HortScience 39(4):xxx–xxx. 2004.

Identification of A Heath-leaved Cypress Cultivar Based on Sequences of Nuclear Ribosomal DNA

Jianhua Li,[1] Jeremy Ledger, and Peter Del Tredici
Arnold Arboretum of Harvard University, 125 Arborway, Jamaica Plain, MA 02130

Donglin Zhang
Landscape Horticulture, University of Maine, Orono, ME 04469-5722

Additional index words. nucleotide, cultivar identification, *Chamaecyparis formosensis*, *Chamaecyparis pisifera*, *Chamaecyparis obtusa*, *Chamaecyparis lawsoniana*, *Fokienia hodginsii*, *Chamaecyparis thyoides* 'Ericoides'

Abstract. **The identity of heath-leaved cypress is controversial. In this study nucleotide sequences of nuclear ribosomal DNA were used to identify heath-leaved cypress (*Chamaecyparis* 'Ericoides') species. Sixteen individuals were sampled representing the five species of *Chamaecyparis*, 'Ericoides', and four other genera of Cupressaceae (*Cupressus*, *Fokienia*, *Juniperus*, and *Thuja*). The results placed 'Ericoides' unequivocally to *Chamaecyparis thyoides*, supporting a conclusion derived from wood anatomy. This study supports the usefulness and integrity of using molecular data to identify the genetic affinity of cultivars that are morphologically different from the parent species.**

One of the most challenging tasks facing horticultural taxonomists and gardeners is to identify cultivars to the species from which they have been derived. This is particularly true for cultivars that have been selected for their dramatically different morphology from the original species. Juvenile forms of conifers represent such a group of cultivars that are difficult to identify and prone to taxonomic confusion.

The heath-leaved cypress, as commonly known, is a juvenile form that lacks the more distinctive adult leaves (see Fig. 1). It is a desirable garden plant with a compact growth habit and heath-like leaves that turn purple in winter. The plant has been widely cultivated in the United States and Europe for >150 years. According to Miller and Meyer (1989), it was first offered in the United States by Fruitland Nursery of Augusta, Ga., in their 1857–58 catalog as *Cupressus ericoides*. Since then, the heath-leaved cypress has been assigned to different genera of conifers, including *Chamaecyparis* Spach, *Cupressus* L., *Juniperus* L., *Callitris* Vent., and *Retinospora* Siebold & Zucc. (Miller and Meyer, 1989). The latter was established to accommodate juvenile-foliaged conifers that are derived from different or even unknown sources of parentage and is no longer recognized (Hornibrook, 1938; Dirr, 1998). Miller and Meyer (1989) compared wood anatomy of the heath-leaved cypress with that of other coniferous genera, in particular with species of *Chamaecyparis* and *Thuja* L. They concluded that the heath-leaved cypress shared wood anatomy with *Chamaecyparis thyoides* (L.) Britton, Stearns & Poggenb. and thus should be identified as *Chamaecyparis thyoides* 'Ericoides'.

In recent years, molecular techniques (e.g., DNA fragment analyses and sequencing) have been increasingly used in botanical and horticultural taxonomy. For example, Li et al. (2002) used sequences of internal transcribed spacers (ITS) of nuclear ribosomal (nr) DNA to examine the species identity of weeping katsuras trees, successfully unraveling a decade-long conundrum (Dosmann, 1999). Sequences of this DNA region have been used routinely in phylogenetic studies at the species level for various plant groups, including conifers (Del Tredici and Li, 2002; Li et al., 2001a, 2001b; Liston et al., 1999). The objective of this study was to identify the genetic affinity of the heath-leaved cypress using sequences of nrDNA ITS region.

