HOW CLOSELY RELATED TURKISH AND NORTH AMERICAN RED BUDS (*CERCIS*-FABACEAE) ARE: EVIDENCE FROM INTERNAL TRANSCRIBED SPACER REGION OF NUCLEAR RIBOSOMAL DNA SEQUENCES

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Keywords: *Cercis*, phylogeny, red bud, ITS nrDNA, DNA sequencing.

ÖZET

Yaygın bir bahçe bitkisi olarak yetiştirilen erguvan, *Cercis*, cinsinin Kuzey Amerika'da, Çin'de, ve Avrasya'da yaygın olan türleri üzerinde bitkilerin ITS ribozomal çekirdek DNA'larının organik baz dizileri kullanılarak, filogenetik sistematik analizi yapılmıştır. ITS marker'ının analizi sonucunda beklenilenden farklı olarak Avrasya'da yaygın olan erguvan ile Kuzey Amerika'daki erguvan türleri arasında çok yakın bir akrabalık olduğu bulunmuştur. Diğer taraftan Çin erguvanının ise Avrasya erguvanına yakın olması beklenirken daha uzak bir akraba olduğu belirlenmiştir. Ayrıca bu çalışmanın verileri Kuzey Amerika'daki yabani erguvan bitkilerinin aynı türün (*Cercis canadensis*) varyeteleri olduğu yönündeki hipotezi desteklemektedir.

Anahtar Kelimeler: *Cercis*, filogenetik, erguvan, ITS ribozomal çekirdek DNAsi, DNA Organik Baz Dizilişi.

ABSTRACT

A phylogenetic systematic analysis using ITS nuclear ribosomal DNA sequences of the plant taxa of the genus *Cercis*, a commonly cultivated ornamental distributed in North America, China, and Eurasia, was performed. Results of the analysis of the ITS marker yielded that Eurasian red bud is closely related with the North American red bud, an unexpected finding. On the other hand, Chinese red bud is found to be remotely related with the Eurasian red bud contrary to the expectation that they should be closely related. Furthermore, this study supports the hypothesis that the North American red buds are varieties of one species, *Cercis canadensis*.

1. INTRODUCTION

The genus *Cercis* (red bud) belongs to subfamily Caesalpinioideae of the large plant family Fabaceae (Leguminosae-pea family). *Cercis* is grown as an ornamental and cultivated widely in Northern Temperate regions of the world (specifically in North America). The bright to reddish pink color of the flowers are especially attractive in early spring in the North American gardens. Cauliflory, the production of flowers on the stem or trunk, is an important character that gives a more or less unique view to the members of this genus in early spring. Flowers open before the growth of the leaves and their color range from lavender-pink to white in different plants. *Cercis* L., consists of about 10 species of shrubs

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or small trees widely scattered across the north temperate zones of Eurasia and North America.

Isely (1) and Wunderlin, Larsen, and Larsen (2) emphasized that *Cercis* species appear to have become adapted to climatic alterations by changing their leaf morphology. The leaf blades of these plants reflect the environmental conditions and habitat of these plants. Most Chinese species and the Canadian variety of the eastern red bud (*C. canadensis* var. *canadensis*) are thin-leaved and dull on the upper surface, indicating that they live in a humid and probably cloudy environment. Nevertheless, Texan (*C. canadensis* var. *texensis*) and Mexican (*C. canadensis* var. *mexicana*) varieties of eastern red bud, the western red *bud (C. occidentalis*), the Mediterranean red bud (*C. siliquastrum*), and the central Asian red bud *(C. griffithii*) have thick-leathery, often pubescent or glossy and sometimes glaucous on the upper surface of the leaves suggesting adaptation to arid or semi-arid environments (3, 2, and 4).

Systematics of the Fabaceae has been studied in detail, and a great majority of the publications has been accumulated in the volumes of "Advances in Legume Systematics" by various authors along with many articles published in journals between 1981 and 1995. Subjects published in those volumes have ranged from morphology, paleobotany, and chemistry to cytogenetics. Volume 7, edited by Crisp and Doyle (5) is of a special importance among the others of these voluminous works because it dealt with the phylogeny of legumes and included relationships between *Cercis* and other genera (i.e., placing *Bauhinia* as the closest relative of *Cercis*).

Li (6) worked on the taxonomy and distribution of the genus *Cercis* in China and constructed a key to the species of the genus based on morphology and geographic distribution. He emphasized that eastern Asia was possibly a center of development for the genus *Cercis* based on geographic range of the genus and number of species present in Asia. Li (6) based his implication on recent descriptions of the *Cercis* species from China. Isely (1) constructed a key to the species of *Cercis* distributed in the United States and gave special references to the other species of this genus distributed in Eurasia known at that time. Although Isely (1) constructed keys to the species of *Cercis*, he did not attempt to classify them into subgenera and/or sections. He also based his key on morphological and geographical data.

2. MATERIAL AND METHODS

2.1. Plant Materials

Taxon sampling and outgroup selection

I was able to obtain five *Cercis* taxa from different geographic regions of the world in which this genus is present (Figure 1 and Appendix 1). Included were three North American *Cercis* species with three varieties accepted widely in the literature: *C. canadensis* var. *canadensis*, *C. canadensis* var. *texensis*, *C. canadensis* var. *mexicana*, and one species from Eurasia (*C. siliquastrum*), and the last one, *C. chinensis*, from China.

