

FINAL REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. E - 56

Endangered and Threatened Species Conservation

**Population Structure and Dynamics of the Navasota Ladies
Tresses, An Endangered Orchid of East-central Texas**

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5 December 2006

FINAL REPORT

STATE: Texas GRANT NUMBER: E - 56

GRANT TITLE: Population Structure and Dynamics of the Navasota Ladies Tresses, An Endangered Orchid of East-central

REPORTING PERIOD: 10/01/04 to 9/30/06

OBJECTIVE(S):

To obtain data on the reproductive biology and population-level genetics of *S. parksii* needed and provide findings/recommendations to the Navasota Ladies' Tresses Recovery Team.

Segment Objectives:

1. Use microsatellite markers, along with field and laboratory observations to define the breeding system of *S. parksii*, and to identify the pattern and distribution of genetic diversity in *S. parksii* populations.
2. Grow accessions of *S. parksii* from seed to determine the extent of sexual reproduction, and to develop protocols for possible eventual reintroduction efforts.
3. Determine levels of gene flow between *S. parksii* and *S. cernua*.
4. Utilize critical biological information in the development of an updated Recovery Plan.

Significant Deviation:

See Attachment A (pp. 50-51).

Preliminary Findings:

See Attachment A.

Location: Lab work: Texas A&M University, College Station, Texas. Field Work: Brazos, Burlason, Freestone, Grimes, Jasper, Leon, Lee, Madison, Robertson, and Washington Counties.

Cost: _____

Prepared by: Craig Farquhar

Date: 5 Dec 2006

Approved by: _____ Date: _____

Neil (Nick) E. Carter

Attachment A

A Genetic Study of the Rare and Endangered Orchid *Spiranthes parksii*
Correll in a Comparative Context.

Contract No 147331

{**Grant Title:** Population Structure and Dynamics of the Navasota Ladies
Tresses, An Endangered Orchid of East-central }

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ABSTRACT

Using four AFLP markers and seven polymorphic microsatellite loci, the genetic structure of the rare and endangered *Spiranthes parksii* Correll (Orchidaceae) was examined. Based on these data *Spiranthes parksii* could not be distinguished from sympatric *S. cernua* (L.) Rich, though low levels of polymorphisms existed within both taxa. These low levels of intra- and interspecific genetic diversity are likely a result of high levels of agamospermic reproduction through adventitious embryony (although there is evidence of occasional sexual reproduction). These results suggest that both *S. parksii*, as well as the sympatric, open flower form of *S. cernua*, are products of the more widely distributed *S. cernua* complex. However, we found no positive evidence to support the merging of *S. parksii* with *S. cernua* or for any other taxonomic revision for *S. parksii*. Further, another local form of *S. cernua*, distinguished by the production of closed flowers, is genetically distinct from both *S. parksii* and the open-flower form of *S. cernua*, as shown by AFLPs and microsatellite loci. This is the first known set of microsatellite primers developed specifically for use in *Spiranthes*. The application of these markers may be used to address questions concerning the populations-genetic status of other species of *Spiranthes*, many of which are also endangered or have populations in decline in the U.S.

INTRODUCTION

Spiranthes is one of a few orchid genera with a cosmopolitan distribution and its members are characterized as a taxonomically difficult group due to substantial variation in traditionally important characters such as plant size and floral morphology as well as reproductive biology.

Depending on the treatment, a few dozen to three hundred species are delimited (Correll 1978). All members are terrestrial and have an inflorescence with flowers arranged in a single to several-ranked spiral around a central axis. Leaves are arranged in a basal rosette and are present during spring in both *S. parksii* Correll and *S. cernua* (L.) Rich, at which time it is impossible to distinguish the two species, but often absent during their fall bloom period. Even when in flower, *S. parksii*, is easily confused with more widely distributed species, particularly *S. cernua*, which is sympatric throughout *S. parksii*'s range. *Spiranthes parksii* was first described (Correll 1950) based on its unique morphology including smaller size, lateral sepal shape and position, and more open spiral of the inflorescence. Its upcurved and long lateral sepals relative to the petals as well as its white-tipped floral bracts most easily distinguish *S. parksii* from *S. cernua*.

Spiranthes parksii, also known as Navasota Ladies' Tresses, is an endemic of the post oak savanna region of east-central Texas. It was first discovered along the Navasota River in 1945 and was named for its collector, H.B. Parks. Many years passed without sightings, prompting the Smithsonian institute to declare it extinct in 1975. However, in October of 1978, twenty plants were located northwest of Navasota, TX (Catling & McIntosh 1979). This species was listed on May 6, 1982 as an endangered species under the Endangered Species Act of 1973 due to low numbers of plants observed during its fall blooming season as well as its specific habitat requirements. The primary threats to this species include habitat loss due to urban development, secondary succession and possibly herbivory.