Materials and Methods

In total, 16 plants were sampled in this study (Table 1). Three accessions of the heath-leaved cypress were obtained from the Lyle E. Littlefield Ornamental Trial Garden of the University of Maine, which were originally acquired from Michael A. Dirr's Ornamental Plant Improvement Program at the University of Georgia. To evaluate the validity of previous taxonomic treatments we sampled species from each of the relevant genera, including *Cupressus*, *Fokienia*, *Juniperus*, and *Thuja*. We did not include *Callitris* since recent phylogenetic analyses have suggested its distant relationship with Northern Hemisphere genera of Cupressaceae (Gadek et al., 2000). For *Chamaecyparis*, which is the most probable genus from which the heath-leaved cypress was derived, five species were examined.

DNA was extracted from silica gel-dried or fresh leaves of these accessions using either Qiagen DNeasy Plant Mini Kit (Santa Clarita, CA) or CTAB method (Doyle and Doyle, 1987). The nrDNA ITS region was amplified using primers ITSLeu (Baum et al., 1998) and ITS4 (White et al., 1990) in Perkin Elmer thermocyclers. PCR products were then purified using the Qiagen PCR purification kit according to the manufacturer's instructions. Sequencing reactions were carried out for PCR products using ABI BigDye terminator Cycle Sequencing Ready Reaction Kit (Foster City, Calif.) following the manufacturer's protocols. Sequences were analyzed using an ABI Genetic Analyzer 3100 in the Biological Laboratories of Harvard University, and edited using the computer software Sequencher (version 3.0, Gene Codes Corp., Ann Arbor, Mich.). Sequences were aligned using Clustal X (Jeanmougin et al., 1998) and adjusted slightly by eye in MacClade 4.0 (Maddison and Maddison, 2000). Delimitations of sequences were determined by comparing published sequences in GenBank (U60750). Gaps were treated as missing data; characters were equally weighted and their states were unordered. Parsimony analyses were conducted in PAUP* (version 4.0b10, Swofford, 2002) using the branch-and-bound tree search and furthest sequence addition. To evaluate the support for individual clades, bootstrap analyses were conducted for 1000 replicates using the same tree search options as above (Felsenstein, 1985).

Results

Sequences of nrDNA ITS ranged from 1000 to 1112 base pairs (bp) in length, and the alignment generated a data set of 1176 characters, 380 of which were parsimony informative. Sequence divergences ranged from 0.02 to 0.12 within genera and from 0.14 to 0.3 between genera (Table 1). Sequences of the three accessions of 'Ericoides' were almost identical to those *of C. thyoides*, but diverged greatly from those of other genera, including *Cupressus*, *Fokienia*, *Juniperus*, and *Thuja*. Parsimony analyses generated six trees of 791 steps and Figure 1 is the strict consensus tree [consistency index (CI) = 0.79, retention index (RI) =0.86]. Species of *Chamaecyparis* formed a well-supported clade (bootstrap = 97%). Three accessions of 'Ericoides' were clustered together with *C. thyoides* (bootstrap = 100%) but were separated from all of the other genera, each of which formed their own clades (Fig. 1).

Discussion

Sequences of nrDNA ITS region show great divergence between and among genera (Table 1), and species of each genus form their own clades in the ITS phylogeny (Fig. 1), supporting the naturalness of these genera. *Cupressus nootkatensis* D. Don which has been treated as a species of *Chamaecyparis*, has a closer relationship with *Cupressus* than *Chamaecyparis*. Our results are consistent with recent phylogenetic studies of Cupressaceae based

Received for publication 6 June 2003. Accepted for publication 17 Nov. 2003. We thank Shiying Hwang for providing leaf material of *Chamaecyparis formosensis* for this study, and Michael Dosmann, Michael Dirr, and two anonymous reviewers for constructive comments on the manuscript.
[1]To whom reprint request should be addressed; e-mail jli@oeb.harvard.edu.

Table 1. Sequence divergence of nrDNA ITS region in Cupressaceae, calculated using Jukes-Cantor method in PAUP*. Vouchers are deposited in A. UME = University of Maine, AA = Arnold Arboretum.

