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) Hornib., 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 monocious. 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 (Nybomb, 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 (Nybomb, 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.) (Krahli 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-
| Accession no. | Plant name<sup>2</sup> and source | Suggested name<sup>2</sup> |
|--------------|----------------------------------|--------------------------|
| 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* ‘Prostrate’ |
| 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* ‘Prostrate’ |
| 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* ‘Prostrate’ |
| 22           | ✧ *C. harringtonia*; Univ. Ga. Bot. Garden, Athens, Ga. | *C. harringtonia* ‘Prostrate’ |
| 23           | ✧ *C. harringtonia*; Univ. Ga. Bot. Garden, Athens, Ga. | *C. harringtonia* ‘Prostrate’ |
| 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* ‘Prostrate’ |
| 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* ‘Prostrate’ |
| 31           | ✧ *C. harringtonia var. drupacea*; Overlook Nursery, Mobile, Ala. | *C. harringtonia* ‘Prostrate’ |
| 32           | ✧ *C. harringtonia var. drupacea*; Woodlanders Nursery, Aiken, S.C. | *C. harringtonia* ‘Prostrate’ |
| 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* ‘Prostrate’ |
| 36           | ✧ *C. harringtonia* ‘Prostrate Form’; Yucca Do Nursery, Waller, Texas | *C. harringtonia* ‘Prostrate’ |
| 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* ‘Prostrate’ |
| 49           | ✧ *C. harringtonia* ‘Gimborn’s Pillow’; Barncroft Nurseries, England | *C. harringtonia* ‘Prostrate’ |
| 50           | ✧ *C. harringtonia* ‘Glassnevin’; 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* ‘Prostrate’ |
| 54           | ✧ *C. harringtonia* ‘Long Leaf Form’; Hill Nursery, Commerce, Ga. | *C. harringtonia* ‘Prostrate’ |
| 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* |
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 plant species identification. He also concluded that the AFLP technique not only had high reproducibility and reliability, but could also 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 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 for 10 min at 4 °C and the supernatant was discarded and the pellet was washed at least 20 °C for 1 h, then centrifuged at 5000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed 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.

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

| Accession no. | Plant name² and source | Suggested name³ |
|---------------|------------------------|-----------------|
| 59            | ? C. harringtonia var. nana; Kew Gardens, England | C. harringtonia var. nana |
| 60            | ? C. harringtonia ‘Ogon’; Atlanta Botanical Garden, Atlanta, Ga. | C. harringtonia var. "Fastigiata" |
| 61            | ♂ C. harringtonia ‘Pedunculata’; Arnold Arb., Jamaica Plain, Mass. | C. harringtonia |
| 62            | ♂ C. harringtonia ‘Prostrata’; Arnold Arb., Jamaica Plain, Mass. | C. harringtonia var. "Prostrata" |
| 63            | ♂ C. harringtonia ‘Prostrata’; Brooklyn Bot. Garden, Brooklyn, N.Y. | C. harringtonia var. "Prostrata" |
| 64            | ? C. harringtonia ‘Prostrata’; M. Dirr’s Garden, Watkinsville, Ga. | C. harringtonia var. "Prostrata" |
| 65            | ? C. harringtonia ‘Prostrata’; McCorkle Nurseries, Deering, Ga. | C. harringtonia var. "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 var. "Prostrata" |
| 68            | ♂ C. harringtonia var. sinensis; Kew Gardens, England | C. harringtonia |
| 69            | ? C. harringtonia ‘Tall Form’; County Line Nursery, Byron, Ga. | C. harringtonia var. "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. x sinensis |
| 79            | ♂ C. sinensis; Arnold Arb., Jamaica Plain, Mass. | C. x sinensis |
| 80            | ♂ C. sinensis; Atlanta Bot. Garden, Atlanta, Ga. | C. x sinensis |
| 81            | ? C. sinensis; Edinburgh Bot. Garden, Scotland | C. x sinensis |
| 82            | ? C. sinensis; Nurseries Caroliniana, N. Augusta, S.C. | C. x sinensis |
| 83            | ♂ C. sinensis; Yucca Do Nursery, Waller, Texas | C. x 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. fortunii var. alpina; Atlanta Bot. Garden, Atlanta, Ga. | C. fortunii var. alpina |
| 89            | ? C. harringtonia; Univ. W. Australia, Perth, Australia | C. harringtonia |
| 90            | ? C. harringtonia ‘Sea Island’; The Cloisters, Sea Island, Ga. | C. harringtonia |