Cercis plant materials collected and used in this study were vouchered as herbarium specimens and were deposited in the Herbarium of the University of North Carolina (NCU) (Appendix 1). Based on Bentham (7), Polhill, Raven, and Stirton (8), and Wunderlin and

Larsen (2), one *Bauhinia* species, *B. faberi*, was sampled as an outgroup that is closely related to *Cercis.*

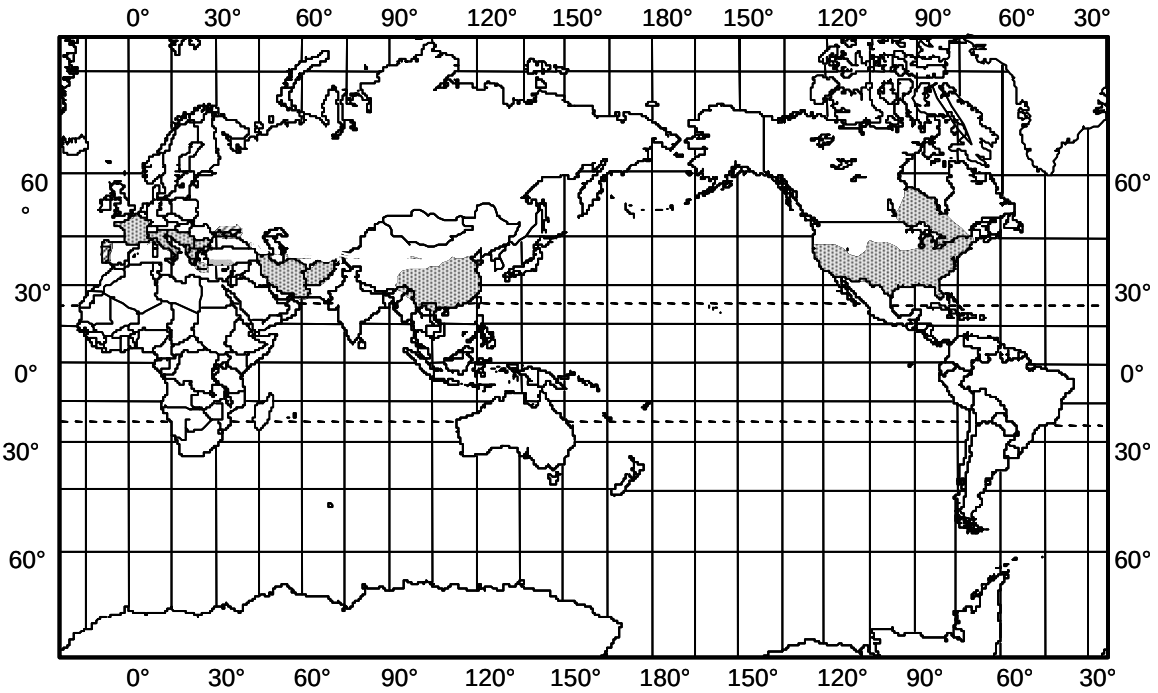

Figure 1. Worldwide distribution map of *Cercis* taxa.

2.2 Methods

Total genomic DNA was initially extracted with a modified version of the 'hot' CTAB method outlined in Doyle and Doyle (9) for all plants included in this work. Either 2 g fresh or 0.5 g silica gel-dried leaf tissue was ground in liquid nitrogen, and added to 20 mL hot (65^0C) 2x CTAB buffer as described in Doyle and Doyle (9). Then the mixture was incubated in 65^0C for ten minutes, and extracted with 24/1 ratio of chloroform/isoamyl alcohol, respectively. The DNA was then precipitated with 2/3 volume isopropyl alcohol at -20^0C overnight. DNA extracts were suspended in 500 to 1000 µL of sterile distilled, deionized water (ddH_2O), and stored at -20^0C. Later, Qiagen company's DNeasy Plant Mini Kit was used to extract plant genomic DNAs following the manufacturer's protocol.

Molecular markers analyzed in this study included ITS nuclear ribosomal DNA (nrDNA) (see Figure 2) for all taxa. Polymerase Chain Reaction (PCR) amplifications of ITS region of nuclear ribosomal DNA (nrDNA) were performed using primer pairs ITS5angiosperm (ITS5a, designed by Kenneth Wurdack) and ITS4 White et al. (10) for all taxa included in this work (see Appendix 2).