Taxon and JLI DNA bank no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Genbank no.	Source or voucher
1 'Ericoides' 3139	---																AY283428	UME JLI3139
2 'Ericoides' 3125	0	---															AY283429	UME JLI3125
3 'Ericoides' 3147	0	0	---														AY283430	UME JLI3147
4 *Chamaecyparis thyoides* 2709	0	0	0	---													AY211248	AA 13047A
5 *Chamaecyparis thyoides* 2710	0	0	0	0	---												AY211249	AA 13047C
6 *Chamaecyparis obtusa* 2701	0.12	0.12	0.12	0.12	0.12	---											AY211252	AA 833-69B
7 *Chamaecyparis lawsoniana* 2702	0.12	0.12	0.12	0.12	0.12	0.09	---										AY211253	AA 1164-71B
8 *Chamaecyparis pisifera* 2708	0.1	0.11	0.1	0.1	0.1	0.08	0.07	---									AY211255	AA 1067-38A
9 *Chamaecyparis formosensis* 2751	0.11	0.11	0.11	0.11	0.11	0.1	0.08	0.05	---								AY211257	Taiwan JLI2751
10 *Thuja occidentalis* 2754	0.25	0.25	0.25	0.25	0.24	0.24	0.23	0.24	0.24	---							AY283431	AA 1594-80B
11 *Thuja koraiensis* 2728	0.26	0.26	0.26	0.26	0.26	0.25	0.24	0.25	0.25	0.02	---						AY283432	AA 412-94B
12 *Cupressus arizonica*	0.26	0.26	0.26	0.26	0.25	0.25	0.26	0.26	0.26	0.29	0.3	---					U77962	Genbank
13 *Cupressus nootkatensis* 2704	0.25	0.25	0.25	0.25	0.25	0.26	0.27	0.26	0.26	0.28	0.28	0.02	---				AY283433	AA 935-62A
14 *Juniperus virginiana* 2717	0.26	0.26	0.26	0.26	0.26	0.26	0.27	0.27	0.27	0.3	0.3	0.06	0.04	-			AY283434	AA 14886A
15 *Juniperus communis* 2713	0.24	0.24	0.24	0.24	0.24	0.25	0.26	0.25	0.25	0.29	0.3	0.06	0.04	0.03	---		AY283435	AA 4176-2A
16 *Fokienia hodginsii* 2730	0.18	0.19	0.18	0.18	0.18	0.16	0.16	0.14	0.15	0.27	0.28	0.24	0.23	0.25	0.23	---	AY211259	AA 1562-80A

on chloroplast DNA sequences (Gadek et al., 2000).

Miller and Meyer (1989) studied wood anatomy of the heath-leaved cypress, and compared its wood attributes with those of relevant coniferous genera. As with *C. thyoides*, this cultivar does not have resin canals, spiral thickenings, ray tracheids, indentures, or nodular end walls. The heath-leaved cypress has a single row of bordered pits on the axial tracheids, cupressoid cross-field pitting, and smooth end walls of the ray parenchyma, as does *C. thyoides* (Phillips, 1948; Kukachka, 1960). This unique combination of characters of wood anatomy in the heath-leaved cypress led Miller and Meyer (1989) to the conclusion that this cultivar was from a juvenile form of *C. thyoides*. In the ITS tree, the three accessions of heath-leaved cypress are clustered as a clade with *C. thyoides* (Fig. 1), and their sequences are almost identical (Table 1). Therefore, our sequence data unequivocally support Miller and Meyer's identification of the heath-leaved cypress as *C. thyoides*.

As admitted by Miller and Meyer (1989), "These features [taxodioid cross-field pitting, indentures, and nodular end walls on the axial parenchyma] separate *Thuja* from the cultivar 'Ericoides', but sometimes the features are not easily discerned." In the ITS tree (Fig. 1), species of *Thuja* and *Chamaecyparis* each form their own clades, showing clear separation. Sequence divergence between *Thuja* and *Chamaecyparis* is >0.2. Compared with wood anatomy, DNA sequence data appear less ambiguous. Nevertheless, the congruent results from these two independent data sets indicate that microscopic morphological and anatomical characters are of significance in reflecting genetic affinity of species within Cupressaceae.