²Plant names were not verified or identified by authors and were listed as they were labeled.
³Suggested name after AFLP analysis.
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 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 µg·mL−1. A DNA stock solution of 20 µL at 100 µg·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 a 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 ‘Fastigia’, 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 range 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). 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 (Sxy) was estimated according to Nei and Li (1979) by Excel (version 6.0, Microsoft). Conversion to genetic distance, Dxy, was obtained by the following equation: Dxy = 1 − Sxy (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 (Dxy) 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 Dxy values among established species and low Dxy values among clones were expected. Dxy 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
At the species level, the AFLP results supported the rbcL 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 complex.

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

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

| Taxon | Blue | Green | Yellow | Total |
|-------|------|-------|--------|-------|
| 01    | 59   | 45    | 68     | 172   |
| 02    | 63   | 68    | 71     | 202   |
| 03    | 69   | 77    | 77     | 223   |
| 04    | 49   | 58    | 74     | 181   |
| 05    | 64   | 60    | 63     | 187   |
| 06    | 62   | 67    | 75     | 204   |
| 07    | 67   | 63    | 71     | 201   |
| 08    | 64   | 63    | 70     | 197   |
| 09    | 65   | 69    | 75     | 209   |
| 10    | 68   | 83    | 80     | 231   |
| 11    | 70   | 72    | 77     | 219   |
| 12    | 53   | 65    | 68     | 186   |
| 13    | 64   | 79    | 70     | 197   |
| 14    | 51   | 56    | 63     | 170   |
| 15    | 40   | 69    | 74     | 183   |
| 16    | 50   | 47    | 60     | 157   |
| 17    | 68   | 71    | 74     | 213   |
| 18    | 66   | 84    | 85     | 235   |
| 19    | 69   | 71    | 80     | 220   |
| 20    | 68   | 77    | 80     | 225   |
| 21    | 66   | 79    | 81     | 226   |
| 22    | 62   | 70    | 78     | 210   |
| 23    | 69   | 79    | 71     | 219   |
| 24    | 67   | 74    | 76     | 217   |
| 25    | 61   | 66    | 75     | 202   |
| 26    | 58   | 75    | 75     | 208   |
| 27    | 62   | 74    | 76     | 212   |
| 28    | 63   | 64    | 72     | 199   |
| 29    | 66   | 76    | 76     | 218   |
| 30    | 63   | 59    | 75     | 197   |
| 31    | 68   | 68    | 76     | 212   |
| 32    | 64   | 79    | 79     | 222   |
| 33    | 66   | 76    | 75     | 217   |
| 34    | 70   | 76    | 73     | 219   |
| 35    | 68   | 73    | 80     | 221   |
| 36    | 65   | 73    | 78     | 216   |
| 37    | 62   | 70    | 77     | 209   |
| 38    | 72   | 77    | 76     | 225   |
| 39    | 59   | 75    | 77     | 211   |
| 40    | 69   | 74    | 77     | 220   |
| 41    | 60   | 73    | 76     | 209   |
| 42    | 68   | 76    | 67     | 211   |
| 43    | 47   | 67    | 59     | 173   |
| 44    | 66   | 76    | 49     | 191   |
| 45    | 65   | 81    | 60     | 206   |
| 46    | 60   | 73    | 66     | 199   |

The Mean 63 73 72 172. Markers 97 139 167 403.
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 widespread 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
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. harringtonia ‘Fastigiata’ (McCorkle Nurseries), 3 C. drupacea (J.C. Raulston Arb.), 41 C. harringtonia ‘Fastigiata’ (Hillier Arb.), 42 C. harringtonia var. fastigiata (Kew Gardens), 38 C. harringtonia ‘Duke Gardens’ (Duke Gardens), 40 C. harringtonia ‘Fastigiata’ (Edinburgh Bot. Garden), 39 C. harringtonia ‘Fastigiata’ (Arnold Arb.), 55 C. harringtonia ‘Short Leaf Form’ (Hill Nursery).