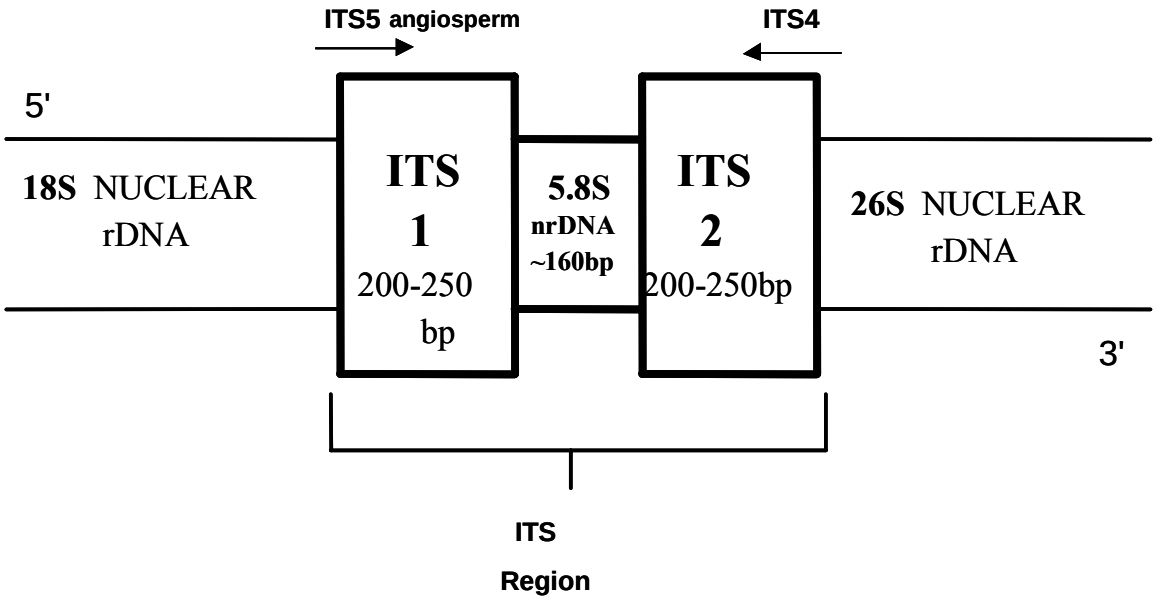

Figure 2. Diagrammatic representation of the Internal Transcribed Spacer (ITS) region of 18S-26S nuclear ribosomal DNA (nrDNA) showing the primer positions and their directions included in this work. Using the entire ITS region, including the 5.8S rDNA sequences, the phylogenetic analyses were performed for this work.

Double stranded DNA amplifications were performed in 35 μL volume containing 28 μL sterile deionized, distilled water, 3.5 μL 10x Taq DNA polymerase PCR buffer (GibcoBRL, Life Technologies or Qiagen companies), 1.05 μL $MgCl_2$ GibcoBRL (Life Technologies or sometimes used 'Q solution' which includes $MgCl_2$, by Qiagen), 0.7 μL 200 μM dNTPs in equimolar ratio (either by Qiagen or GibcoBRL), 2 μL of each 10 μM primer, 0.175 μL Taq DNA polymerase enzyme (either Qiagen or GibcoBRL). For some amplifications of the GC-rich DNA templates, 0.5 to 3 μL 10% Bovine Serum Albumine (BSA) and/or DiMethylSulfOxide (DMSO) were added to the total reaction volume depending on the experience of initial trials of the PCR amplifications. During amplification of ITS nrDNA region, the following PCR amplification protocols were performed in the thermal cycler machine (Perkin-Elmer Applied Biosystems, Inc. model 377): the first cycle was at 95^0 C for 1 minute and 15 seconds for denaturation of double stranded DNA. The following 30 more cycles were performed using 1 minute at 94^0 C for more denaturation time, 1 minute at 55^0 C for annealing, and 2 minutes and 30 seconds for primer extension; an additional 8 minutes of extension time was the final cycle. In order to check whether PCR Master Mix was contaminated with any DNA or not, negative controls were used in all PCR amplifications. In order to judge the fact that optimum PCR amplification conditions were provided, positive controls were also included in most sets of amplifications.

PCR products were purified using 'Qiaquick PCR purification Kit' (Qiagen) and followed the instructions directed by the company. Both strands of DNAs were sequenced

for all taxa and the sequences were generated from two or three different individuals for each taxon.

Initially, cycle sequencing reactions were performed at Parks Lab., (in the Department of Biology at UNC-Chapel Hill, N.C., U.S.A.) using Perkin-Elmer Applied Biosystems, Inc. according to manufacturer's protocols (i.e., Cycle sequencing 1: at 96^0C for 4 min.; Cycle sequencing 2: at 96^0C for 30 sec., at 50^0C for 15 sec., and at 60^0C for 4 min. in total of 30 cycles). Then cycle-sequenced products were cleaned by using Sephadex columns and vacuum dried and mailed to Iowa State University's DNA Sequencing Facility for final automated sequencer-generated data collection. Later, purified PCR products were sent to UNC-Chapel Hill DNA Sequencing Facility for cycle sequencing reactions and automated sequencer-generated data collection. Sequence data generated through automated methods were manually edited for each DNA marker for each taxon using the commercial software Sequencher version 3.1 for Macintosh computers, 1998 (Gene Codes Corporation) and assembled into consensus sequences (contigs).

2.3. Data Analysis

The ITS region of nrDNA (Figure 2) consensus sequences were first aligned using the software "MultAlin" by Corpet (11), available free on Internet at the address below: http://prodes.toulouse.inra.fr/multalin/multalin.html. Then they were visually checked and manually edited, if necessary.

The data analysis followed using PAUP* Version 4.0b8 for Macintosh (PPC), (Phylogenetic Analysis Using Parsimony and Other Methods) by Swofford (12). Pairwise distances using Jukes-Cantor model as estimator were generated using PAUP* software. A complete aligned data matrix of *Cercis* ITS nrDNA region can be seen in Appendix 4. All informative base-pair differences were used in the analysis, and gaps were coded as missing data.

Exhaustive searches were executed to find the most parsimonious ITS nrDNA trees of *Cercis*. Exhaustive searches followed keeping 'minimal trees only' with 'collapse' option in effect and saving all trees. Branch-and-Bound search computed via "stepwise addition sequence" using "furthest" option, keeping minimal trees only, and saving all trees. Heuristic search for Bootstrap Analyses used stepwise addition with "simple" addition sequence, 'swapping on best trees only' option, and employing the 'Tree Bisection-Reconnection (TBR)' algorithm for branch swapping. Parsimony analyses included following search options: General search options with collapsing branches if maximum length is zero. Character state optimization followed Accelerated transformation (ACCTRAN). Stepmatrix options utilized allowing assignment of states not observed in terminal taxa to internal nodes using all states in stepmatrix. Multistate taxa were interpreted as "uncertainty" and gaps were treated as "missing data".

During the analyses, several statistical measures were utilized including: bootstrap (13) with 1000 replicates; consistency indices (14); retention indices (15), homoplasy indices (14), and Hillis and Huelsenbeck's g1 statistic (16) which was obtained by generating 1,000,000 random trees using the PAUP* 4.0b10 (2002).

