

Phylogeny of *Hibiscus* sect. *Muenchhusia* (Malvaceae) Based on Chloroplast *rpL16* and *ndhF*, and Nuclear ITS and GBSSI Sequences

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ABSTRACT. *Hibiscus* section *Muenchhusia* is a North American taxon with five species as recognized in the most recent taxonomic revision: *H. coccineus*, *H. dasycalyx*, *H. grandiflorus*, *H. laevis*, and *H. moscheutos*. To investigate the monophyly of *Hibiscus* section *Muenchhusia*, its relationship to other *Hibiscus* species, and the phylogenetic relationships among its species, DNA sequence data were obtained. To investigate the placement of section *Muenchhusia* within *Hibiscus*, chloroplast *ndhF* gene and *rpL16* intron sequences were generated and added to a recently published dataset. To investigate relationships within section *Muenchhusia* three DNA sequence data sets were generated: a non-coding region of the chloroplast genome (*rpL16* intron), nuclear ribosomal ITS, and a nuclear gene encoding granule-bound starch synthase (GBSSI). Analyses of *ndhF* + *rpL16* data indicated that section *Muenchhusia* is indeed monophyletic and is embedded in a clade that includes representatives of *Hibiscus* section *Trionum* sensu lato plus other genera segregated from *Hibiscus* (*Abelmoschus*, *Fioria*), and the tribe Malvaceae. Within section *Muenchhusia* little to no phylogenetically informative variation was detected in the ITS or *rpL16* sequences. The GBSSI data, on the other hand, provided sufficient information to resolve relationships among species. The species of section *Muenchhusia* fall into two primary clades, one consisting of *H. grandiflorus* and *H. moscheutos* sensu lato, the other including *H. coccineus*, *H. dasycalyx*, and *H. laevis*. These phylogenetic data corroborate earlier biosystematic studies that also placed the species into these same two groups. Sequence polymorphism was observed in one accession each of *H. dasycalyx* and *H. grandiflorus* and resolution of the underlying alleles indicates that gene flow has occurred from *H. moscheutos* into both *H. dasycalyx* and *H. grandiflorus*.

Hibiscus L. sect. *Muenchhusia* (Heister ex Fabricium) O. Blanchard (Malvaceae), colloquially known as the Rose Mallows, is a North American taxon that includes five species as circumscribed in the most recent taxonomic revision (Blanchard 1976; see Table 1): *Hibiscus coccineus* Walter, *Hibiscus dasycalyx* Blake & Shiller, *Hibiscus grandiflorus* Michaux, *Hibiscus laevis* Allioni, and *Hibiscus moscheutos* L. Within *H. moscheutos* two subspecies were recognized by Blanchard (1976): *H. moscheutos* subsp. *moscheutos*, and *H. moscheutos* subsp. *lasiocarpus* (Cavanilles) O. J. Blanchard. A number of additional taxa associated with *H. moscheutos* have been variously recognized historically either as varieties, subspecies, or distinct species. Primary among these are *Hibiscus moscheutos* subsp. *palustris* L. of the northeastern US (synonymized with *H. moscheutos* subsp. *moscheutos* by Blanchard [1976]) and *Hibiscus moscheutos* subsp. *incanus* Wendl. of the southeastern coastal plain (synonymized with *H. moscheutos* subsp. *lasiocarpus* by Blanchard [1976]).

The recognition of *Hibiscus* sect. *Muenchhusia* as a group was resurrected by Blanchard (in Fryxell 1988); previously these species had been placed in the large and heterogeneous *Hibiscus* sect. *Trionum* (e.g., Hochreutiner 1900). The monophyly of sect. *Muenchhusia* is suggested by the overall morphological similarity of its included species, a shared chromosome number of $n=19$ (Wise and Menzel 1971), similar ecological affinities (all species are primarily wetland species, either in marshes or along river, drainage ditch, or lake edges), a common growth habit being perennial herbs that grow back from a rootstock each year, and finally their shared geographic distribution with natural popula-

tions generally confined to eastern and central North America.

The taxonomic work of Blanchard (1976) serves as a foundation for further systematic work on this group. The results of his study were two-fold. First, the species of sect. *Muenchhusia* were segregated from sect. *Trionum* in recognition of the clear affinities of these species to each other, but no clear relationship between them and sect. *Trionum*. Second, Blanchard (1976) collected extensively throughout the range of these species and catalogued the extensive intraspecific variation present within those species. Blanchard's (1976) work, however, did not address the question of species relationships within sect. *Muenchhusia*, thus the phylogeny of sect. *Muenchhusia* and the relationship of sect. *Muenchhusia* to the rest of *Hibiscus* remains unclear.