Morphological, cytogenetic and reproductive studies have centered on the closely related and broadly distributed *Spiranthes cernua* (L.) Rich (Sheviak 1982, Schmidt 1992, Catling 1982 & 1983), yet the genetics, population dynamics and ecology of *S. parksii* remain poorly understood. Major goals of this study were to 1) examine the genetic structure of both *S. parksii* and *S. cernua* using chloroplast sequence data, AFLPs and microsatellite loci, 2)

determine the reproductive mode and define the breeding system for *S. parksii*, and 3) determine the evolutionary origins of *S. parksii*.

Species Descriptions

Spiranthes parksii is commonly known as Navasota Ladies' Tresses and has distinguishing morphology characterized by a truncated labellum, up-curved lateral sepals, and flowers ascending to perpendicular to the axis of the inflorescence rather than perpendicular or nodding as in the open flower form of *S. cernua*. Additional supporting morphological characters include more pubescence on the bracts and flowers and a white-tipped bract typically curved away from the flower (Fig.1). Chromosome counts for this species are $2n=4X=60$ indicating it is a tetraploid (C. Sheviak, unpublished report).



Fig. 1 Images of *Spiranthes parksii*. Flowers shown have a white tipped bract, a truncated lip, and lateral sepals which curve upwards and are longer than the petals.

Spiranthes cernua is a highly polymorphic compilospecies which occurs in many geographically variable forms. Chromosome counts indicate that most individuals are tetraploid ($2n=4X=60$), though numerous aneuploids ($2n=4x=60 \pm 2$) as well as triploids ($2n=3x=45$) are known to occur. In east-central Texas, there are two general forms described by Sheviak, which are discussed below.

The woodland form has open flowers and an expanded labellum, which is as long as the lateral sepals. It was described based on its occurrence in more shaded areas in a woodland habitat. The orientation of single flowers ranges from perpendicular to the axis of inflorescence or nodding, but is never ascending. Additional characters, which are less pronounced to varying degrees, include a brighter, whiter color of the petals and sepals and a lesser degree of pubescence on floral structures compared to *S. parksii*. Floral bracts are green-tipped and typically curve in towards the flower (Fig. 2). Here, this form will be referred to as *S. cernua* (of).

The second form is the closed-flowered or peloric form, which was described based on the labellum's non-distinct appearance as a third petal. It is also referred to as cleistogamous form for its suspected method of reproduction as well as the southern prairie race for its association with more open prairie areas of the southern U.S. Here this form will be referred to as *S. cernua* (cf) to avoid assumptions about breeding system or habitat. For this study, this form was identified based on the presence of closed, ascending flowers which are greenish-white to white (Fig. 3). However, plants may occasionally produce several open flowers on an inflorescence or produce an inflorescence with entirely open flowers (Fig. 4). The degree that flowers open is reportedly temperature dependent, with flowers closed in warmer temperatures

and open in cooler temperatures. Mature plants of this form often produce plump ovaries on all of the flowers of the inflorescence, which suggests either agamospermic or autogamic reproduction occurs in these individuals.



Fig. 2 Images of *Spiranthes cernua* (of). Flowers are white with green-tipped bracts and lateral sepals are as long as petals.



Fig. 3 Images of *Spiranthes cernua* (cf). Flowers shown are closed while seeds are developing inside the ovary.



Fig. 4 Images of *Spiranthes cernua* (cf) with flower slightly open.

Reproductive Biology

The breeding systems of *Spiranthes* are quite variable and include agamospermy (through adventitious embryony), autogamy, outcrossing and mixed-breeding systems involving various levels and combinations of the above (Catling 1982 & 1983). Unlike many orchid species, *Spiranthes* do not have species-specific pollinators. *Bombus* species and solitary leaf-cutting bees of the Megachilidae are reported to be the most common pollinators for most species of this genus. Though pollinia have on rare occasion been observed on petals and sepals of *S. parksii* flowers, indicating some level of insect activity, the pollinators for the species are unknown. Small flower size in *S. parksii* may prevent bumblebees from accessing pollinia. Bumblebees have been reported on *S. parksii* (H. Wilson, unpublished report) however, it is not clear whether they serve as effective pollinators. Protandry and early development of flowers positioned lower on the spike are thought to promote outcrossing in the genus, however, many species of *Spiranthes* deviate from this mode of reproduction.