3. RESULTS

The total length of the aligned ITS sequence matrix was 715 nucleotides. There were a total of 114 variable characters of which 8 were parsimony informative and 593 characters were constant. Analyses of the ITS region of nrDNA showed pairwise DNA sequence differences ranging from 0.724 % between *C. canadensis* var. *canadensis* and *C. canadensis* var. *texensis* to 0.868% between *C. canadensis* var. *canadensis* and *C. siliquastrum* (see Appendix 2). On the other hand, the highest DNA sequence difference was observed between *C. canadensis* var. *mexicana* and *C. chinensis* (2.49%). A complete and aligned data matrix of ITS nrDNA region of *Cercis* taxa can be seen in Appendix 4. The Exhaustive search of the ITS nrDNA data generated five equally most parsimonious (MP) phylogenetic trees with a 0.984 consistency index (CI) value including uninformative characters (Figure 3). CI excluding uninformative characters was 0.818. The resulting tree length of these five equally MP trees was 126 steps.

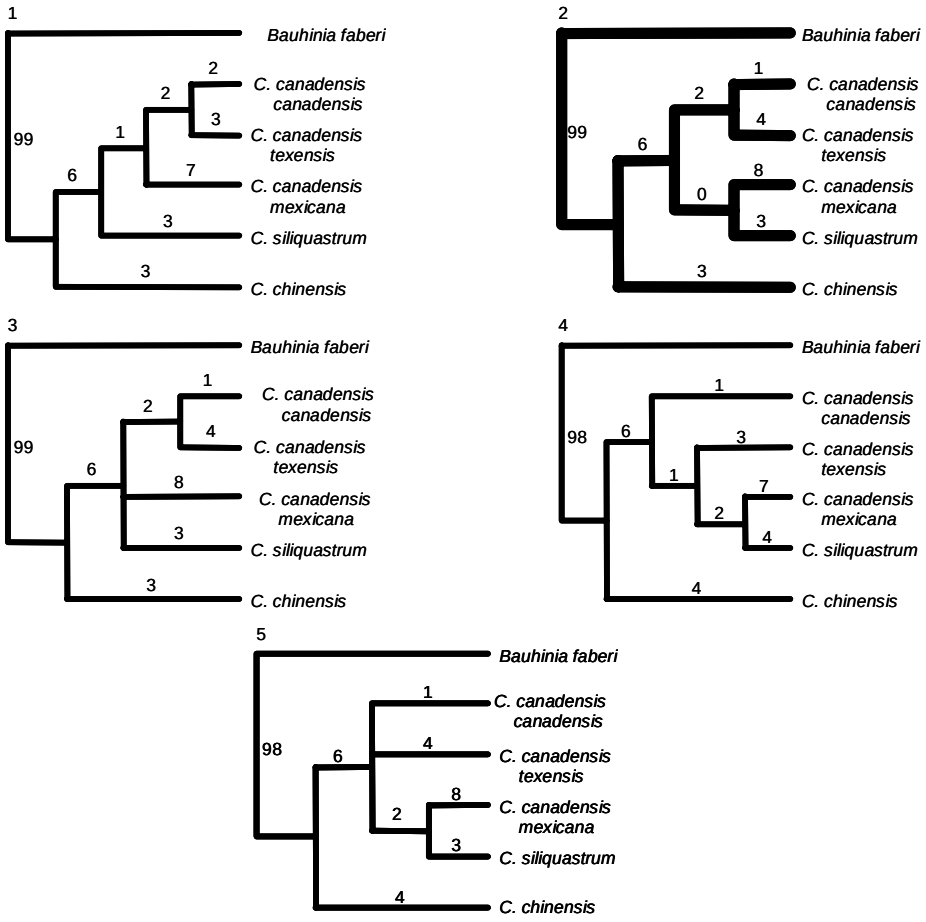

Figure 3. Five equally most parsimonious ITS trees following an exhaustive search with branch lengths shown above branches.

Figure 4 shows strict consensus of these five equally MP trees and one of these MP trees with Bootstrap statistical supports and other statistical indices highlighted.