Wise and Menzel (1971) took a biosystematic approach to understanding species relationships in sect. *Muenchhusia* by performing crossing studies within and between species. These analyses resulted in the recognition of two primary crossing groups, Group I consisting of *H. grandiflorus* and *H. moscheutos* s.l., and Group II consisting of *H. coccineus* and *H. laevis* (*H. dasycalyx* was not included in the analyses of Wise and Menzel [1971]). In all crosses between species within groups, fertile hybrids were formed. Crosses between groups, however, were generally unsuccessful in setting fruit. These data suggest that these two groups may represent two distinct natural groups, although the alternative that one group gave rise to the other with a single evolution of reproductive isolation in the derived group cannot be ruled out.

Pfeil et al. (2002) used chloroplast DNA (cpDNA)

TABLE 1. Plant materials used for DNA sequencing, with voucher information and GenBank accession numbers. All voucher specimens are deposited at the University of Tennessee herbarium (TENN). All sequences are newly reported except for *rpL16* from *H. trionum* which was taken from GenBank.

<i>H. coccineus</i> —Missouri Botanical Garden, <i>R. Small s.n.</i> ; <i>ndhF</i> AY341395, <i>rpL16</i> AY341407, ITS AY341386, GBSSI AY341420. FL, St. John's Co., <i>R. Small 187</i> ; GBSSI AY341421. <i>H. dasycalyx</i> —TX, Houston Co., <i>O. Blanchard 249</i> ; <i>ndhF</i> AY341397, <i>rpL16</i> AY341406, ITS AY341388, GBSSI AY341419. TX, Trinity Co., <i>R. Klips s.n.</i> ; GBSSI AY341417, AY341418. <i>H. grandiflorus</i> —FL, Gulf Co., <i>R. Small 190</i> ; <i>ndhF</i> AY341398, <i>rpL16</i> AY341400, ITS AY341389, GBSSI AY341410. FL, St. John's Co., <i>K. Siripun s.n.</i> ; GBSSI AY341408, AY341409. <i>H. laevis</i> —NE, county unknown, <i>R. Small s.n.</i> ; <i>ndhF</i> AY341396, <i>rpL16</i> AY341405, ITS AY341387, GBSSI AY341416. TN, Polk Co., <i>J. Shaw s.n.</i> ; GBSSI AY341415. <i>H. moscheutos</i> subsp. <i>incanus</i> —FL, Alachua Co., <i>R. Small 189</i> ; <i>rpL16</i> AY341401, ITS AY341391, GBSSI AY341411. <i>H. moscheutos</i> subsp. <i>lasiocarpus</i> —IL, Lawrence Co., <i>R. Small 203</i> ; <i>rpL16</i> AY341403, ITS AY341392, GBSSI AY341413. <i>H. moscheutos</i> subsp. <i>moscheutos</i> —TN, Knox Co., <i>R. Small 173</i> ; <i>ndhF</i> AY341399, <i>rpL16</i> AY341402, ITS AY341390, GBSSI AY341412. <i>H. moscheutos</i> subsp. <i>palustris</i> —NY, Cayuga Co., <i>R. Small 172</i> ; <i>rpL16</i> AY341404, ITS AY341393, GBSSI AY341414. <i>H. trionum</i> —South Africa, <i>R. Small s.n.</i> ; <i>ndhF</i> AY341394, <i>rpL16</i> AF384612, ITS AY341385, GBSSI AY341422.

sequences of the gene *ndhF* and the intron of the *rpL16* gene to address phylogenetic relationships of the genera ascribed to the tribe Hibisceae, and the genus *Hibiscus* itself. Their results indicate that several genera that have been placed in Hibisceae are basal within the family or part of a large polytomy near the base of the family (e.g., *Radyera*, *Hovittia*, *Lagunaria*) making Hibisceae a paraphyletic grade. In addition, within the large grade of the genus *Hibiscus* are embedded several genera that have been segregated from *Hibiscus* (e.g., *Abelmoschus*, *Fioria*, *Macrostelia*) as well as the tribes Malvaceae and Decaschistaeae. A final result of the work of Pfeil et al. (2002) has been the recognition of potentially monophyletic groups within the large and heterogeneous grade of *Hibiscus* (including segregate genera). Some members of *Hibiscus* sect. *Trionum* s.l. were included in the analyses of Pfeil et al. (2002) including *H. trionum* L. (maintained in sect. *Trionum*) and *H. striatus* Cav. (segregated into sect. *Striati*). No members of sect. *Muenchhusia* were included in the analyses of Pfeil et al. (2002), so the placement of this section relative to sect. *Trionum* and the other sections segregated from sect. *Trionum* by Blanchard remains unclear.

