Hillier Arb., England	*C. fortunei* 'Grandis'
15	? *C. fortunei* 'Prostrate Spreader'; Hillier Arb., England	*C. fortunei* 'Prostrate Spreader'
16	➚ *C. harringtonia*; Atlanta Bot. Garden, Atlanta, Ga.	*C. harringtonia*
17	? *C. harringtonia;* Earth Shade Nursery, Warne, N.C.	*C. harringtonia* 'Prostrata'
18	? *C. harringtonia*; Edinburgh Bot. Garden, Scotland	*C. harringtonia*
19	? *C. harringtonia* (94-1497A); Edinburgh Bot. Garden, Scotland	*C. harringtonia*
20	? *C. harringtonia* (tissue culture); J. Frett, Newark, Del.	*C. harringtonia*
21	✚ *C. harringtonia*; Kew Gardens, England	*C. harringtonia* 'Prostrata'
22	➚ *C. harringtonia*; Univ. Ga. Bot. Garden, Athens, Ga.	*C. harringtonia*
23	✚ *C. harringtonia*; Univ. Ga. Bot. Garden, Athens, Ga.	*C. harringtonia* 'Prostrata'
24	➚ *C. harringtonia*; Univ. Ga. Campus, Athens, Ga.	*C. harringtonia* 'Duke Gardens'
25	➚ *C. harringtonia* 'Augusta Upright'; Natl. Golf Course, Augusta, Ga.	*C. harringtonia*
26	? *C. harringtonia* 'Dirr Clone'; M. Dirr's Garden, Watkinsville, Ga.	*C. harringtonia* 'McCorkle'
27	? *C. harringtonia* var. *drupacea*; Edinburgh Bot. Garden, Scotland	*C. harringtonia* 'Prostrata'
28	➚ *C. harringtonia* var. *drupacea* (1978); Hillier Arb., England	*C. harringtonia*
29	? *C. harringtonia* var. *drupacea* (1980); Hillier Arb., England	*C. harringtonia*
30	✚ *C. harringtonia* var. *drupacea*; Kew Gardens, England	*C. harringtonia* 'Prostrata'
31	✚ *C. harringtonia* var. *drupacea*; Overlook Nursery, Mobile, Ala.	*C. harringtonia* 'Prostrata'
32	? *C. harringtonia* var. *drupacea*; Woodlanders Nursery, Aiken, S.C.	*C. harringtonia* 'Prostrata'
33	? *C. harringtonia* var. *drupacea*; Yucca Do Nursery, Waller, Texas	*C. harringtonia*
34	? *C. harringtonia* 'Duke Gardens'; Yucca Do Nursery, Waller, Texas	*C. harringtonia* 'Duke Gardens'
35	? *C. harringtonia* 'Mary Flemming'; Yucca Do Nursery, Waller, Texas	*C. harringtonia* 'Prostrata'
36	➚ *C. harringtonia* 'Prostrate Form'; Yucca Do Nursery, Waller, Texas	*C. harringtonia* 'Prostrata'
37	? *C. harringtonia* 'Duke Gardens'; Bernheim Arb., Clermont, Ky.	*C. harringtonia* 'Duke Gardens'
38	? *C. harringtonia* 'Duke Gardens'; Duke Gardens, Durham, N.C.	*C. harringtonia* 'Duke Gardens'
39	? *C. harringtonia* 'Fastigiata'; Arnold Arb., Jamaica Plain, Mass	*C. harringtonia* 'Fastigiata'
40	? *C. harringtonia* 'Fastigiata'; Edinburgh Bot. Garden, Scotland	*C. harringtonia* 'Fastigiata'
41	? *C. harringtonia* 'Fastigiata'; Hillier Arb., England	*C. harringtonia* 'Fastigiata'
42	? *C. harringtonia* var. *fastigiata*; Kew Gardens, England	*C. harringtonia* 'Duke Gardens'
43	? *C. harringtonia* 'Fastigiata'; McCorkle Nurseries, Dearing, Ga.	*C. harringtonia* 'Fastigiata'
44	? *C. harringtonia* 'Fastigiata'; Overlook Nursery, Mobile, Ala.	*C. harringtonia* 'Fastigiata'
45	? *C. harringtonia* 'Fastigiata'; Univ. Ga. Bot. Garden, Athens, Ga.	*C. harringtonia* 'Duke Gardens'
46	? *C. harringtonia* var. *fastigiata*; Yucca Do Nursery, Waller, Texas	*C. harringtonia* 'Fastigiata'
47	➚ *C. harringtonia*; Nurseries Caroliniana, N. Augusta, S.C.	*C. harringtonia*
48	? *C. harringtonia* 'Fritz Huber'; Yucca Do Nursery, Waller, Texas	*C. harringtonia* 'Prostrata'
49	? *C. harringtonia* 'Gimborn's Pillow'; Barncroft Nurseries, England	*C. harringtonia* 'Prostrata'
50	? *C. harringtonia* 'Glasnevin'; Natl. Bot. Garden, Ireland	*C. harringtonia* var. *nana*
51	? *C. harringtonia* 'Gnome'; Hillier Arb., England	*C. harringtonia* 'Duke Gardens'
52	? *C. harringtonia* 'Goodyear'; Goodyear Garden, Aiken, S.C.	*C. harringtonia* 'Goodyear'
53	? *C. harringtonia* 'H.W. Sargent'; J.C. Raulston Arb., Raleigh, N.C.	*C. harringtonia* 'Prostrata'
54	? *C. harringtonia* 'Long Leaf Form'; Hill Nursery, Commerce, Ga.	*C. harringtonia* 'Prostrata'
55	? *C. harringtonia* 'Short Leaf Form'; Hill Nursery, Commerce, Ga.	*C. harringtonia* 'Duke Gardens'
56	? *C. harringtonia* 'Korean Gold'; Atlanta Bot. Garden, Atlanta, Ga	*C. harringtonia* 'Fastigiata'
57	? *C. harringtonia* 'Drupacea'; McCorkle Nurseries, Dearing, Ga.	*C. harringtonia* 'McCorkle'
58	➚ *C. harringtonia* var. *nana*; Arnold Arboretum, Jamaica Plain, Mass.	*C. harringtonia* var. *nana*

Table 1. Continued (♂ = male, ✚ = female, ? = unknown).