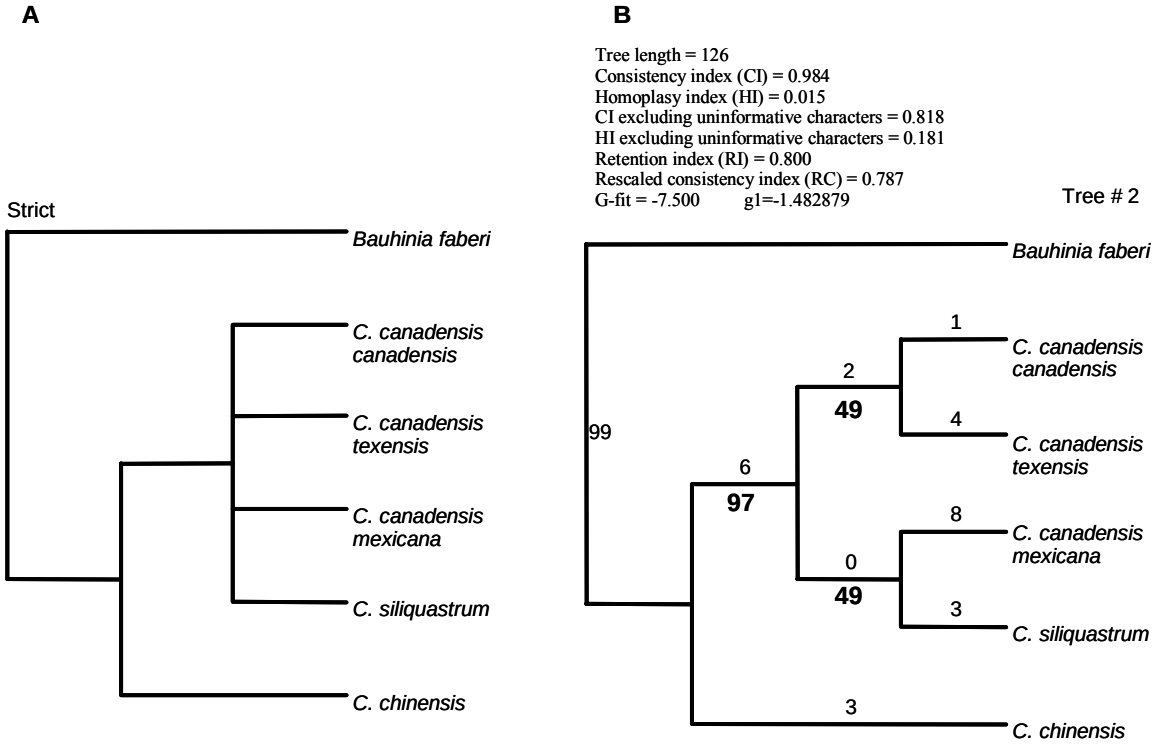

Figure 4. A. Strict consensus of 5 equally most parsimonious trees. **B.** Tree #2 of the five equally most parsimonious ITS trees of *Cercis* taxa following an Exhaustive Search. Branch lengths are shown above the branches and Bootstrap values below the branches in bold.

The analysis of the ITS sequences supported the genus *Cercis* as a monophyletic sister group to *Bauhinia* (see Figures 3, 4B, and Appendix 3) and related the Eurasian red bud, *C. siliquastrum*, and Mexican red bud, *C. canadensis* var. *mexicana*, each other. Canadian and texan red buds were found to be sister taxa (*C. canadensis* var. *canadensis* and *C. canadensis* var. *texensis*, respectively). The branch yielding the common ancestor of North American and Eurasian red buds received very high Bootstrap support (Figure 4B). Canadian and Texan red buds formed a moderately supported monophyletic group (49% bootstrap). On the other hand, the clade containing Mexican and Eurasian red buds received moderate support (49% bootstrap).

4. DISCUSSION AND CONCLUSION

Analyses of the data set indicated that ITS region of nrDNA could be used as a molecular marker to estimate the *Cercis* phylogeny (Figure 3). It resolved well for most of

the relationships among the *Cercis* taxa. In all analyses, *C. chinensis* is sister to the rest of *Cercis*. A well-supported clade consisting of all varieties of *C. canadensis* showed close affinity with *C. siliquastrum*. In particular, the Mexican variety, *C. canadensis* var. *mexicana*, was found closely related with the Eurasian red bud, *C. siliquastrum*, on one hand (49% bootstrap support) and Canadian and Texan varieties of *C. canadensis* formed another clade on the other (49% bootstrap support, see Figure 4B). Although one of the five equally MP trees displayed common ancestry between *C. siliquastrum* and *C. canadensis* var. *canadensis* and another tree showed close affinity between *C. siliquastrum* and *C. canadensis* var. *texensis* (Figure 3), none of them received bootstrap support (Figure 4B). These results suggest that all *Cercis* taxa from North America are closely related and have shared a common ancestor along with the Eurasian red bud, *C. siliquastrum*. Thus ITS data suggests the recognition of the North American *Cercis* taxa as varieties of one species, *C. canadensis*. McVaugh (17) suggested the same argument as well. The data from this analysis, at least, indicates that *Cercis* taxa collected from Canada and Texas be considered as varieties of the same species, *C. canadensis* (Figures 3 and 4, and Appendix 3). Otherwise, all the plants collected from North America should be considered as varieties of the same species. However, this conclusion may require more data to support the argument at this point.

Anderson (18) has proposed that dispersed introgression between the varieties of *C. canadensis* took place extending from northeastern Texas to southwestern Illinois. Ballenger (19) has also discussed the presence of intermediate characters between all the varieties of *C. canadensis* collected from Sierra Madre Oriental region of Mexico. Davis et al. (4) collected and analyzed *Cercis canadensis* taxa from the same area in Mexico and concluded that all accessions of *Cercis canadensis* from their analysis had grouped together. All accessions of North American *Cercis* taxa for my work also group together indicating that there was no introgression amongst the North American *Cercis* taxa. Hence there is no evidence of introgression by this analysis as well. More data employing more taxa and characters are needed to further confirm the possibility of introgression between the varieties of North American *Cercis* taxa.

Based on the phylogenetic data analysis, it appears that *C. canadensis* var. *canadensis*, *C. canadensis* var. *texensis*, *C. canadensis* var. *mexicana*, and *C. siliquastrum* have shared the same common ancestor supported very highly (97%) by bootstrap analysis (Figure 4B). Data from this analysis suggests that ancestor of *Cercis* species appeared first in Asia since the basal-most taxon in the phylogenetic tree is *C. chinensis* within ingroup taxa (Figures 3 and 4). However, Polhill, Raven, and Stirton (8) and Crisp and Doyle (5) argued that *Bauhinia* was more modern than *Cercis* (diverged from *Cercis* later), based on morphological data. If that is true, then *C. chinensis* would be younger than rest of the *Cercis* taxa based on our molecular data since Chinese red bud showed sister relationship with *Bauhinia* in the unrooted trees following the Exhaustive search in this analysis (Figure not shown). This assumption leaves the varieties of *C. canadensis* and *C. siliquastrum* as older ones, and *C. chinensis* as being the younger divergent taxon. Thus it may also be postulated that first the common ancestor of *C. canadensis* and *C. siliquastrum* might have appeared then *C. chinensis* and *Bauhinia* were derived from that common ancestor.