Given these taxonomic, biosystematic, and phylogenetic studies, the current work was undertaken to address three primary questions. First, is *Hibiscus* sect. *Muenchhusia* monophyletic? Second, what is the phylogenetic placement of *Hibiscus* sect. *Muenchhusia* within the genus *Hibiscus* and tribe Hibisceae? Third, what are the phylogenetic relationships among the species of *Hibiscus* sect. *Muenchhusia*? DNA sequence data from three sources were explored for this project: cpDNA, including the gene *ndhF* and the *rpL16* intron for higher level analysis and *rpL16* alone for lower level analysis; the internal transcribed spacer region of nuclear ribosomal DNA (ITS); and a nuclear encoded granule-bound starch synthase gene (GBSSI). Chloroplast DNA noncoding regions and ITS sequences are often applied to systematic problems at low taxonomic levels because they generally show greater levels of sequence variation than cpDNA or nuclear rDNA genes. Low-

copy nuclear genes such as GBSSI are used less often than cpDNA or ITS sequences due the greater difficulty in isolating and characterizing low-copy genes. Nonetheless, such genes may provide phylogenetically informative characters where other sequences such as cpDNA or ITS do not (e.g., Small et al. 1998; Sang 2002). GBSSI sequences have been applied to both species-level and higher phylogenetic questions in a variety of plant families (e.g., Mason-Gamer et al. 1998; Miller et al. 1999; Evans et al. 2000; Mason-Gamer 2001; Peralta and Spooner 2001; Walsh and Hoot 2001; Evans and Campbell 2002; Mathews et al. 2002; Smedmark et al. 2003). Most studies thus far have found GBSSI to be single-copy in diploid species although there are two copies per diploid genome in Rosaceae (Evans et al. 2000). Gene structure appears to be conserved and consists of one untranslated and 13 translated exons (van der Leij et al. 1991). Different groups of researchers have used different parts of the GBSSI gene for phylogenetic studies. For example, in Poaceae (Mason-Gamer et al. 1998; Mason-Gamer 2001) and *Ipomoea* (Miller et al. 1999) the 3' end of the gene (exons 8–13 or 9–11 respectively) has been sequenced, whereas in Malvaceae, Rosaceae, and Solanaceae (this paper; Evans et al. 2000; Peralta and Spooner 2001; Evans and Campbell 2002; Smedmark et al. 2003) the 5' end of the gene (exons 1–8 or 9) have been sequenced. Further, different subsets of the data have been used to address different questions. For higher level studies (e.g., genera within families) often only exon sequences are used because intron sequences are difficult to align (e.g., Mason-Gamer et al. 1998; Evans et al. 2000; Evans and Campbell 2002), while at lower levels (e.g., species within genera) both exon and intron sequences are used (e.g., Miller et al. 1999; Mason-Gamer 2001; Peralta and Spooner 2001; Walsh and Hoot 2001; Smedmark et al. 2003).

MATERIALS AND METHODS

Plant Materials. Specimens for these analyses were from wild-collected populations, botanical gardens, or USDA germplasm maintenance facilities (Table 1). All five species of sect.

TABLE 2. Amplification and sequencing primers for GBSSI, ITS, *rpL16*, and *ndhF* used in this study.

Primer	Sequence (5' to 3')	Amp/Seq	Reference
GBSSI			
1F	CTG GTG GAC TCG GTG ATG TTC TTG	Amp	Evans et al. 2000
9R	CTC TTC TAG CCT GCC AAT GAA CC	Amp	Evans et al. 2000
3R	TCR AGG AAC AYR GGG TGA TC	Seq	this paper
3F	ACT GTY CGR TTC TTC CAC	Seq	this paper
6R	AGA GCA GTG TGC CAA TCA TTG	Seq	this paper
8R	TCA CCR GAW ACA AGC TCC TG	Seq	this paper
8F	CCT GTC AAG GGA AGG AAA AT	Seq	this paper
ITS			
ITS4	TCC TCC GCT TAT TGA TAT GC	Amp/Seq	White et al. 1990
ITS5	GGA AGT AAA AGT CGT AAC AAG G	Amp/Seq	White et al. 1990
5.8SF	TCG ATG AAG AAC GTA GCG AAA T	Seq	this paper
5.8SR	TGA CAC CCA GGC AGA CGT GC	Seq	this paper
<i>rpL16</i>			
F71	GCT ATG CTT AGT GTG TGA CTC GTT G	Amp	Small et al. 1998
R400	CTT CAA TGA ACR GAT ATT CAT CTA A	Seq	this paper
F627	CGG AAC AAA CCA GAG ACC AC	Seq	Pfeil et al. 2002
R699	TCG CGG GCG AAT ATT TAC	Seq	Pfeil et al. 2002
R1516	CCC TTC ATT CTT CCT CTA TGT TG	Amp/Seq	Small et al. 1998
<i>ndhF</i>			
F1	GAA TAT GCA TGG ATC ATA CC	Amp/Seq	Pfeil et al. 2002
R1318	CGA AAC ATA TAA AAT GCR GTT AAT CC	Amp/Seq	Pfeil et al. 2002

Muenchhusia were included, as were the four primarily recognized subspecies of *H. moscheutos*. Selection of *H. trionum* as the outgroup was based initially on the fact that sect. *Muenchhusia* had previously been included within *Hibiscus* sect. *Trionum* (Hochreutiner 1900). The appropriateness of this selection was later corroborated by phylogenetic analyses of cpDNA sequences in the context of a broader sampling of *Hibiscus* species (Pfeil et al. 2002; see below).