Accession no.	Plant name[z] and source	Suggested name[y]
59	? *C. harringtonia* var. *nana*; Kew Gardens, England	*C. harringtonia* var. *nana*
60	? *C. harringtonia* 'Ogon'; Atlanta Botanical Garden, Atlanta, Ga.	*C. harringtonia* 'Fastigiata'
61	♂ *C. harringtonia* 'Pedunculata'; Arnold Arb., Jamaica Plain, Mass.	*C. harringtonia*
62	♂ *C. harringtonia* 'Prostrata'; Arnold Arb., Jamaica Plain, Mass.	*C. harringtonia* 'Prostrata'
63	♂ *C. harringtonia* 'Prostrata'; Brooklyn Bot. Garden, Brooklyn, N.Y.	*C. harringtonia* 'Prostrata'
64	? *C. harringtonia* 'Prostrata'; M. Dirr's Garden, Watkinsville, Ga.	*C. harringtonia* 'Prostrata'
65	? *C. harringtonia* 'Prostrata'; McCorkle Nurseries, Dearing, Ga.	*C. harringtonia* 'Prostrata'
66	? *C. harringtonia* 'Ridge Spring'; Watson's Garden, Ridge Spring, S.C.	*C. harringtonia* 'Ridge Spring'
67	? *C. harringtonia* 'Short Form'; County Line Nursery, Byron, Ga.	*C. harringtonia* 'Prostrata'
68	✚ *C. harringtonia* var. *sinensis*; Kew Gardens, England	*C. harringtonia*
69	? *C. harringtonia* 'Tall Form'; County Line Nursery, Byron, Ga.	*C. harringtonia* 'Prostrata'
70	? *C. harringtonia* 'Weeping'; Bransford Road, Augusta, Ga.	*C. harringtonia*
71	✚ *C. koreana* ; Arnold Arb., Jamaica Plain, Mass.	*C. harringtonia* var. *koreana*
72	? *C. koreana*; Atlanta Bot. Garden, Atlanta, Ga.	*C. harringtonia* var. *koreana*
73	♂ *C. koreana* (75.20552); Kew Gardens, England	*C. harringtonia* var. *koreana*
74	? *C. koreana*; J.C. Raulston Arb., Raleigh, N.C.	*C. harringtonia* var. *koreana*
75	? *C. koreana*; Nurseries Caroliniana, N. Augusta, S.C.	*C. harringtonia* var. *koreana*
76	♂ *C. koreana*; Yucca Do Nursery, Waller, Texas	*C. harringtonia* var. *koreana*
77	? *C. oliveri* ; Piroche Plants, Pitt Meadows, BC, Canada	*C. oliveri*
78	? *C. sinensis* (hybrid); Arnold Arb., Jamaica Plain, Mass.	*C.* ×*sinensis*
79	♂ *C. sinensis*; Arnold Arb., Jamaica Plain, Mass.	*C.* ×*sinensis*
80	✚ *C. sinensis*; Atlanta Bot. Garden, Atlanta, Ga.	*C.* ×*sinensis*
81	? *C. sinensis*; Edinburgh Bot. Garden, Scotland	*C.* ×*sinensis*
82	? *C. sinensis*; Nurseries Caroliniana, N. Augusta, S.C.	*C.* ×*sinensis*
83	♂ *C. sinensis*; Yucca Do Nursery, Waller, Texas	*C.* ×*sinensis*
84	? *C. sinensis* 'Dogwoodhills'; Yucca Do Nursery, Waller, Texas	*C. harringtonia*
85	? *C. wilsoniana* (93-3497B); Edinburgh Bot. Garden, Scotland	*C. harringtonia* var. *wilsoniana*
86	? *C. wilsoniana* (93-4074B); Edinburgh Bot. Garden, Scotland	*C. harringtonia* var. *wilsoniana*
87	? *C. wilsoniana* (93-3497D); Edinburgh Bot. Garden, Scotland	*C. harringtonia* var. *wilsoniana*
88	? *C. fortunei* var. *alpina*; Atlanta Bot. Garden, Atlanta, Ga.	*C. fortunei* var. *alpina*
89	? *C. harringtonia*; Univ. W. Australia, Perth, Australia	*C. harringtonia*
90	? *C. harringtonia* 'Sea Island'; The Cloisters, Sea Island, Ga.	*C. harringtonia*

[z]Plant names were not verified or identified by authors and were listed as they were labeled.
[y]Suggested name after AFLP analysis.

ever, RAPD analysis is sensitive to experimental conditions (primer selections, magnesium concentration, and PCR conditions) and can be subject to low reproducibility if the DNA concentration is below a threshold value or if amplification conditions are alternated (Lin et al., 1996).