Accession 51, C. harringtonia ‘Gnome’ (Hillier Arb.), 56 C. harringtonia ‘Korean Gold’ (Atlanta Bot. Garden), 57 C. harringtonia var. drupacea (Hillier Arb.), 33 C. harringtonia var. drupacea (Yucca Do Nursery) had <10.0% genetic difference while 71 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 semiwheuld 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 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,
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., 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 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 regions in the world have been collected and cultivated in the same area. Hybridization has possibly occurred among species, varieties, and cultivars. 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.

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

| Primer color | Size (bp) | C. fortunei | C. sinensis | C. harringtonia |
|--------------|----------|-------------|-------------|----------------|
| Blue         | 80       | —           | —           | —              |
| Yellow       | 113      | —           | —           | —              |
| Green        | 115      | —           | —           | —              |
| Blue         | 121.5    | —           | —           | —              |
| Yellow       | 132.5    | —           | —           | —              |
| Green        | 140      | —           | —           | —              |
| Green        | 145      | —           | —           | —              |
| Yellow       | 168.5    | —           | —           | —              |
| Yellow       | 178      | —           | —           | —              |
| Yellow       | 187      | —           | —           | —              |
| Blue         | 189.5    | —           | —           | —              |
| Yellow       | 189.5    | —           | —           | —              |
| Green        | 190      | —           | —           | —              |
| Yellow       | 204      | —           | —           | —              |
| Yellow       | 211      | —           | —           | —              |
| Yellow       | 221      | —           | —           | —              |
| Yellow       | 223.5    | —           | —           | —              |
| Yellow       | 238      | —           | —           | —              |
| Blue         | 281      | —           | —           | —              |
| Green        | 297      | —           | —           | —              |
| Yellow       | 297      | —           | —           | —              |
| Yellow       | 383.5    | —           | —           | —              |
| Green        | 450.5    | —           | —           | —              |

*Accession number in Table 1.*
Literature Cited

Becker, J., P. Vos, M. Kuiper, F. Salamini, and M. Heun. 1995. Combined mapping of AFLP and RFLP markers in barley. Mol. Gen. Genet. 249:65–73.

Cheng, W.C. and L.K. Fu. 1978. Flora reipublicae popularis sinicae, Tomus 7 (Gymnospermae). Science Press, Beijing. People’s Republic of China.

Dallimore, W. and A.B. Jackson. 1967. A handbook of Coniferae and Ginkgoaceae. St. Martin’s Press, New York.

Dirr, M.A. 1999. Manual of woody landscape plants: Their identification, ornamental characteristics, culture, propagation, and uses. Stipes Publishing Co., Champaign, Ill.

Dirr, M.A. 1992. Cephalotaxus harringtonia, the Japanese plum yew: Superbly tolerant of heat, drought, sun, and cold dipping to –15° to –20°. Nursery Manager 8(4):24–25.

Dirr, M.A. and C.W. Heuser, Jr. 1987. The reference manual of woody plant propagation: From seed to tissue culture. Varsity Press, Athens, Ga.

Elder, J.K. and E.M. Southern. 1987. Computer-aided analysis of one-dimensional restriction fragment gels. p. 165–175. In: M.J. Bishop and J. Rawlings (eds.). Nucleic acid and protein sequence analysis—A practice approach. IRL Press, Oxford, U.K.

Felsenstein, J. 1995. PHYLIP (Phylogeny inference package) Version 3.57c. Univ. Wash., Seattle (http://evolution.genetics.washington.edu/phylip.html).

Fu, L.K. 1984. A study on the genus Cephalotaxus Sieb. et Zucc. Acta Phytotaxon Sin. 24:277–288.

Guillemaut, P. and L. Marechal-Drouard. 1992. Isolation of plant DNA: A fast, inexpensive, and reliable method. Plant Mol. Biol. Rpt. 10:60–65.

Harlass, S. 1994. Georgia names 4 outstanding plants. Greenhouse Mgr. 13(1):79–82.

Hillier Nurseries. 1995. The Hillier manual of trees and shrubs. David and Charles, Inc., North Pomfret, Vt.

Hubbard, M., J. Kelly, S. Rajapakse, A. Abbott, and R. Ballard. 1992. Restriction fragment length polymorphisms in rose and their use for cultivar identification. HortScience 27:172–173.