DNA Extraction, PCR, and Sequencing. DNA was extracted from young leaves of wild-collected or greenhouse grown plants using the DNeasy Plant Mini Kit (Qiagen). Sequence data was obtained from four separate loci: the chloroplast *ndhF* gene, the chloroplast *rpL16* intron, the nuclear ribosomal DNA internal transcribed spacer (ITS) region, and a nuclear-encoded granule-bound starch synthase I gene (GBSSI). PCR and sequencing primers are given in Table 2. PCR reactions were performed in 50 μ L volumes with the following reaction components: 1 μ L template DNA (~10–100 ng), 1X *ExTaq* buffer (TaKaRa), 200 μ M each dNTP, 3.0 mM MgCl₂, 0.1 μ M each primer, and 1.25 units *ExTaq* (TaKaRa). Some reactions included bovine serum albumin at a final concentration of 0.2 μ g/ μ L, which improved amplification from difficult templates. PCR cycling conditions for *ndhF*: 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec, primer extension at 72°C for 2 min. A final extension step consisted of 5 min at 72°C. PCR cycling conditions for *rpL16*: 30 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, followed by a slow ramp of 0.3°C/sec to 65°C, and primer extension at 65°C for 4 min. A final extension step consisted of 5 min at 65°C. PCR cycling conditions for ITS: 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, primer extension at 72°C for 1 min. A final extension step consisted of 5 min at 72°C. PCR cycling conditions for GBSSI: 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, primer extension at 72°C for 2 min. A final extension step consisted of 5 min at 72°C. All PCR and sequencing reactions were performed in Eppendorf Mastercycler gradient or Mastercycler personal thermal cyclers. PCR products were cleaned prior to sequencing with the QIAquick PCR Purification Kit (Qiagen). Two accessions surveyed in this study displayed sequence polymorphism at phylogenetically informative sites in the GBSSI data set. To deconstruct these polymorphic sequences into individual al-

les, the PCR products were cloned into pGEM-T (Promega) as described (Small et al. 1998). Multiple clones from each accession were screened by sequencing with a single primer to identify clones representative of each of the underlying alleles. Complete sequences of one clone representing each allele were then used in the phylogenetic analyses. Sequences of the clones were additive with respect to the initial polymorphic sequence indicating that no PCR-mediated base mutations or recombination occurred. DNA sequencing was performed with either the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, electrophoresed and detected on an ABI Prism 373 automated sequencer; or with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, v. 2.0, electrophoresed and detected on an ABI Prism 3100 automated sequencer (University of Tennessee Molecular Biology Resource Facility). All newly generated DNA sequences have been deposited in GenBank (Table 1).

Phylogenetic Analyses. For analysis of the placement of *Hibiscus* sect. *Muenchhusia* within the phylogenetic context of *Hibiscus* and Hibisceae, *ndhF* and *rpL16* sequences from all five *Muenchhusia* species were added to a trimmed *ndhF* + *rpL16* data set from Pfeil et al. (2002) that included representatives of the major clades in *Hibiscus* and Hibisceae. Maximum parsimony analysis was conducted using PAUP* v. 4.0 b10 (Swofford 2002) via a heuristic search with 100 random sequence addition replicates, ACCTRAN character-state optimization, gaps coded as missing data, and saving all most parsimonious trees. Of the 2,668 bp data set, 138 bp of the *rpL16* data were excluded from the analysis because complex and overlapping indels made alignment ambiguous. Relative support for clades recovered was assessed via bootstrap analysis using the "fast heuristic" option with 10,000 replicates.

For analyses within *Hibiscus* sect. *Muenchhusia* alignment of DNA sequences was initially performed with ClustalX (Thompson et al. 1997), with subsequent refinement by eye. In all cases, alignments were unambiguous, and few gaps were required. Phylogenetic analyses were performed under the optimality criterion of maximum parsimony using PAUP* v. 4.0 b10 (Swofford 2002). Due to the paucity of phylogenetically informative characters in the *ndhF*, *rpL16*, and ITS data sets (see below), phylogenetic analyses were not performed for these data sets. Searching of tree space for the GBSSI data set was performed using the Branch and Bound

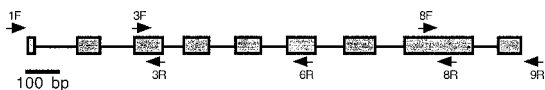


FIG. 1. Diagrammatic representation of the GBSSI gene in *Hibiscus* sect. *Muenchhusia*. Shaded boxes represent exons and intervening lines represent introns. Approximate locations of amplification and sequencing primers (designated by exon number and direction; F = forward, R = reverse) are shown. A 100 bp scale bar is shown for reference.

algorithm of PAUP*, with ACCTRAN character state optimization, and gaps treated as missing data. Relative measures of support for clades were estimated using bootstrap analysis (1,000 replicates with full heuristic searches); jackknife analysis (50% character deletion, 1,000 replicates with full heuristic searches); and decay analysis (all trees up to six steps longer than the most parsimonious tree were saved and evaluated; decay values represent the number of extra steps a given clade survives in the strict consensus tree).