Amplified fragment length polymorphism (AFLP) was developed by Kengene in Wageningen, The Netherlands (Vos et al., 1995; Zabeau and Vos, 1993); a combination of RFLP analysis and PCR, which results in highly informative fingerprints. Compared with RFLP or RAPD markers, analyzing AFLPs is the most useful, reliable, and promising molecular marker technique for genotypic comparisons within species (Becker et al., 1995; Lin et al., 1996; Meksem et al., 1995). Zhang (1997) reported 99.9% reproducibility among three replications of 27 bermudagrass [*Cynodon dactylon* (L.) Pers.] 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 identification and protection. The present study uses AFLPs to determine the genetic distinctness of *Cephalotaxus* taxa.

Materials and Methods

Plant materials. In this study, 90 accessions of *Cephalotaxus* were collected and grown in environmentally controlled greenhouses, outdoor lath areas, and field trials at the University of Georgia, Athens (Table 1).

DNA extraction. Total genomic DNA was isolated from leaves following the acidic extraction protocol (modified from Guillemaut and Marechal-Drouard, 1992) which can be summarized as follows. One gram of fresh mature leaf tissue (0.5 g for young and silica gel dried leaves) was ground in liquid nitrogen, then mixed with 10 mL of extraction buffer (100 mM NaOAc at pH 4.8, 50 mM EDTA, 500 mM NaCl, 2% polyvinylpyrrolidone (PVP), 1.4% sodium dodecyl sulfate (SDS), 50 mM cysteine, and pure sodium hydroxide beads to adjust pH to 5.5) and incubated at 65 °C for 10 min with occasional swirling. Samples were centrifuged at 5000 g_n for 10 min. The supernatant was decanted into a new tube and 4 mL 3 M potassium acetate at pH 5.4 was added. Tubes were incubated in an ice-water bath for at least 30 min, centrifuged at 5000 g_n for 10 min at 4 °C and, the supernatant was decanted into a new tube. One volume of pure ice-cold isopropanol was added and the tubes were placed at –20 °C for 1 to 2 h, then centrifuged at 5000 g_n for 10 min at 4 °C. The supernatant was discarded and the pellet was dried for 24 h, then redissolved in 500 to 600 µL TE buffer (10 mM Tris, 1 mM EDTA) at pH 8.0. DNAse-free RNAse was added to the dissolved pellet at 8 µg·mL^{-1} and incubated at 37 °C for 39 min or more. The

Table 2. Oligonucleotide adaptors and primers used for AFLP analysis of *Cephalotaxus* accessions.

Adaptor or primer	Oligonucleotide
EcoRI adaptors	CTCGTAGACTGCGTACC
	CATCTGACGCATGGTTAA
MseI adaptors	GACGATGAGTCCTGAG
	TACTCAGGACTCAT
AFLP primers	
EcoRI + 1	AGACTGCGTACC + AATTC + A
MseI + 1	GATGAGTCCTGAG + TAA + C
EcoRI + 3	GACTGCGTACC + AATTC + ACT
	GACTGCGTACC + AATTC + ACG
	GACTGCGTACC + AATTC + AGC
MseI + 3	GATGAGTCCTGAG + TAA + CTC

solution was transferred to 1.5 mL tubes, then 500 µL phenol at pH 8.0 was added. The sample was mixed and centrifuged in a microcentrifuge at 5000 g_n for 3 to 5 min. The upper layer was transferred to another tube and 500 µL of pure ice-cold isopropanol was added. DNA was pelleted by centrifugation, then stored in a freezer at –20 °C in deionized distilled water.

All isolated DNAs were quantified using a DNA Fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). The original DNA concentrations were between 107 and 574 $\mu g \cdot mL^{-1}$. A DNA stock solution of 20 µL at 100 $\mu g \cdot mL^{-1}$ was used for the AFLP experiment.

Aflp procedure. Perkin Elmer (Foster City, Calif.) Large Plant Genome Kit and Small Plant Genome Kit were purchased to conduct the research. Amplified fragment length polymorphism reactions were conducted as recommended by the Perkin Elmer AFLP Plant Mapping Protocol (PE Applied Biosystems, 1996) except for the following modifications.

1) Restriction–ligation reactions: Master Mix I for digestion of template DNA and Master Mix II for AFLP adaptor ligation to target sequences were prepared separately, then combined in a single tube.

2) Preselective amplifications of target sequences: Preselective primer pairs were primers complementary to the DNA sequences of the AFLP adaptor oligonucleotides (Table 2).

Polymerase chain reactions (PCRs) were performed on the GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.) as follows: one cycle at 72 °C for 2 min, then 20 cycles at 94 °C for 1 s, 56 °C for 30 s, and 72 °C for 2 min. The preselective PCR products (10 µL for each sample) were verified by electrophoresis on an 1.5% agarose gel stained with ethidium bromide. Smears of the PCR products from 100 to 1500 bps were clearly visible.

3) Selective amplification: Initially, four accessions (*C. harringtonia, C. harringtonia* 'Duke Gardens', *C. harringtonia* 'Fastigiata', and *C. harringtonia* 'Prostrata') were tested with 32 primer combinations including 16 from a Perkin Elmer Large Plant Genome Kit and 16 from a Small Plant Genome Kit. Based on the results obtained, such as band patterns and average band numbers, three of these 32 combinations were selected and used against all 90 *Cephalotaxus* samples. The three primer pairs were MseI adaptor sequence plus CTC combining with EcoRI adaptor sequence plus ACT, ACG, and AGC (Table 2). In all reactions, only EcoRI selective primers were labeled with a fluorescent dye (Perkin-Elmer, Foster City, Calif.). PCRs were performed on the same machine mentioned above with a touchdown cycle profile as follows: 94 °C for 2 min, 65 °C for 30 s, and 72 °C for 2 min, then 94 °C for 1 s, 64 °C (–1 °C/cycle) for 30 s, and 72 °C for 2 min until reaching the optimal annealing temperature of 56 °C. At this temperature, 27 more cycles were carried out for all EcoRI primers.

