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A possible new Arkansas endemic plant revealed by DNA sequence analysis

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Abstract

Cardamine angustata var. *ouachitana*, a wildflower in the mustard family (Brassicaceae), was described by Smith in 1982 to include a form of *Cardamine* found only in the Ouachita Mountains of Arkansas. This variety is morphologically very similar to typical *Cardamine angustata*. The major difference noted by Smith for the two varieties was the complete lack of leaf hairs (trichomes) in the new variety, whereas typical *Cardamine angustata* normally possesses trichomes. However, Al-Shehbaz rejected the variety *ouachitana* and reduced it to synonymy with the typical *C. angustata*. The recommendation of Al-Shehbaz has been followed — and the taxon *Cardamine angustata* var. *ouachitana* is currently not accepted by most plant taxonomists. We performed a preliminary evaluation of the status of *Cardamine angustata* var. *ouachitana* by comparing ribosomal internal transcribed spacer region DNA sequences from specimens of *Cardamine angustata* var. *ouachitana* with sequences of *Cardamine angustata* from the main range of the species and other related species of *Cardamine*. Phylogenetic analyses of these data produced an unexpected result; specimens of *C. angustata* var. *ouachitana* were actually closely related to *C. concatenata*, rather than the expected close relationship with *C. angustata*. However, *C. angustata* var. *ouachitana* is morphologically distinct from *C. concatenata.* These results suggest that *Cardamine angustata* var. *ouachitana* is actually a new species found only in the Ouachita Mountains of Arkansas.

Key words. Brassicaceae, *Cardamine*, endemic species, internal transcribed spacer, Phylogeny, sequence analysis.

Introduction

The toothworts, a group of spring-flowering perennials of the genus *Cardamine* (subgenus *Dentaria*) (Brassicaceae), include 3 recognized species found in Arkansas. These are the common, widespread species, *C. concatenata* (Michx.) O. Schwarz, and 2 species found in Arkansas only in the Ouachita Mountains, *C. angustata* O.E. Schulz and *C. dissecta* (Leavenw.) Al-Shehbaz. However, Smith (1982) proposed that the Arkansas populations of *Cardamine angustata* should be recognized as a distinct taxon, *C. angustata* var. *ouachitana*. According to Smith (1982), *C. angustata* var. *angustata* is known from the United States east of the Mississippi River, but is unknown from Arkansas. The major difference noted for the two varieties was the complete lack of marginal leaf hairs (trichomes) in the new variety, whereas typical *Cardamine angustata* var. *angustata* normally possesses trichomes. However, Al-Shehbaz (1988) asserted that the presence or absence of the marginal leaf hairs was variable within typical *C. angustata* and he rejected *C. angustata* var. *ouachitana*, reducing it to synonymy with typical *C. angustata*. The recommendation of Al-Shehbaz has been followed by taxonomists and the taxon *Cardamine angustata* var. *ouachitana* is not generally accepted.

We were curious about the actual status of *Cardamine angustata* var. *ouachitana* because of its disjunct distribution and the fact that specimens of *C. angustata* var. *ouachitana* are much smaller than typical *C. angustata*, a difference not noted by Smith (1982). The University of Arkansas at Monticello Regional Flora class of Spring Semester, 2011 performed a preliminary evaluation of this problem by producing sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA cistron (including the 5.8S rDNA) from specimens of *Cardamine angustata* var. *ouachitana* and comparing those sequences to sequences of *Cardamine angustata* from the main range of the species and other related species of *Cardamine*. An analysis of these data indicates that *C. angustata* var. *ouachitana* is likely closely related to the widely distributed species, *C. concatenata*, rather than *C. angustata* var. *angustata*.