The data sets and phylogenetic trees described in this study have been deposited in TreeBASE (matrix accession numbers M1790, M1791, study accession number S1050).

RESULTS

Sequence Variation in *Hibiscus* Sect. *Muenchhusia*.

For the *ndhF*, *rpL16*, and ITS data sets, little to no sequence variation was detected among the taxa of sect. *Muenchhusia*. The *ndhF* data set consisted of 1,265 aligned nucleotides from the 5' end of the *ndhF* gene. No nucleotide substitutions or indels were observed and 0% of the data were scored as missing. The *rpL16* data set consisted of a total of 1,121 aligned nucleotides. No nucleotide substitutions were detected among the ingroup sequences, and three indels were inferred during alignment. A total of 0.6% of the *rpL16* data set (excluding indels) were scored as missing data. The ITS data set consisted of a total of 683 aligned nucleotides (ITS1=291 bp; 5.8S rDNA gene=164 bp; ITS2=228–229 bp). No indels were required to align ingroup sequences. A total of 17 nucleotide substitutions were observed within the ingroup although the majority (9/17) were due to polymorphic sites within a single sequence. Only two potentially phylogenetically informative characters were present in ITS, one of which was polymorphic in three sequences. A total of 0.7% of the ITS data set (excluding indels) were scored as missing data.

The GBSSI gene in most plants evaluated to date consists of one untranslated exon, 13 translated exons, and 13 introns (van der Leij et al. 1991; Mason-Gamer et al. 1998; Evans et al. 2000). The primers used in this study were designed for Rosaceae (Evans et al. 2000) and are placed in translated exon 1 and exon 9 (Fig. 1). Studies of the GBSSI gene family in Malvaceae indicate that most diploid Malvaceae have three GBSSI loci (unpublished data); however, only a single locus was amplified from the *Hibiscus* sect. *Muenchhusia* species using the amplification conditions described above. BLAST searches using the sect. *Muenchhusia*

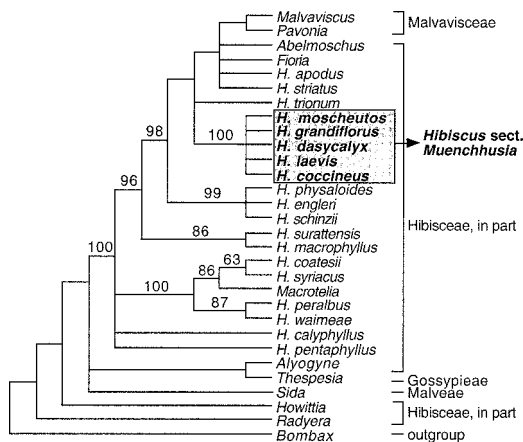


FIG. 2. Strict consensus of 683 equally parsimonious trees showing phylogenetic relationships among the tribes of Malvaceae, with emphasis on tribe Hibisceae and the genus *Hibiscus* and the placement of *Hibiscus* sect. *Muenchhusia* inferred from analysis of chloroplast *ndhF* and *rpL16* sequences (Pfeil et al. 2002). Tree length = 337, CI = 0.91, RI = 0.90. Bootstrap values greater than 50% are shown above each branch.

GBSSI sequences revealed high sequence identity with GBSSI sequences from several other plant families (e.g., Rosaceae, Solanaceae, Fabaceae, Euphorbiaceae, Convolvulaceae, Poaceae). Exons in the sect. *Muenchhusia* GBSSI sequences were identified by alignment of exon sequences from *Solanum tuberosum* GBSSI (GenBank accession X58453) with the sect. *Muenchhusia* sequences. These alignments revealed conservation of exon length, and intron boundary dinucleotides 5' "GT" and 3' "AG".

The GBSSI data set for sect. *Muenchhusia* consisted of a total of 1,972 aligned nucleotides; 842 nucleotides of exon sequence and 1,130 nucleotides of intron sequence. A total of 44 characters were variable among ingroup sequences, and 28 of these were phylogenetically informative. Only a single indel (a 6 bp deletion apomorphic for *H. coccineus* in intron 4) was inferred in aligning ingroup sequences. A total of 0.08% of the GBSSI data set (excluding indels inferred during alignment) were scored as missing data.