4) Multiplex, gel, and electrophoresis conditions: Although single PCRs were performed for each primer combination, the products from all three primer combinations were loaded simultaneously on a 5.0% long ranger gel [19 acrylamide : 1 bisacrylamide (v/v)] in 1×TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA). Samples were electrophoresed (2500 V) for 4 h at 48 °C in 1× TBE buffer, on an automated DNA sequencer (model ABI377, Perkin-Elmer, Applied Biosystems) equipped with GeneScan Analysis software (version 2.0.2. Perkin-Elmer, Applied Biosystems). Fragment sizes were calculated automatically using the local Southern sizing algorithms (Elder and Southern, 1987).

5) AFLP data analyses: Combined data files containing sizing data for all DNAs were created using Genotyper (version 1.1, Perkin-Elmer, Applied Biosystems) for each primer combination. The threshold value for fragment detection was 50. Pairwise comparisons were done for all genotypes, and the number of shared fragments for each comparison was calculated with the aid of Mathematica (version 2.2, Wolfram Research, Champaign, Ill.). Relative genetic similarity coefficient (S_{xy}) was estimated according to Nei and Li (1979) by Excel (version 6.0, Microsoft). Conversion to genetic distance, D_{xy}, was obtained by the following equation: $D_{xy} = 1 – S_{xy}$ (data not presented) and unweighted pair group method with arithmetic average (UPGMA) and neighbor-joining (NJ) phenograms were derived using molecular evolutionary genetics analysis (MEGA) (Sudhir et al., 1993), numerical taxonomy and multivariate analysis system (NTSYS) (Rohlf, 1993), and phylogeny inference package (PHYLIP) (Felsenstein, 1995).

Results and Discussion

A total of 403 useful markers between 75 and 500 bps fragment sizes were generated from three primer–pair combinations of 90 *Cephalotaxus* accessions. The average number of markers for each accession was 208 and each primer–pair combination produced ≈70 useful markers for each taxon (Table 3). Little variation (<10%) was observed among primer–pair combinations for the number of useful markers except for accessions 16 (157 markers) and 84 (141 markers) (Tables 1 and 3).

Based on all useful AFLP markers, genetic dissimilarity coefficients (D_{xy}) were calculated (data not presented). Dissimilarity coefficients ranged from 0.0043 to 0.4253. Both male clones of *C. harringtonia* 'Prostrata' collected from the Arnold Arboretum and Brooklyn Botanical Garden showed minimal genetic difference while *C. oliveri* from Piroche Plants (Pitt Meadows, British Columbia, Canada) had the highest genetic distance compared to *C. harringtonia* (male) from the Atlanta Botanical Garden (Atlanta, Ga.). High D_{xy} values among established species and low D_{xy} values among clones were expected. D_{xy} values of varieties and cultivars were intermediate to values among species and clones.

Two UPGMA phenograms (trees) and two NJ phenograms were generated from MEGA (Sudhir et al., 1993) and NTSYS (Rohlf, 1993), respectively. Also, one consensus phenogram was generated from the phylogeny inference package (PHYLIP) (Felsenstein, 1995). Although each phenogram showed different genetic relationships among clusters, most clusters consisted of the same accessions (the detailed discussions follow). Based on

Table 3. AFLP markers generated from three primer pairs of 90 *Cephalotaxus* accessions.

Taxon	Blue	Green	Yellow	Total	Taxon	Blue	Green	Yellow	Total
01	59	45	68	172	47	68	80	75	223
02	63	68	71	202	48	60	71	75	206
03	69	77	77	223	49	61	74	73	208
04	49	58	74	181	50	50	64	68	182
05	64	60	63	187	51	64	75	73	212
06	62	67	75	204	52	64	73	73	210
07	67	63	71	201	53	64	78	70	212
08	64	63	70	197	54	60	70	61	191
09	65	69	75	209	55	68	73	67	208
10	68	83	80	231	56	68	76	76	220
11	70	72	77	219	57	49	75	76	200
12	53	65	68	186	58	66	75	65	206
13	64	69	60	193	59	65	72	61	198
14	51	56	63	170	60	57	69	76	202
15	40	69	74	183	61	58	66	72	196
16	50	47	60	157	62	71	83	75	229
17	68	71	74	213	63	71	84	74	229
18	66	84	85	235	64	57	73	62	192
19	69	71	80	220	65	71	78	70	219
20	68	77	80	225	66	57	67	68	192
21	66	79	81	226	67	69	76	73	218
22	62	70	78	210	68	68	78	75	221
23	69	79	71	219	69	58	76	67	201
24	67	74	76	217	70	56	71	74	201
25	61	66	75	202	71	55	72	74	201
26	58	75	75	208	72	73	77	79	229
27	62	74	76	212	73	59	71	68	198
28	63	64	72	199	74	71	76	78	225
29	66	76	76	218	75	74	77	76	227
30	63	59	75	197	76	72	75	73	220
31	68	68	76	212	77	39	74	78	191
32	64	79	79	222	78	69	77	74	220
33	66	76	75	217	79	50	75	86	211
34	70	76	73	219	80	71	86	75	232
35	68	73	80	221	81	70	81	79	230
36	65	73	78	216	82	70	88	78	236
37	62	70	77	209	83	71	87	79	237
38	72	77	76	225	84	41	58	42	141
39	59	75	77	211	85	65	81	66	212
40	69	74	77	220	86	57	77	63	197
41	60	73	76	209	87	66	76	63	205
42	68	76	67	211	88	55	71	63	189
43	47	67	59	173	89	54	69	44	167
44	66	76	49	191	90	53	78	63	194
45	65	81	60	206	Mean	63	73	72	208
46	60	73	66	199	Markers	97	139	167	403

the NTSYS phenograms, a consensus tree was generated with reference of MEGA phenograms and PHYLIP consensus phenogram (Fig. 1).