Davis et al. (4) have worked on the phylogeny of *Cercis* and found similar but not necessarily the same results by my analysis. The differences between the two analyses are

likely to be arising from using different number of sampled taxa and using different forward primer of the same DNA marker, ITS. The data from my analysis used longer DNA sequences, but less number of taxa than Davis et. al. (4). The basal status of *C. chinensis* and a close affinity between *C. siliquastrum* and varieties of *C. canadensis* have been attained by the results of both analyses. Same unresolved portions of the phylogenetic tree in the strict consensus of equally MP trees were also found in both analyses.

In conclusion, sequence divergences between the North American *Cercis* taxa and Eurasian *Cercis* taxon, *C. siliquastrum*, are relatively low in comparison with the divergence either between the North American *Cercis* taxa and eastern Asian *Cercis* taxon, *C. chinensis*, or between Eurasian *Cercis* taxon and eastern Asian *Cercis* taxon (Appendix 3). This result indicates that Eurasian *Cercis* taxon and North American *Cercis* taxa have shared the same common ancestory.

5. REFERENCES

[1] . Isely, D., "Leguminosae of the United States: I. Subfamily Caesalpinioidea". Mem. N. Y. Bot. Gard., 25(2): 1-228, (1975).

[2] . Wunderlin, R.P., K.Larsen, and S.S.Larsen, Tribe 3. CERCIDEAE, "**Advances in Legume Systematics**", Part 1, Polhill, R. M. and P. H. Raven, Editors. Royal Botanic Gardens, Kew, England (1981).

[3] . Raven, P. H., The relationships between 'Mediterranean Floras", "**Plant life of south-west Asia**", P. H. Davis, P. C. Harper, and I. C. Hedge, Editors. Aberdeen: Botanical Society of Edinburgh, s: 119-134 (1971).

[4] . Davis, C. C., P. W. Fritsch, J. Li, and M. J. Donoghue, "Phylogeny and biogeography of Cercis (Fabaceae): Evidence from nuclear ribosomal ITS and chloroplast *ndh*F sequences" **Systematic Botany**, 27(2): 289-302, (2002).

[5] . Crisp, M. D. and J. J. Doyle, Editors, "**Advances in Legume Systematics**", Part 7: Phylogeny, Royal Botanic Gardens, Kew, England (1995).

[6] . Li, H-L. "Taxonomy and distribution of the genus *Cercis* in China" **Bulletin of the Torrey Botanical Club**, 71(4): 419-425, (1944).

[7] . Bentham, G., "Enumeration of plants collected by Mr. Schomburgk in British Guiana" **Hook. Journ. Bot.**, 2: 38-99, (1840).

[8] . Polhill, R. M., P. H. Raven, and C. H. Stirton, Evolution and systematics of the Leguminosae, "**Advances in legume systematics**", Part 2 R. M. Polhill and P. H. Raven Editors, Royal Botanic Gardens, Kew, England (1981).

[9] . Doyle, J. J., and J. Doyle, "A rapid DNA isolation procedure for small quantities of fresh leaf tissue" **Phytochemical Bulletin**, 19: 11-15, (1987).

[10]. White, T.J., T. Bruns, S. Lee and J. Taylor, Amplifications and direct sequencing of fungal ribosomal RNA genes for phylogenetics, "**PCR protocols: a guide to methods and applications**", M. Innis, D. Gelfand, J. Sninsky, and T. White, Editors, Academic Press, San Diego, California, USA s: 315-322 (1990).

[11] . Corpet, Florence, "Multiple sequence alignment with hierarchical clustering" **Nucl. Acids Res.**, 16 (22): 10881-10890, (1988).

[12] . Swofford, D. L., "**PAUP*. *Phylogenetic Analysis Using Parsimony* (*and Other Methods). Version 4.0b8**" Sinauer Associates, Sunderland, Massachusetts (2001).

[13] . Felsenstein, J., "Confidence limits on phylogenies: An approach using bootstrap" **Evolution**, 39: 783-791, (1985).

[14] . Kluge, A. G. and J. S. Farris., "Quantitative phyletics and the evolution of Anurans" **Systematic Zoology** (currently named as **Systematic Biology**)18: 1-32, (1969).

[15] . Farris, J. S., "The retention index and the rescaled consistency index" **Cladistics**, 5: 417-419, (1989).

[16] . Hillis, D. M., and J. P. Huelsenbeck, "Signal, noise, and reliability in molecular phylogenetic analysis" **Journal of Heredity**, 83: 189-195, (1992).

[17] . McVaugh, R., "Suggested phylogeny of Prunus serotina and other wide-ranging phylads in North America" **Brittonia**, 7 (5): 317-346, (1952).

[18] . Anderson, E. A., "Introgressive hybridization" **Biological Reviews**, 28: 280-307, (1953).

[19]. Ballenger, J. A., "**A biosystematic revision of the genus *Cercis* L. (Leguminosae) in North America**", Unpublished Ph.D. dissertation. Miami University, Miami, Ohio. (1992).

6. APPENDICES

Appendix 1. Voucher information for the taxa used in this study including their worldwide distribution.

Species	Collection #s	Source	Worldwide Distribution
Cercis canadensis var. *canadensis*	FC-032a	J.C. Raulston Arboretum, NC	Canada and Eastern U.S.A.
Cercis canadensis var. *texensis*	FC-033a	J.C. Raulston Arboretum, NC	Eastern United States of America
Cercis canadensis var. *mexicana*	FC-034a	J.C. Raulston Arboretum, NC	Eastern United States and Mexico
Cercis chinensis	FC-035a	J.C. Raulston Arboretum, NC	China
Cercis siliquastrum	FC-042a	J.C. Raulston Arboretum, NC	Europe and south west Turkey
Cercis siliquastrum	FC-055a	Fatih Coskun (Antalya, Turkey)	Europe and south west Turkey
Bauhinia faberi	FC-095a	Clifford R. Parks, C.Hill, NC	

Appendix 2. ITS nrDNA primers used in this study and their designers.