Phylogenetic Analyses. Phylogenetic analyses of the sect. *Muenchhusia* *ndhF* + *rpL16* sequences along with representative *Hibiscus* and Hibisceae sequences of Pfeil et al. (2002) resulted in 683 equally parsimonious trees, the strict consensus of which is shown in Fig. 2. The topology of this tree mirrors the results of Pfeil et al. (2002). All five species of sect. *Muenchhusia* were included in this analysis and the monophyly of sect. *Muenchhusia* is strongly supported by the cpDNA data (100% bootstrap support). Section *Muenchhusia* is placed in a clade that includes *H. trionum* (sect. *Trionum*), *H. striatus* (sect. *Striati*), *H. apodus* (sect. *Panduriformes*), members of two segregate genera (*Fioria* and

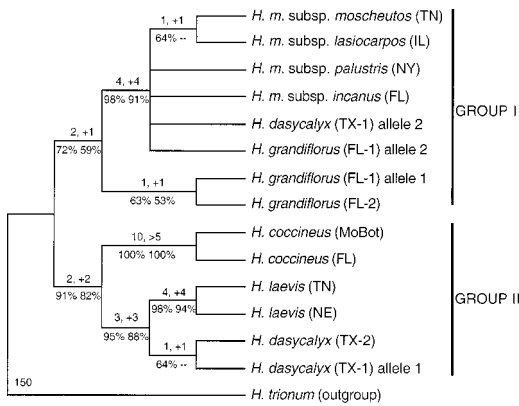


FIG. 3. Single most-parsimonious tree showing phylogenetic relationships of *Hibiscus* sect. *Muenchhusia* inferred from GBSSI sequences. Number of character-state changes and decay values (indicated by a +) are shown above, and bootstrap followed by jackknife values below each internal branch. Each accession is identified by species and state of collection. Two accessions (*H. dasycalyx* TX-1; *H. grandiflorus* FL-1) showed significant heterozygosity and individual GBSSI alleles (identified as "allele 1" and "allele 2") were isolated from these individuals. Tree length = 194, CI = 1.0, RI = 1.0.

Abelmoschus), as well as members of two genera of the tribe Malvaceae (*Paeonia* and *Malva*).

Within *Hibiscus* sect. *Muenchhusia*, because of the complete lack of phylogenetically informative characters in the *ndhF* and *rpL16* data sets, and the very low number of phylogenetically informative characters in the ITS data set, phylogenetic analyses were not performed with these data. Phylogenetic analysis of the GBSSI data set found a single most-parsimonious tree (Fig. 3). This tree separates the taxa of *Hibiscus* sect. *Muenchhusia* into two primary clades, designated on the tree as Group I (*H. grandiflorus* and *H. moscheutos* s.l., plus one allele from *H. dasycalyx* [Trinity Co.]) and Group II (*H. coccineus*, *H. dasycalyx*, and *H. laevis*). Group I has a bootstrap value of 72%, a jackknife value of 59%, and a decay value of +1. Group II has a bootstrap value of 91%, a jackknife value of 82%, and a decay value of +2. Although character support for these groups is relatively low, the data are completely free of homoplasy (both consistency index and retention index values of 1.0).

Where multiple accessions were sampled per taxon, the accessions either formed monophyletic pairs (*H. coccineus* and *H. laevis*), or polymorphism was detected in one accession of the taxa (*H. grandiflorus* and *H. dasycalyx*). Specifically, in *H. grandiflorus* (St. John's Co., FL = FL-1 in Fig. 3) and *H. dasycalyx* (Trinity Co., TX = TX-1 in Fig. 3) two different alleles were recovered from these accessions and the alleles fell into different clades (Fig. 3). One of the *H. grandiflorus* (FL-1) alleles was sister to the other *H. grandiflorus* (FL-2) accession while the second allele fell into the *H. moscheutos* s.l.

clade. Likewise, one of the *H. dasycalyx* (TX-1) alleles was sister to the other *H. dasycalyx* accession (TX-2), while the other allele fell into the *H. moscheutos* s.l. clade.

DISCUSSION

Placement of *Hibiscus* sect. *Muenchhusia* within *Hibisceae*. Phylogenetic analysis of the cpDNA data for *Hibisceae* (Fig. 2) places sect. *Muenchhusia* as a monophyletic group within a clade with several other *Hibiscus* species as well as members of the tribe Malvaceae (*Malva*, *Paeonia*), and other genera of *Hibisceae* (*Fioria*, *Abelmoschus*). Among the *Hibiscus* species in this clade are *Hibiscus striatus* (sect. *Striati*), and *Hibiscus trionum* (sect. *Trionum*) and *Hibiscus apodus* (sect. *Panduriformes*). Sections *Muenchhusia* and *Striati* (along with sections *Venusti* and *Clypeati*, not represented in the cpDNA data set) were segregated from sect. *Trionum* by Blanchard (in Fryxell 1988). The monophyly of sect. *Muenchhusia* is supported in the cpDNA analysis by six character state changes in all equally parsimonious trees, and by a 100% bootstrap value. Additionally, the monophyly of sect. *Muenchhusia* is supported by shared geography, habit, habitat, and chromosome number. While base chromosome numbers in *Hibiscus* vary widely (Fryxell 1997) a base number of $n=19$ is found only in sect. *Muenchhusia*. Species potentially closely related to sect. *Muenchhusia* have chromosome numbers of $n=28$ (*H. trionum*), $n=26$ (*H. striatus*), $n=10$ (*H. clypeatus*), $n=ca. 46$ (*H. mutabilis*) (Index to Plant Chromosome Numbers: <http://mobot.mobot.org/W3T/Search/ipcn.html>) suggesting the $n=19$ count for sect. *Muenchhusia* is synapomorphic.