At the species level, the AFLP results supported the *rbc*L gene sequence conclusions (Zhang, 1998). *Cephalotaxus oliveri* is a distinct species in all phenograms and showed high genetic dissimilarity (average distance 34.4%) compared to the other taxa. Six accessions of *C. fortunei* taxa were grouped as a cluster except accession 6 from Aiken, S.C.; 7 from Aiken, S.C. (natural seedling); 9 from Edinburgh Botanical Garden (687276), Scotland; 10 from F.W. Schumacher Inc., Sandwich, Mass.; and 11 from Kew Gardens (69.16245), England which were morphologically similar to *C. harringtonia*. Genetic differences of <10% were observed within three U.S. accessions of *C. fortunei* species (Fig. 1). Six accessions of *C. sinensis* formed a cluster, but their genetic relationships with other taxa were confusing. The cluster was grouped either with *C. oliveri* and *C. fortunei* or with *C. harringtonia*. It is possible that *C. sinensis* is a hybrid complex (Tripp, New York Botanical Garden, personal communication). By comparing band patterns of *C. fortunei, C. sinensis,* and *C. harringtonia*, *C. sinensis* shared six bands with *C. fortunei* and 12 bands with *C. harringtonia* (Table 4). All three taxa shared >80 bands in the three primer–pair combinations (data not presented). This result supports the hypothesis that *C. sinensis* is a hybrid

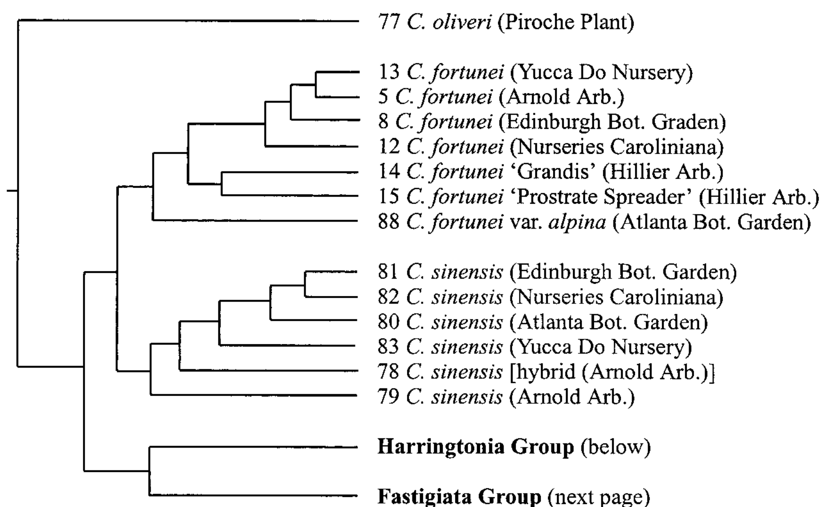

Fig. 1. Phenogram of *Cephalotaxus* accessions based on AFLP data.