Materials and Methods

DNA was extracted from herbarium specimens (Table 1) using a modification of the procedure of Fawley and Fawley (2004). A piece of leaf tissue approximately 5mm x 5mm was placed in a 2 mL screw-top tube with 200 µL of extraction buffer (1M NaCl, 70 mM Tris, 30 mM Na_2EDTA, pH 8.6) and 25 µL of 10% dodecyl-trimethylammonium bromide (DTAB, Sigma Chemical Co., St. Louis). Glass beads (Sigma G-8772) were added to fill the conical portion of the tube. Lastly, 200 µL of chloroform was added. Cells were disrupted using a MiniBeadBeater (Biospec Products, Bartlesville, OK) at highest speed for 40 seconds. The resulting slurry was centrifuged for 2 minutes at 5,000 rpm to achieve phase separation. One hundred µL of the upper aqueous phase was removed and the DNA was purified using the GeneClean Turbo cartridge system (MP Biomedicals, LLC, Solon, OH).

The polymerase chain reaction (PCR) was used to amplify the ITS region of the rDNA using the primers ITS-1 and ITS-5 (White et al. 1990) with annealing at 55°C (Carlsen et al. 2009). The PCR product was purified with the QIAQuick PCR Purification Kit (QIAGN Sciences, Valencia, CA) and quantified using the Qubit fluorometer HS assay (Invitrogen, Eugene, OR). Sequencing was performed with the same primers used for PCR by the DNA Resource Center at the University of Arkansas, Fayetteville. Sequences were assembled and edited with the Staden Package (http://staden.sourceforge.net/). All sequences generated for this project have been submitted to GenBank (Table 1).

The software Muscle (Edgar 2004) was used to align a sequence data set of our new sequences and published sequences from the closely related *Cardamine* species, *C. concatenata*, *C. dissecta*, *C. angustata*, and *C. diphylla*, as well as outgroup *Cardamine* species (Table 1) chosen according to Carlsen et al. (2009). Additional manual editing of the alignment was performed with MacClade 4.08 (Maddison and Maddison 2000). The final alignment included 620 characters of which 95 were variable and 48 were parsimony informative. Maximum Parsimony (MP) analysis was performed with PAUP* 4.0 (Swofford 2002), with gaps treated as missing data. Maximum Likelihood (ML) analysis used the GARLI software (Zwickl 2006) with the GTR+I+G model and 20 random searches. Maximum Parsimony analysis was bootstrapped 1000 times and the ML analysis was bootstrapped 100 times with 2 searches for each replicate. Pairwise distances among the sequences were determined with PAUP* (Swofford 2002).

Table 1. Specimens included in the analyses of ITS sequences from *Cardamine* spp., including GenBank accession numbers. Sequences generated in this study are shown in bold face. All newly examined specimens were from the UAM Herbarium.

Specimen	Accession Number	Geographic Location
C. angustata		
Kral 61398	**JQ901416**	**Wayne Co., TN**
Kral 61453	**JQ901415**	**Colbert Co., AL**
Kral 61453 (MO)	FJ464465-FJ464488	Colbert Co., AL
C. ang. var. *ouachitana*		
Fawley 2011-1	**JQ901417**	**Polk Co. , AR**
McCallie 24	**JQ901418**	**Polk Co. , AR**
Lay 108	**JQ901422**	**Polk Co. , AR**
Nunn 5346	**JQ901419**	**Howard Co. , AR**
Crossett 200	**JQ901421**	**Scott Co. , AR**
Morse 2899	**JQ901420**	**Scott Co. , AR**
Stone 7	**JQ901423**	**Montgomery Co. , AR**
C. concatenata		
Stone 8	**JQ901414**	**Montgomery Co. , AR**
"TC03"	EU819319	Canada
"TC05"	EU819320	Canada
Beyersdorfer 48	DQ005988	Maryland
C. conferta		
"TC03_1"	EU819321	Russia
C. constancei		
"TC03_712"	EU819322	USA
C. diphylla		
"TC03_714"	EU819331	
C. dissecta		Canada
"WU"	FJ464470-FJ464491	Kentucky
C. fragariifolia		
"TC05_4"	EU819336	China
C. yunnanensis		
"TC05_15"	EU819384	China

Results

Excellent ITS sequence data were produced with most of the *Cardamine* specimens used in the study. Only the *C. angustata* specimens, Kral 61398 and Kral 61453, produced significant background noise that

resulted in some ambiguous data. These *C. angustata* specimens were collected in 1978, so some degradation of the DNA was to be expected. The Nunn 5346 specimen of *C. angustata* var. *ouachitana* displayed some evidence of length heterogeneity, but the consensus sequence was easy to determine. However, a small amount of probable heterogeneity was observed in the sequencing results from most of the specimens. Here we used the consensus sequences, with ambiguous sites coded using the IUPAC-IUB codes when there was no clear consensus.