Phylogeny of *Hibiscus* sect. *Muenchhusia*. Analysis of the GBSSI data provides a well-resolved phylogenetic hypothesis for the species of *Hibiscus* sect. *Muenchhusia*. The species are divided into two primary clades, here denoted Group I and Group II (Fig. 3). Group I includes *H. grandiflorus* and *H. moscheutos* s.l., while Group II includes *H. coccineus*, *H. dasycalyx*, and *H. laevis*. Although character support for some of the inferred relationships is limited as indicated by the short branch lengths and low decay values, bootstrap and jackknife values are relatively high, and the lack of homoplasy indicates that the data are completely internally consistent with respect to the inferred relationships.

The phylogenetic results generated here corroborate earlier biosystematic studies in grouping sect. *Muenchhusia* species into two primary groups. The artificial hybridization studies of Wise and Menzel (1971) resulted in grouping the species into the same two groups based on interfertility. *Hibiscus grandiflorus* and *H. moscheutos* s.l. (Group I) were entirely interfertile, and *H. coccineus* and *H. laevis* (Group II) were also in-

terfertile, but crosses between groups were generally unsuccessful. While Wise and Menzel (1971) did not include *H. dasycalyx* in their studies, subsequent work has shown that *H. dasycalyx* is closely related to *H. laevis* (Klips 1995; this study) and clearly belongs in Group II. Although crossing studies can provide important information for assessing the possibility of gene flow between species, they cannot unambiguously be used as a phylogenetic character because the inability to cross may be due to a synapomorphic gain of crossing barriers in one or both of the lineages in question. Thus, intersterility may be due to synapomorphic gain(s) of crossing barriers, but interfertility may be a plesiomorphic condition, and thus not necessarily reflect species relationships. In the present case, however, the biological and phylogenetic analyses agree that the species of sect. *Muenchusia* can be divided into two primary clades.

SPECIES OF GROUP I. Within Group I, *H. grandiflorus* is sister to a mostly unresolved clade that includes all four of the segregates of *H. moscheutos* s.l. sampled in this study. With respect to the *H. moscheutos* segregates there is strong support for grouping them together (4 character state changes, decay of +4, 98% bootstrap, 91% jackknife), but little resolution or genetic divergence among them despite the wide geographic distribution and the different morphologies represented. The single resolved node within this clade places the *H. moscheutos* subsp. *moscheutos* (TN) and *H. moscheutos* subsp. *lasiocarpus* (IL) accessions together, although this relationship is supported by only a single nucleotide change and has low support (decay = +1, bootstrap = 64%, jackknife < 50%). This low genetic divergence between subspecies of *H. moscheutos* s.l. indicates that the divergence between them is recent, and thus molecular phylogenetic studies are unlikely to recover sufficient data to address the origin, evolution, and divergence of these taxa. A population-level approach is indicated to address these questions, and studies of both molecular and morphological data are being conducted to assess species boundaries and relationships among the taxa of *H. moscheutos* s.l.

SPECIES OF GROUP II. Within Group II the initial divergence is between *H. coccineus* and *H. laevis* + *H. dasycalyx*. The two accessions of *H. coccineus* form a strongly supported monophyletic pair (10 character state changes, decay >5, bootstrap and jackknife 100%). *Hibiscus laevis* and *H. dasycalyx* form a strongly supported (3 character state changes, decay +3, bootstrap 95%, jackknife 88%) monophyletic species pair, and the two accessions of each species are monophyletic. This close relationship between *H. laevis* and *H. dasycalyx* is not unexpected given their strong morphological resemblance. Analyses of allozyme data in *H. laevis* and *H. dasycalyx* (Klips 1995) indicated that there is very little genetic divergence between these two spe-

cies, and concluded that *H. dasycalyx* might best be considered a variety of *H. laevis*. Phylogenetic analysis of the GBSSI data, however, suggest (albeit with very limited sampling) that each species is monophyletic. Further sampling of *H. laevis* GBSSI sequences from a broader geographic sampling (especially including Texas populations) is necessary to determine if *H. laevis* is paraphyletic to *H. dasycalyx* (i.e., a progenitor-descendant species pair) or if they are indeed reciprocally monophyletic.