Janssen, P., R. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau, and K. Kersters. 1996. Evaluation of DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142:1881–1893.

Kamalay, J.C. and D.W. Carey. 1995. Application of RAPD–PCR markers for identification and genetic analysis of american elm (Ulmus americana L.) selections. J. Environ. Hort. 13:155–159.

Krhal, K.H., M.A. Dirr, T.M. Halward, G.D. Kochert, and W.M. Randle. 1993. Use of single-primer DNA amplification for the identification of red maple (Acer rubrum L.) cultivars. J. Environ. Hort. 11:89–92.

Krusmann, G. 1985. Manual of cultivated conifers. Timber Press, Portland, Ore.

Lin, J.J., J. Kuo, J. Ma, J.A. Saunders, H.S. Beard, M.H. MacDonald, W. Kenworthy, G.N. Ude, and B.F. Mathews. 1996. Identification of molecular markers in soybean comparing RFLP, RAPD and AFLP mapping techniques. Plant Mol. Biol. Rpt. 14:156–169.

Mabberly, D.J. 1993. The plant-book. Cambridge Univ. Press, Cambridge, U.K.

Meksem, K., D. Leister, J. Peleman, M. Zabeau, F. Salamini, and C. Gebhardt. 1995. A high resolution map of the vicinity of the R1 locus on chromosome V of potato based on RFLP and AFLP markers. Mol. Gen. Genet. 249:74–81.

Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specified genomic regions using segregating populations. Proc. Natl. Acad. Sci. USA 88:9828–9832.

Miller, J.C. and S.D. Tanksley. 1990. RFLP analysis of phylogenetic relationships and genetic variation in the genus Lycopersicon. Theor. Appl. Genet. 80:437–448.

Nei, M. and W.H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. Proc. Natl. Acad. Sci. USA 76:5269–5273.

Nybom, H. 1994. DNA fingerprinting—A useful tool in fruit breeding. Euphytica 77:59–64.

Paul, S., F.N. Wachira, W. Powell, and R. Waugh. 1997. Diversity and genetic differentiation among populations of Indian and Kenyan tea ([Camellia sinensis (L.) O. Kuntze] revealed by AFLP markers. Theor. Appl. Genet. 94:255–263.

PE Applied Biosystems. 1996. AFLP plant mapping protocol. Perkin-Elmer Corp., Foster City, Calif.

Rohlf, F. J. 1993. NTSYS: Numerical taxonomy and multivariate analysis system (version 1.80). State Univ. N.Y., Stony Brook.

Rushforth, K.D. 1987. Conifers. Christopher Helm Publishers, Ltd., London.

Sharma, S.K., M.R. Knox, and T.H.N. Ellis. 1996. AFLP analysis of the diversity and phylogeny of Lens and its comparison with RAPD analysis. Theor. Appl. Genet. 93:751–758.

Silba, J. 1984. Phytologia memoirs VII: An international census of the Coniferae, I. Molsdenke Publisher, Plainfield, NJ.

Staub, J.E. and V. Meglic. 1993. Molecular genetic markers and their legal relevance for cultivar discrimination: A case study in cucumber. HortTechnology 3:291–300.

Sudhir, K., K. Tamura, and M. Nei. 1993. MEGA: Molecular evolutionary genetics analysis (version 1.01). Pa. State Univ., Univ. Park, Pa.

Tripp, K. 1994. A plum yew primer. American Nurseryman 180(9):28–37.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407–4414.

Wang, Z.Y., G. Second, and S.D. Tanksley. 1992. Polymorphism and phylogenetic relationships among species in the genus Oryza as determined by analysis of nuclear RFLPs. Theor Appl. Genet. 83:565–581.

Zabeau, M. and P. Vos. 1993. Selective restriction fragment amplification: A general method for DNA fingerprinting. European Patent Application No. 92042629.7.

Zhang, D. 1998. Classification of Cephalotaxus species based on rbcL sequences. PhD diss., Univ. Georgia, Athens.

Zhang, L. 1997. Genotype identification of bermudagrass (Cydonon spp.) by AFLP analysis. MS thesis, Univ. Georgia, Athens.