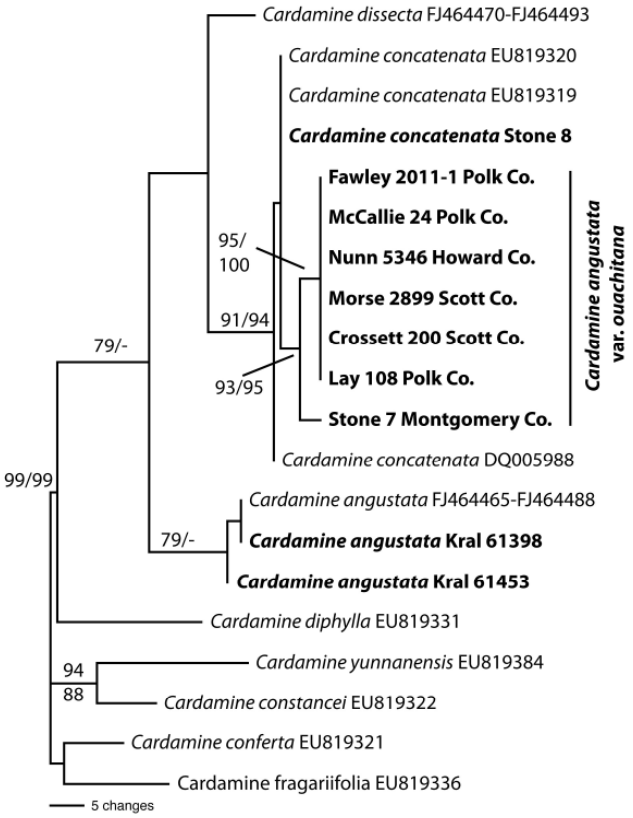

Figure 1. Phylogram resulting from a Maximum Parsimony analysis of ITS sequence data from *Cardamine* species. Bootstrap values for Maximum Parsimony (1000 reps) and Maximum Likelihood (100 reps) are shown. Only bootstrap values of 70 or greater are shown. *Cardamine yunnanensis*, *C. constancei*, *C. conferta* and *C. fragariifolia* are included as outgroup taxa. New sequences generated in this study are shown in bold face.

All of the sequences produced from specimens of *C. angustata* var. *ouachitana* were identical with the exception of the Stone 7 specimen from Montgomery County, AR. The sequence from this specimen differed from the other *C. angustata* var. *ouachitana* sequences by 3 substitutions in the ITS1 region and 3 substitutions and 1 insertion in the ITS2 region (Table 2). The sequences of *C. angustata* var. *ouachitana* differed from the Arkansas *C. concatenata* by 1 or 2 substitutions in the ITS1 region and 5 or 4 substitutions in the ITS2 region for the standard sequences or for the Stone 7 sequence, respectively. All *C. angustata* var. *ouachitana* sequences also differed by a single substitution from the sequence of *C. concatenata* for the 5.8S rRNA region.

On the other hand, the ITS region sequence divergence of the *C. angustata* var. *ouachitana* specimens from the *C. angustata* specimens was 19-20 substitutions. Five substitutions were synapomorphic for the alliance of *C. angustata* var. *ouachitana* and *C. concatenata*, whereas there were no synapomorphies linking *C. angustata* var. *ouachitana* to *C. angustata*.