GBSSI POLYMORPHISM AS EVIDENCE OF HYBRIDIZATION. For most accessions of the species sampled in this investigation little to no sequence polymorphism (i.e., heterozygosity) was detected for GBSSI. Two accessions, however, revealed significant polymorphism at phylogenetically informative sites: *H. grandiflorus* from St. John's Co., FL, and *H. dasycalyx* from Trinity Co., TX (the type locality of the species). These polymorphic sequences were deconstructed into individual alleles by cloning the PCR products, sequencing individual clones, and including these sequences in the phylogenetic analysis. In both cases, one allele of each species grouped with the other accession of that species, while the second allele grouped within the *H. moscheutos* s.l. clade (Fig. 3). Given the sympatric ranges of these species (the limited ranges of both *H. grandiflorus* and *H. dasycalyx* are encompassed within the wide range of *H. moscheutos* s.l.) and evidence of hybridization by occasionally finding morphologically intermediate individuals (Wise and Menzel 1971; Blanchard 1976; Klips 1995; personal observation of herbarium materials) the most likely explanation for the observation of divergent alleles within the polymorphic accessions is gene flow between species. The phylogenetic evidence from the GBSSI sequences suggests gene flow from *H. moscheutos* s.l. into both *H. grandiflorus* and *H. dasycalyx*. This inference is especially notable in the case of *H. dasycalyx* where the polymorphic accession was from the type locality at Apple Springs, Trinity Co., TX. Allozyme and morphological evidence presented by Klips (1995) also indicated that individuals of hybrid origin between *H. dasycalyx* and *H. moscheutos* occurred at the Apple Springs locality. The artificial hybridization studies of Wise and Menzel (1971) suggested little to no interfertility between species of group I and group II raising the question of how gene flow could occur between these species. The study of Klips (1995), however, showed that while fruit set was indeed lower in crosses between *H. moscheutos* and *H. dasycalyx* (54%) than between *H. laevis* or *H. dasycalyx* and *H. dasycalyx* (78% and 73% respectively) these species are clearly capable of cross fertilization.

Relative Utility of cpDNA, ITS, and GBSSI Sequences. At low taxonomic levels, noncoding cpDNA and ITS sequences are the usual tools of choice for phylogenetic analyses. As studies accumulate, however, it has be-

come clearer that these data sources are often inadequate for robust phylogenetic resolution. Chloroplast noncoding sequences, while evolving more quickly than chloroplast gene sequences, still evolve at a rate too slow to resolve recent species divergences (Small et al. 1998; Sang 2002). ITS sequences generally evolve more quickly than cpDNA sequences, but are limited by their short sequence length (generally less than 600 bp for the noncoding portion of the amplicon). Thus, even with greater rates of character transformation insufficient data are often obtained. In addition, sequence polymorphism is often encountered in ITS data suggesting that concerted evolution may incompletely homogenize rDNA sequences, leaving potentially phylogenetically informative characters to be treated as polymorphic characters which further decreases the utility of these sequences. Low-copy nuclear-encoded genes, on the other hand, offer the advantages of both higher substitution rates and longer sequences that may combine to provide adequate phylogenetic signal (Sang 2002). A dramatic example of the differences in relative utilities of these tools is demonstrated by the data presented here. Over 1,100 bp of noncoding cpDNA *rpL16* intron sequences provided no phylogenetically informative characters for the species of sect. *Muenchhusia*. ITS sequences (ca. 500 bp of noncoding sequence) provided slightly greater levels of sequence divergence, but only two phylogenetically informative sites, one of which was polymorphic in three accessions. Neither the cpDNA nor the ITS data set provided sufficient phylogenetic signal to resolve relationships among the species of sect. *Muenchhusia*. The nuclear-encoded GBSSI gene, on the other hand, provided sufficient signal to produce a well-resolved hypothesis of relationships among the species, and identified putative incidences of gene flow between species. While signal strength was relatively low in these data, it was nevertheless stronger than either the cpDNA or ITS data sets, and was additionally both strongly internally consistent and corroborated by independent data from a previous biosystematic study.

Future Directions. The results described here point to two areas in need of further research. First, the lack of genetic divergence within *H. moscheutos* s.l. in all data sets suggests that it is a young taxon despite its wide geographic and morphological range. Efforts are currently underway to begin to assess species boundaries and population relationships within this taxon. Second, the relationship and status of *H. dasycalyx* suggested by the present data indicate that it is closely related to *H. laevis*, but that each is monophyletic. Clearly further sampling from both species from throughout their ranges will be required to clarify the origin of *H. dasycalyx* which is of current conservation concern. Finally, the results presented in this paper highlight the continuing need to pursue additional

data sets beyond the commonly employed tools of cpDNA and ITS sequences for phylogenetic studies at low taxonomic levels.

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