Primer Name	5' to 3' Primer Sequence	Primer Designed by	Based on (the Source Publication)
Forward			
ITS5A (Angiosperm)---	CCTTATCATTTAGAGGAAGGAG	Kenneth J. Wurdack, 1999	White et al.,1990
Reverse			
ITS4----------	TCCTCCGCTTATTGATATGC	Bruce G. Baldwin, 1992	White et al.,1990

Appendix 3. ITS distance matrix showing DNA sequence divergence between taxa using Jukes-Cantor model as estimator.

	1	2	3	4	5	6
1 *Bauhinia faberi*	-					
2 *C. canadensis*	0.17535	-				
3 *C. chinensis*	0.16930	0.01604	-			
4 *C. canadensis* var. *texensis*	0.18064	0.00724	0.02043	-		
5 *C. canadensis* var. *mexicana*	0.18843	0.01603	0.02491	0.01748	-	
6 *C. siliquastrum*	0.17889	0.00868	0.01747	0.01305	0.01604	-

Appendix 4. Complete aligned ITS nrDNA Sequence Data Matrix of *Cercis* Taxa and *Bauhinia faberi*.

```
Taxon/Node                          1                                                                          72
-------------------------------------------------------------------------------------------------------------------
Bauhinia faberi                     AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTCAACAAAA--ACCACCAG
C. canadensis var. canadensis       AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTCACAAACAGCACGACCCG
C. chinensis                        A-TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTCACAAACAGCACGACCCG
C. canadensis var. texensis         AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTCACAAACAGCACGACCCG
C. canadensis var. mexicana         AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTCACAAACAGCACGACCCG
C. siliquastrum                     A-TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGCATCATTGTCGAGACCTCACAAACAGCACGACCCG

Taxon/Node                          73                                                                         144
-------------------------------------------------------------------------------------------------------------------
Bauhinia faberi                     CGAACTTGTTWTTCACACACCGGGGGAGGCGGGGGGTGTCTTCACCCCCGGYCGCCCCCGTATGT--CCTGG
C. canadensis var. Canadensis       CGAATCTGTTT-ATG-ATATTGGGTG--GCGGAGGGCACATTCTGCCCCGAG--CCCCCC-ACGT---CTGG
C. chinensis                        TGAATCTGTTT-ATG-ACATTGGGTG--GCGGAGGGCACATTCTGCCCCGAG--CCCCCC-ACGT---CTGG
C. canadensis var. texensis         CGAATCTGTTT-ATG-ATATTGGGTG--GCGGAGGGCACATTCTGCCCCGAG--CCCCCC-ACGT---CTGG
C. canadensis var. mexicana         CGAATCTGTTT-ATG-ATATTGGGTG--GCAGAGGGCACATTCTGCCCCGAG--CCCCCC-ACGT---CTGG
C. siliquastrum                     CGAATCTGTTT-ATG-ATATTGGGTG--GCGGAGGGCACATTCTGTCCCGAG--CCCCCC-ACGT---CTGG

Taxon/Node                          145                                                                        216
-------------------------------------------------------------------------------------------------------------------
Bauhinia faberi                     CGGGG-ACGCGTCGGGCCAACCCCCCTCGCGTGCTCGTCCGGGCGAACGAACAAACCCACGGCGCCAGACGC
C. canadensis var. canadensis       ATGGG-ACACAGCAGGCC--TTGCCTGTGCGTGCTCGTCCTGGCAAACAACGAAACCC-CGGCGCCAGTCGC
C. chinensis                        ATGGG-ACACAGCAGGCC--TTGCCTGTGCGTGCTCGTCCGGGCAAACAACGAAACCC-CGGCGCCAGTCGC
C. canadensis var. texensis         ATGGG-ACACAGCAGGCC--TTGCCTGTGCGTGCTCGTCCTGGCAAACAACGAAACCC-CGGCGCCAGTCGC
C. canadensis var. mexicana         ATGGG-ACACAGCAGGCC--TTGCCTGTGCGTGCTCGTCCCGGCAAACAACGAAACCC-CGGCGCCAGTCGC
C. siliquastrum                     ATGGG-ACACAGCAGGCC--TTGCCTGTGCGTGCTCGTCCCGGCAAACAACGAAACCC-CGGCGCCAGTCGC

Taxon/Node                          217                                                                        288
-------------------------------------------------------------------------------------------------------------------
Bauhinia faberi                     GCCAAGGAC-TATTAACAACGAGGCACGCCCCCGYCGGCCCGGCAACGGTGACCGCGCGGGAAGCGTCGCAA
C. canadensis var. canadensis       GCCAAGGAA-CTCTAACATAACAGCGTGCCCCTGTCGGCCCGGGAACGGTGCCC-TACAGGGTGCGTCGCGA
C. chinensis                        GCCAAGGAAACTCTAACATAACAGCGTGCCCCTGTCGGCCCGGGAACGGTGCCC-TGCAGGGTGCGTCGCGA
C. canadensis var. texensis         GCCAAGGAA-CTCTAACATAACAGCGTGCCCCTGTCGGCCCGGGAACGGTGCCC-TACAGGGTGCGTCGCGA
C. canadensis var. mexicana         GCCAAGGAA-CTCTAACATAACAGCGTGCCCCTGTCGGCCCGGGAACGGTGCCC-TACAGGGTGTGTCGCGA
C. siliquastrum                     GCCAAGGAA-CTCTAACATAACAGCGTGCCCCTGTCGGCCCGGGAACGGTGCCC-TACAGGGTGCGTCGCGA
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Appendix 4. Complete aligned ITS nrDNA Sequence Data Matrix of *Cercis* Taxa and *Bauhinia faberi* (Continued).

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Taxon/Node                          289                                                                                  360
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Bauhinia faberi                     CGTATTTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