Maximum Parsimony analysis of the nuclear ribosomal ITS sequence data resulted in 2 most parsimonious trees with 124 steps, which differed only in the relative positions of *C. angustata* and *C. dissecta* among the ingroup taxa. Figure 1 presents one of these phylograms. The results indicate a monophyletic lineage in which *Cardamine angustata* var. *ouachitana* is allied with *C. concatenata*, not *C. angustata* (Fig. 1). This alliance has high bootstrap support with MP analysis. Maximum Likelihood analysis resulted in a phylogram (not shown) similar to Fig. 1, with high bootstrap support for the alliance of *C. angustata* var. *ouachitana* and *C. concatenata* (Fig. 1).

Discussion

The results of our sequence analysis and phylogenetic analyses of the DNA sequence from the ITS region indicate that *C. angustata* var. *ouachitana* is allied with *C. concatenata* instead of *C. angustata*. This result is compelling given the strong support for monophyly of the *C. angustata* var. *ouachitana* and *C. concatenata* alliance, which is probably due to the 5 synapomorphic characters defining this lineage. On the other hand, there are no synapomorphic characters that support the placement of *C. angustata* var. *ouachitana* with *C. angustata*.

Our results provide strong evidence that the Arkansas specimens referred to as *C. angustata* var. *ouachitana* should probably be considered either a new variety of *C. concatenata* or a new species of *Cardamine*. *Cardamine concatenata* and *C. angustata* var. *ouachitana* are in some ways similar plants. Specimens of both taxa are smaller than *C. angustata*. However, the leaf shape of *C. angustata* var. *ouachitana* is more similar to that of *C. angustata* than the shape is to leaves of *C. concatenata* and the leaf

Table 2. Pairwise distances among the *Cardamine* ITS sequences used in this study. Only values for unique sequences are included.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
1. *C. dissecta*											
2. *C. diphylla*	20										
3. *C. concatenata* DQ005988	5	22									
4. *C. concatenata* Stone 8	6	23	1								
5. *C. angustata* Kral 61453	10	19	13	14							
6. *C. angustata* Kral 61398	9	18	12	13	3						
7. *C. ang.* var. *ouachitana* Fawley 2011-1	9	27	7	6	20	19					
8. *C. ang.* var. *ouachitana* Stone 7	11	29	7	6	20	19	6				
9. *C. yunnanensis*	47	48	48	49	51	52	53	51			
10. *C. constancei*	28	36	35	36	36	37	40	42	32		
11. *C. conferta*	28	33	33	32	34	35	36	38	33	24	
12. *C. fragariifolia*	37	34	39	38	41	42	42	42	45	32	25

margins of *C. concatenata* always have spreading trichomes, whereas the leaf margins of *C. angustata* var. *ouachitana* are always glabrous. In our experience, leaf shape varies considerably within all of these taxa, so it may not be possible to clearly separate the species on this character. The difference in the number of leaves is a character that does appear useful for separating *C. angustata* var. *ouachitana* from *C. concatenata*. *Cardamine concatenata* typically has 3 leaves whereas *C. angustata* var. *ouachitana* always has just 2 leaves. We have started sampling Arkansas populations of *C. angustata* var. *ouachitana* and *C. concatenata* more intensively in order to better evaluate the morphologies of these two taxa.

A curious finding of this study was the anomalous specimen, Stone 7, from Montgomery County, AR. The sequence from this specimen differed markedly from other *C. angustata* var. *ouachitana* specimens, although morphologically it is grouped with this taxon. In the future, we will visit the site of this specimen's collection to verify our findings.

Our results also extend the range of *C. angustata* var. *ouachitana* to Scott Co., in the area along the Fourche La Fave River east and west of Boles. Specimens of *C. angustata* var. *ouachitana* from this area were found in the UAM herbarium previously identified as *C. concatenata* (Morse 2899 and Crossett 200).

Finally, we offer a note of caution. Although our results are strongly indicative of the alliance between *C. angustata* var. *ouachitana* and *C. concatenata*, taxonomic revision is ill advised based on only a single locus. We will continue these studies by examining additional specimens of several species of *Cardamine* and adding other loci, especially highly variable plastid loci, to the sequence analysis. In the meantime, specimens should be referred to *C. angustata* var. *ouachitana*.

Acknowledgments

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