The development of molecular systematics has improved the understanding of phylogenetic relationships of plant groups at different taxonomic levels. Many genetic markers have been developed and proven to be useful at the species level (Baldwin et al., 1995; Taberlet et al., 1991). Results from this study further show that DNA sequence data can be useful in identifying cultivars that are morphological dissimilar to the original species. Nevertheless, only when a complete database containing all known species of a specific genus is obtained can the full use of DNA data be made to determine the identity of cultivars. Therefore, collaborative efforts should be undertaken to obtain sequences of appropriate markers for horticulturally important plant genera.

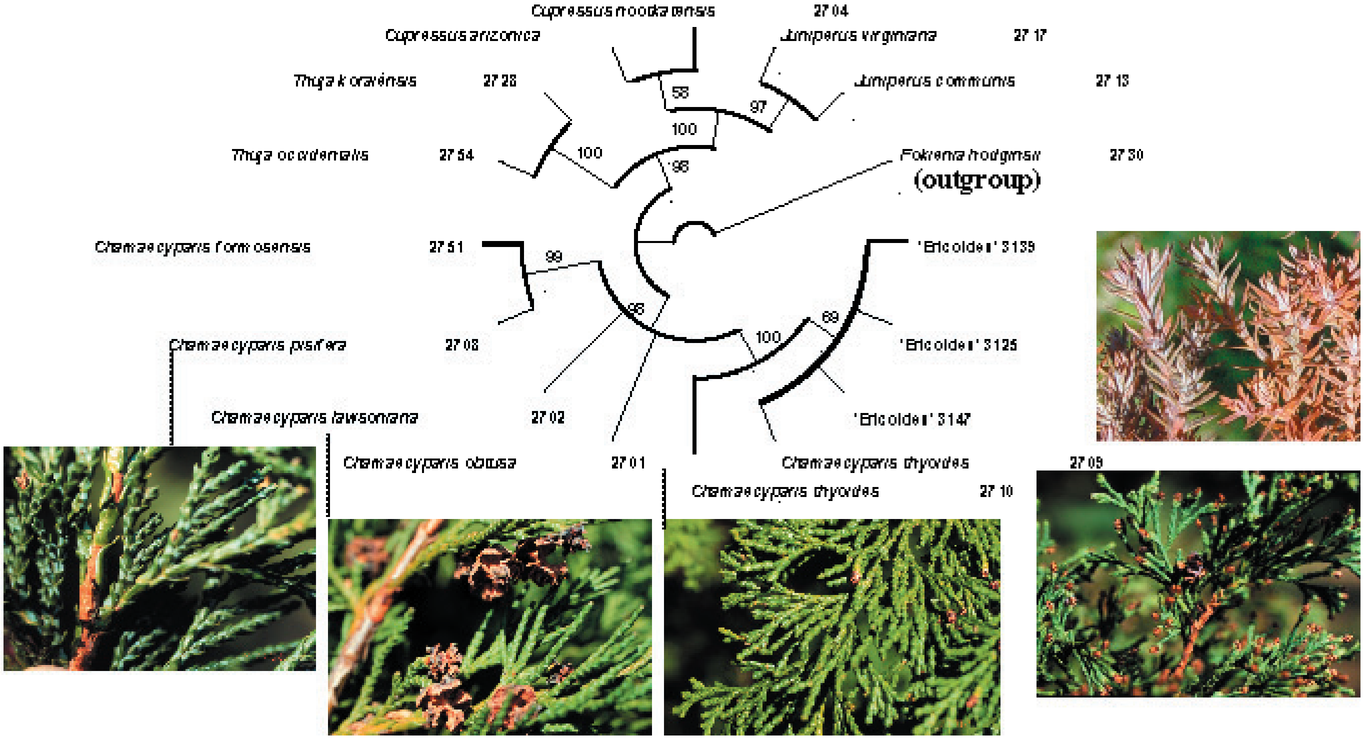

Fig. 1. Strict consensus of six trees of 791 steps based on nrDNA ITS data set (CI = 0.79, RI = 0.86). Numbers at branches are bootstrap percentages, and those following each sample name refer to JLI's DNA bank accession numbers.