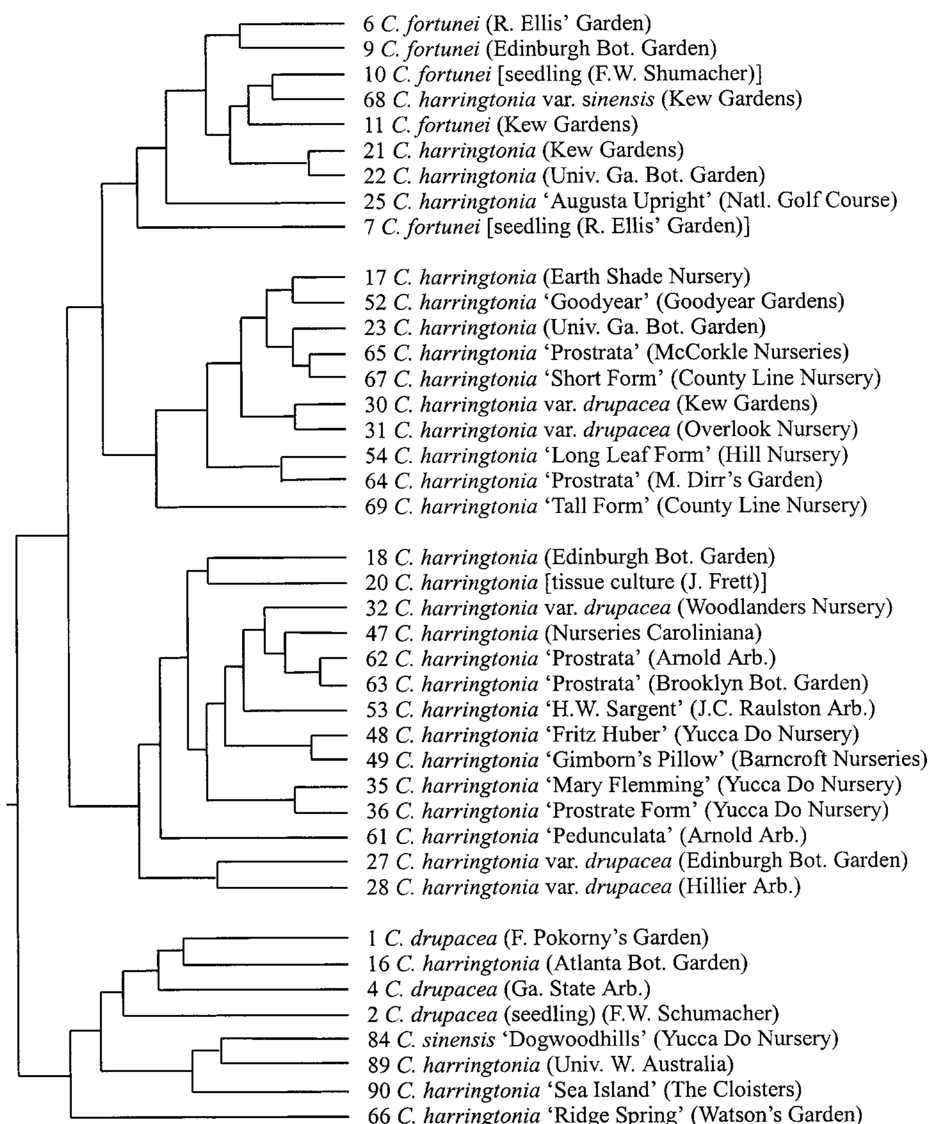

Fig. 1 (continued). **Harringtonia Group.**

species derived from *C. fortunei* and *C. harringtonia*. All other taxa (accessions) were grouped under *C. harringtonia* (Fig. 1).

The taxa subordinate to species, especially cultivars, are very important to the nursery industries. Normally, a plant with unique horticultural trait(s) can be described morphologically as a new cultivar if the trait(s) can be reproduced asexually or sexually. The plant can be patented or trademarked for protection and marketing purposes. However, no genetic information is needed for patenting or registering a new cultivar. In this study, no intraspecific taxa were available under *C. oliveri* and only two cultivars and one variety under *C. fortunei*. *Cephalotaxus fortunei* 'Prostrate Spreader' had 16.7% genetic distance compared to *C. fortunei* 'Grandis' (Fig. 1). Morphologically, 'Grandis' is a female with bush-like habit and longer leaves, while 'Prostrate Spreader' originated from a side shoot (plagiotropic) and developed into a ground cover plant with widespreading branches (Hillier Nurseries, 1995). Compared with the other four accessions of *C. fortunei*, the above cultivars had at least 16.8% genetic distance. *Cephalotaxus fortunei* var. *alpina* averaged 15.2% genetic difference from the species and the above two cultivars (Fig. 1). Morphologically, the leaves of *C. fortunei* var. *alpina* are much wider. Based on genetic and morphological differences, the two *C. fortunei* cultivars and the variety should be accepted.

One cultivar, *C. sinensis* 'Dogwoodhills', was listed under *C. sinensis*. An average 26.6% genetic distance was recorded as compared to the other accessions and the lowest value of 15.0% was found compared to *C. harringtonia* from Australia in the **harringtonia** group. Thus, it is apparently misclassified under the wrong species. For the relationship to other taxa, it follows the same trend as its species and may be a hybrid cultivar. With only one rooted cutting available in our collection, little morphological information can be used to reach a conclusion for this taxon.

Two distinguishable groups, **harringtonia** and **fastigiata**, were separable under the species *C. harringtonia* (Fig. 1). Morphologically, the **harringtonia** group is characterized by uniform two-ranked leaves, upright (only terminal growth with spirally arranged leaves) or prostrate habits, while the **fastigiata** group bears spirally arranged or semi-whorled leaves, with leaves absent between new and old growth, leaves arranged in more or less two ranks, and columnar (no lateral branches) or vase-shaped, upright growth habits (morphological characters of *C. koreana, C. harringtonia* var. *nana,* and *C. wilsoniana* will be discussed later).

Four subgroups were reconciled from the **harringtonia** group, i.e, harringtonia, goodyear, prostrata, and drupacea (Fig. 1). The