C. canadensis var. canadensis       CATTTGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
C. chinensis                        CATTTGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
C. canadensis var. texensis         CATTTGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
C. canadensis var. mexicana         CATTTGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
C. siliquastrum                     CATTTGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC


Taxon/Node                          361                                                                                  432
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Bauhinia faberi                     GATACTTGGTGTGAATTGCAGAATCCCGTGAATCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTA
C. canadensis var. canadensis       GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTA
C. chinensis                        GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCAAAGCCATTA
C. canadensis var. texensis         GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTA
C. canadensis var. mexicana         GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTA
C. siliquastrum                     GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTA


Taxon/Node                          433                                                                                  504
-------------------------------------------------------------------------------------------------------------------------------
Bauhinia faberi                     GGCCGAGGGCACGTCTGCCTGGGCGTCAAACAACGTTGCCC--------------CCCACACGAATCGTGCG
C. canadensis var. canadensis       GGCCGAGGGCACGTCTGCTTGGGTGTCAAACATCGTTGCCCAAACACAATGTCCTTCTCCGGGCATCATGTG
C. chinensis                        GGCCGAGGGCACGTCTGCCTGGGTGTCAAACATCGTTGCCCAAACACAATGTCCTTCTCCGGGCATCATGTG
C. canadensis var. texensis         GGCCGAGGGCACGTCTGCCTGGGTGTCAAACATCGTTGCCCAAACACAATGTCCTTCTCCGGGCATCATGTG
C. canadensis var. mexicana         GGCCGAGGGCACGTCTGCCTGGGTGTCAAACATCGTTGCCCAAACACAATGTCCTTCTCCGGGCATCATGTG
C. siliquastrum                     GGCCGAGGGCACGTCTGCCTGGGTGTCAAACATCGTTGCCCAAACACAATGTCCTTCTCCGGGCATCATGTG


Taxon/Node                          505                                                                                  576
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Bauhinia faberi                     GGGGGCGGAGATTGGCCTCCCGTGAGCGAGACTCGCGGTTGGCCTAAATGCGAGTCCGTGGTGGCGAGCACC
C. canadensis var. canadensis       AAGGGCGGATGCTGGCCTCCCGTGAGCACGCCTCGCGGTTGGCCTAAATGCGAGTCCACGGTGGCGAGCACC
C. chinensis                        AAGGGCGGATGCTGGCCTCCCGTGAGCACGCCTCGCGGTTGGCCCAAATGCGAGTCCGTGGTGGCGAGCACC
C. canadensis var. texensis         AAGGGCGGATGCTGGCCTCCCGTGAGCACGCCTCGCGGTTGGCCTAAATGCGAGTCCACGGTGGCGAGCACC
C. canadensis var. mexicana         AAGGGCGGATGCTGGCCTCCCGTGAGCACGCCTCGCGGTTGGCCCAAATGCGAGTCCACGGTGGCGAGCACC
C. siliquastrum                     AAGGGCGGATGCTGGCCTCCCGTGAGCAGGCCTCGCGGTTGGCCCAAATGCGAGTCCACGGTGGCGAGCACC
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Appendix 4. Complete aligned ITS nrDNA Sequence Data Matrix of *Cercis* Taxa and *Bauhinia faberi* (Continued).

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Taxon/Node                          577                                                                  648
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Bauhinia faberi                     AAGGCACGAGGTGGTTGAGTCACGCTCGAAGCCAAGTCCTGAGTGCCTCG-CCCGTYGAACGGCTCCCTGAA
C. canadensis var. canadensis       ACGACGCACGGTGGTTGAGTAACGCTCGAAGCCA-GTCCTGCGTGTCTCGTCCCAATACACGGCTCCTTGA-
C. chinensis                        ACGACGCACGGTGGTTGAGTAACACTCGAAGCCA-GTCCTGCGTGTCTCGTCCCAATACACGGCTCCTTGA-
C. canadensis var. texensis         ACGACGCACGGTGGTTGAGTAACGCTCGAAGCCA-GTCCTGCGTGTCTCGTCCCAATACACGGCTCCTTGA-
C. canadensis var. mexicana         ACGACGCACGGTGGTTGAGTAACGCTCGAAGCCA-GTCCTGCGTGTCTCGTCCCAATACACGGCTCCTTGA-
C. siliquastrum                     ACGACGCACGGTGGTTGAGTAACGCTCGAAGCCA-GTCCTGCGTGTCTCGTCCCAATACACGGCTCCTTGA-


Taxon/Node                          649                                                                  715
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Bauhinia faberi                     CCCCCTCGCATCCCACGGATGCTTCCAACGCGACCCCAGGTCAGGCGGGACTACCCGCTGAATTTAA
C. canadensis var. canadensis       CCCTCTTGCATCCCACGGATGCTTCCAACGCGACCCCAGGTCAGGCGGGGCTACCCGCTGAATTT??
C. chinensis                        CCCTCTTGCATCCCACGGATGCTTCCAACGCGACCCCAGGTCAGGCGGGGCTACCCGCTGAATTTAA
C. canadensis var. texensis         CCCTCTTGCATCCCACGGATGCTTCCAACGCGACCCCAGGTCAGGCCGGGCATCCCGCTGAATTAA?
C. canadensis var. mexicana         CCCTCTTGCATCCCACGGATGCTTCCAACGCGACCCCAGGTCAGGCGGG--TTACCCCCTGATTTAA
C. siliquastrum                     CCCTCTTGCATCCCACGGATGCTTCCAACGCGACCCCAGGTCAGGCGGGGCTACCCGCTGAATTTA?
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