Literature Cited

Baldwin, B.G., M.J. Sanderson, J.M. Porter, M.F. Wojciechowski, C.S. Campbell, and M.J. Donoghue. 1995. The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylgoeny. Ann. Missouri Bot. Gard. 82:247–277.

Baum, D. A., R. L. Small, and J. F. Wendel. 1998. Biogeography and floral evolution of Baobabs (*Adansonia*, Bombacaceae) as inferred from multiple data sets. Syst. Biol. 47:181–207.

Del Tredici, P. and J. Li. 2002. *Stewartia* × 'Scarlet Sentinel'. HortScience 37:412–414.

Dirr, M. 1998. Manual of woody landscape plants. 5th ed. Stipes Publ., Champaign, Ill.

Dosmann, M.S. 1999. Katsura: A review of *Cercidiphyllum* in cultivation and in the wild. New Plantsman 6:52–62.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bul. Bot. Soc. Amer. 19:11–15.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

Gadek, P.A., D.L. Alpers, M.M. Heslewood, and C.J. Quinn. 2000. Relationships within Cupressaceae *sensu lato*: A combined morphological and molecular approach. Amer. J. Bot. 87: 1044–1057.

Hornibrook, M. 1938. Dwarf and slow-growing conifers. 2nd ed. Charles Scribner's Sons, New York.

Jeanmougin, F., J.D. Thompson, M.M. Gouy, D.G. Higgins, and T.J. Gibson. 1998. Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23:203–205.

Kukachka, B.F. 1960. Identification of coniferous woods. Tappi 43:887–896.

Li, J., C.C. Davis, P. Del Tredici, and M.J. Donoghue. 2001a. Phylogeny and biogeography of *Taxus* (Taxaceae) inferred from sequences of the internal transcribed spacer region of nuclear ribosomal DNA. Harvard Pap. Bot. 6:267–274.

Li, J., C.C. Davis, S. Kelley, P. Del Tredici, and M.J. Donoghue. 2001b. Phylogenetic relationships of *Torreya* (Taxaceae) inferred from sequences of nuclear ribosomal ITS region. Harvard Pap. Bot. 6:275–281.

Li, J., M.S. Dosmann, P. Del Tredici, and S. Andryns. 2002. Systematic relationship of weeping katsura based on nuclear ribosomal DNA sequences. HortScience 37:595–598.

Liston, A., W. A. Robinson, D. Pinero, and E. R. Alvarez-Bullya. 1999. Phylogenetics of *Pinus* (Pinaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. Mol. Phyl. Evol. 11:95–109.

Maddison, D.R. and W.P. Maddison. 2000. MacClade 4: Analysis of phylogeny and character evolution. Version 4.0. Sinauer Assoc., Sunderland, Mass.

Miller, R.B. and F.G. Meyer. 1989. Identification of the heath-leaved Cypress, *Chamaecyparis thyoides* 'Ericoides' (Cupressaceae). Baileya 23:57–67.

Phillips, E.W.J. 1948. The identification of coniferous woods by their microscopic structure. For. Products Res. Bul. 22:1–56.

Swofford, D.L. 2002. PAUP*, Phylogenetic Analysis Using Parsimony (and other methods). Version 4.0 b10. Sinauer Associates, Sunderland, MA.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17:1105–1109.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. In: M. Innis, D. Gelfand, J. Sninsky, and T. White (eds.). PCR protocols: A guide to methods and applications. Academic Press, San Diego.