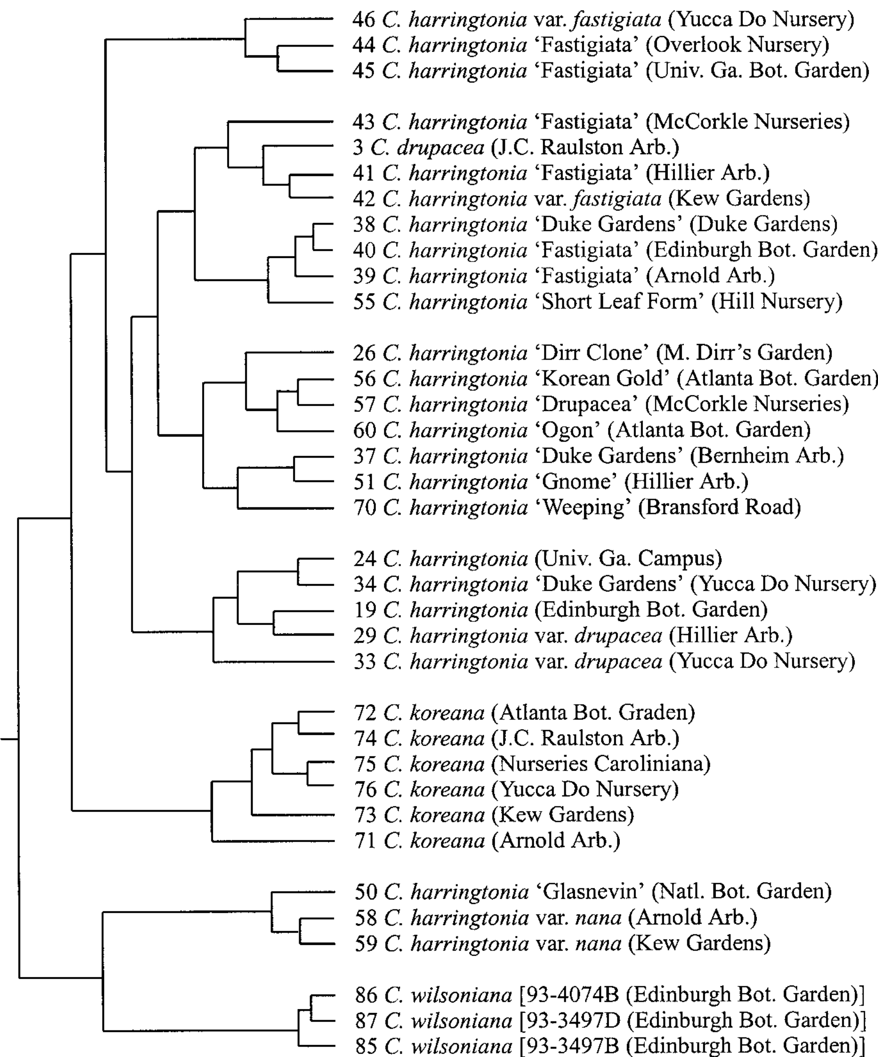

Fig. 1 (continued). **Fastigiata Group**.

harringtonia subgroup consisted of nine accessions in which five of them were originally named *C. fortunei*. Genetically, <14% genetic distance was observed among accessions. Morphologically, all accessions were similar with upright or shrub-like growth habits and uniform two-ranked leaves. Accession 9, *C. fortunei* from Edinburgh Botanical Garden (687276), Scotland, and the four other accessions (6, 7, 10, and 11) were either misidentified or mislabeled. They are allied with *C. harringtonia*.

All plants in the goodyear subgroup were similar with <10.0% genetic distance except 69 *C. harringtonia* 'Tall Form' from County Line Nursery, Byron, Ga. Plants developed spreading growth forms akin to ground covers. With the exception of a fast growing clone from Goodyear Garden, Aiken, S.C., *C. harringtonia* 'Prostrata' is the correct name for this subgroup. The prostrata subgroup had slightly over 10.0% genetic distance among accessions except 61, *C. harringtonia* 'Pedunculata', from the Arnold Arboretum, Jamaica Plain, Mass. Accession 35, *C. harringtonia* 'Mary Flemming', and 36, *C. harringtonia* 'Prostrate Form', (both from Yucca Do Nursery, Waller, Texas) had 5.7% genetic differences. Morphologically, the extremely recurved needles described in the Yucca Do catalog were not found on the 230-cm tall plant that was received from the nursery. It is possible that a chimeral clonal mother plant shows different morphological characters without distinguished genetic difference. However, it is logical to list both accessions as *C. harringtonia* 'Prostrata'. All other accessions had <10.0% genetic difference while comparing with both 62 and 63, *C. harringtonia* 'Prostrata' (Fig. 1). Morphologically, most plants in the prostrate subgroup were prostrate or shrub-like forms with dark green foliage.

Compared to the above three subgroups, the drupacea subgroup had >20.0% genetic distance except questionable 84, *C. sinensis* 'Dogwoodhills', and 89, *C. harringtonia*. Accession 66, *C. harringtonia* 'Ridge Spring', was a mutation of *C. harringtonia* found by Robert McCartney in Watson's garden in Ridge Spring, S.C. The plant has an upright growth form with scalelike (<1 cm long) leaves and pendulous lateral branches. All other accessions were similar morphologically to the above three subgroups.

The **fastigiata** group can be also separated into four subgroups (Fig. 1). A total of 23 accessions comprised the top subgroup with <15.0% genetic distance. Morphologically, cultivars in this subgroup did somehow relate to *C. harringtonia* 'Fastigiata' with the exception of 70, *C. harringtonia* 'Weeping', from Augusta, Ga.; 19, *C. harringtonia*, from Edinburgh Botanical Garden, Scotland; 29, *C. harringtonia* var. *drupacea*, from the Hillier Arboretum, England; and 33, *C. harringtonia* var. *drupacea* from Yucca Do Nursery, Waller, Texas. *Cephalotaxus harringtonia* 'Duke Gardens' is a branch sport of *C. harringtonia* 'Fastigiata' with two-ranked leaves. *Cephalotaxus harringtonia* 'McCorkle', a clone grown by McCorkle Nurseries, Inc., Dearing, Ga., possesses greater vigor with the V-shaped habit and semiwhorled leaves, characteristics intermediate between *C. harringtonia* 'Fastigiata' and *C. harringtonia* 'Duke Gardens'. Accession 51, *C. harringtonia* 'Gnome', from Hillier Arboretum, England is morphologically identical to *C. harringtonia* 'Duke Gardens'. Accessions 56 and 60, *C. harringtonia* 'Korean Gold' and 'Ogon' are *C. harringtonia* 'Fastigiata' with yellow new growth. All other accessions in this subgroup were probably mislabeled or misidentified. Although the genetic distances were not significantly distinguished, three morphological distinct cultivars, *C. harringtonia* 'Fastigiata', 'McCorkle', and 'Duke Gardens' should be accepted.

The six accessions of *C. koreana* formed a subgroup. Less than 10.0% genetic distances were observed except for 71 *C. koreana* from the Arnold Arboretum, Jamaica Plain, Mass. Although plants were morphologically variable, a variety, *C. harringtonia* var. *koreana*, was suggested based on AFLP data. Further studies should be designed to address this question.

Cephalotaxus harringtonia 'Glasnevin' and *C. harringtonia* var. *nana* clustered together with <15.0% genetic differences. Plants of three accessions shared compact growth habits, shining dark green foliage, needles inverted (apex pointing down) with prominent silver bands underneath. Based on genetic and morphological characteristics, a variety, *C. harringtonia* var. *nana*, should be considered.

The three accessions from three different populations of *C. wilsoniana* collected by Edinburgh Botanical Garden, Scotland,

Table 4. Representive AFLP band patterns of *C. fortunei, C. sinensis,* and *C. harringtonia.*

Primer	Dye color	Size (bp)	*C. fortunei* 5[z]	*C. fortunei* 8	*C. sinensis* 79	*C. sinensis* 81	*C. harringtonia* 20	*C. harringtonia* 22
CTC+ACT	Blue	80	—	—	—	—	—	—
CTC+AGC	Yellow	113			—	—	—	—
CTC+ACG	Green	115	—	—	—	—		
CTC+ACT	Blue	121.5			—	—	—	—
CTC+AGC	Yellow	132.5			—	—	—	—
CTC+ACG	Green	140	—	—	—	—		
CTC+ACG	Green	145	—	—	—	—	—	—
CTC+AGC	Yellow	168.5			—	—	—	—
CTC+AGC	Yellow	178			—	—	—	—
CTC+AGC	Yellow	187	—	—	—	—		
CTC+ACT	Blue	189.5	—	—	—	—	—	—
CTC+AGC	Yellow	189.5			—	—	—	—
CTC+ACG	Green	190			—	—	—	—
CTC+AGC	Yellow	204			—	—	—	—
CTC+AGC	Yellow	221			—	—	—	—
CTC+AGC	Yellow	223.5	—	—	—	—		
CTC+AGC	Yellow	238			—	—	—	—
CTC+ACT	Blue	281	—	—	—	—		
CTC+ACG	Green	297			—	—	—	—
CTC+AGC	Yellow	297	—	—	—	—		
CTC+AGC	Yellow	383.5			—	—	—	—
CTC+ACG	Green	450.5	—	—	—	—	—	—

[z]Accession number in Table 1.

were included in this study. Regardless of the clustering methods (UPGMA or NJ) and programs (NTSYS, MEGA, and PHYLIP), the three accessions always formed a subgroup. Less than 10.0% genetic difference was recorded. Literature descriptions and molecular data (Cheng and Fu, 1978; Fu, 1984; Zhang, 1998) indicated that the variety *C. harringtonia* var. *wilsoniana* should be accepted.

The large number of fragments amplified from the *Cephalotaxus* genome shows the potential of the AFLP technique for cultivar discrimination and genetic analyses. Sharma et al. (1996) reported that the AFLP method detected 10 times more informative bands per primer than the RAPD method with *Lens*. Also reproducibility and reliability of the AFLP technique had been reported as being very high (Janssen et al., 1996; Vos et al., 1995; Zabeau and Vos, 1993), especially when the semiautomated fluorescence-based AFLP method was used (Zhang, 1997). In this preliminary study, the genetic differences among 90 *Cephalotaxus* accessions are documented (data not presented). For cultivar discrimination, 90 accessions could be distinguished as four species, four varieties, and eight cultivars (Table 1).

The origin of *Cephalotaxus* is complicated, particularly for the cultivated taxa (Dirr, 1990; Tripp, 1994). Different relationships occurred if the AFLP data were analyzed using different cluster methods and different computer programs. Since *Cephalotaxus* was introduced to cultivation, plants from different geographical regions in the world have been collected and cultivated in the same area. Hybridization has possibly occurred among *Cephalotaxus* taxa. If so, it is impossible to determine the relationships among *Cephalotaxus* taxa with this AFLP data.

Although the clustering results varied using different methods, some accessions were 100% associated within a formed single subgroup. The three accessions of *C. wilsoniana* provide a good example, which also indicated that the AFLP method can be used for investigating genetic diversity among *Cephalotaxus* populations.

Morphological changes may not follow the same trend as the genetic changes because morphological characteristics may be regulated by environmental factors. Also, some genetic changes might not be reflected by morphological characteristics. In this study, three cultivars in the **harringtonia** group can be explained by these factors. If the dark green short needles or yellow new growth are considered stable morphological characteristics, then *C. harringtonia* 'Fritz Huber' or 'Korean Gold', respectively, should be accepted. In nursery production, most *Cephalotaxus* are propagated by stem cuttings. Topophysis is another factor determining the growth habits of the rooted cuttings (Dirr and Heuser, 1987). Prostrate growing plants can be rooted from the horizontal branches (plagiotropic) while upright plants result from rooting cuttings from vertical (orthotropic) branches. If both types of cuttings were collected from a single plant, two or more growth forms can be produced without any genetic difference. Although AFLP is a highly informative method, it would be virtually impossible to detect such genetic differences.

Conclusion

Based on results of AFLP data combined with morphological characteristics, three species, *C. oliveri, C. fortunei,* and *C. harringtonia*, and one hybrid species, *C. ×sinensis*, four varieties, *C. fortunei* var. *alpina, C. harringtonia* var. *koreana, C. harringtonia* var. *nana,* and *C. harringtonia* var. *wilsoniana*, and eight cultivars, *C. fortunei* 'Grandis' and 'Prostrate Spreader', *C. harringtonia* 'Duke Gardens', 'Fastigiata', 'Goodyear', 'McCorkle', 'Prostrata', and 'Ridge Spring' should be accepted. The data in this study serve as a guide to researchers and growers for identification and genetic distance and a model to establish a cultivar library against which later introductions or nomenclatural irregularities within *Cephalotaxus* can be cross-referenced.

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