Polyembryony has long been recognized in *Spiranthes* (Leavitt 1901; Swamy 1948) and was further associated with a form of agamospermy known as adventitious embryony in which embryos are formed mitotically, without pollination, from the inner integument rather than an unreduced egg cell. This mode of reproduction resembles vegetative reproduction in that all offspring produced carry the maternal genotype. Agamospermy has been considered to be an adaptation in pollinator-limited populations as it may be more common among more peripheral individuals of a population in *S. cernua*. (Schmidt 1992). Polyembryonic seeds (Fig. 5) typically contain between two and six embryos. However, adventitious embryony does not exclude the possibility of sexually produced embryos within the same seed. While *S. cernua* exists in sexual, agamospermic and mixed races (Sheviak 1982; Catling 1982), the mode of reproduction in *S. parksii* is not defined, though polyembryonic seeds are reportedly as high as 80-90% (Catling & McIntosh 1979). There is evidence for apomixis for this species; however, the frequency of this mode of reproduction is unknown.

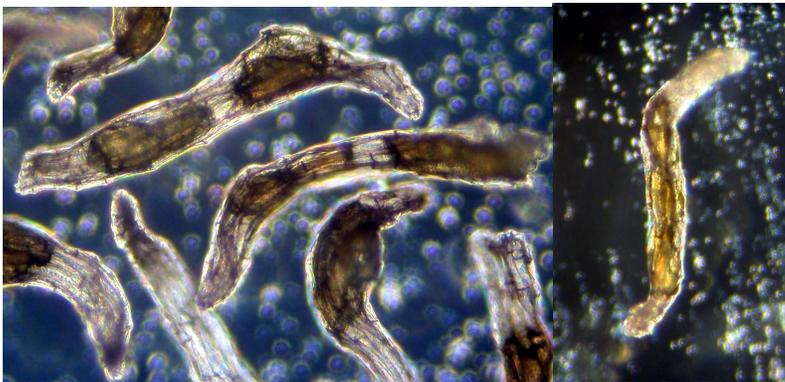


Fig. 5 Polyembryonic seeds of *Spiranthes cernua* (cf).

Though the focal species of this study is *S. parksii*, other closely related species were incorporated, providing a framework for comparative biology of this species. These species include *S. cernua*, *S. magnicamporum* Sheviak as well as *S. vernalis* Engelm. & Gray. While *S. cernua* is suspected to exist in outcrossing, agamospermic and mixed races, *S. magnicamporum*, a diploid relative of and one of the putative progenitor of *S. cernua*, is thought to be mostly outcrossing with other diploid relatives. *Spiranthes vernalis*, though not associated with the *S. cernua* complex, is potentially useful for comparison due to the fact it is an obligate outcrossing species in which agamospermy is not known to occur. In this species, seeds are regular in shape and either lacking an embryo entirely, indicating failed fertilization, or contain a single embryo positioned centrally within the seed (Fig. 6).



Fig. 6 Monoembryonic seeds of *Spiranthes vernalis*.

There is little known about the biology of S. parksii, other than its presumed close evolutionary relationship to the more widely distributed and morphologically variable S. cernua. The need exists to learn more about the basic biology of S. parksii so that this information can be incorporated into the new Recovery Plan. Some critical questions to be addressed in the proposed study include the following:

- Is there gene flow from S. cernua to S. parksii that impacts the long-term viability of S. parksii?
- What is the breeding system of S. parksii? Does it reproduce by outcrossing, selfing or apomixis?
- How genetically diverse is this species? How is its genetic diversity partitioned among metapopulations, populations and individuals?
- To what extent is there gene flow between populations? How is this gene flow effected by development and other anthropogenic factors?

- What are the genetically effective population sizes (relative to the census population sizes) for this species?
- How adequately does protection of the large "core" populations in Brazos and Grimes counties preserve the global genetic diversity of the species? How important are small, "peripheral" populations to the long-term viability of the species?

Numerous technological advances with proven utility in species conservation have occurred since the initial Recovery Plan (1984), including the use of molecular-genetic markers to address questions about the reproductive and population biology of species (for reviews see Lambert, 1995; Haig, 1998; Remy et al. 1998). Recent investigations of other rare members of the genus Spiranthes using molecular-genetic tools (McClaran and Sundt, 1992; Sipes and Tepedino, 1995; Szalanski et al., 2001) have provided important biological information for conservation planning and management. For example, Szalanski et al. (2001) demonstrated that the threatened Ute ladies-tresses (S. diluvialis) harbors very low levels of genetic diversity, despite a wide geographic distribution. This study further demonstrated that S. diluvialis is probably a species of hybrid origin.

OBJECTIVE

We propose to obtain critical information on the reproductive biology and population-level genetics of S. parksii needed for an effective, science-based Recovery Plan for this species.

LOCATION

Most known populations are centered along a narrow band in Brazos and Grimes counties near the Navasota River and can often be found in open wooded areas near small, upland streams and drainages (Fig. 1). Additional outlying plants were found in the Angelina National Forest, approximately 100 miles east of the bulk of the distribution (Bridges & Orzell 1989), as well as in Limestone County (2004) and in Bastrop County at the Stengl Biological Research Station (2004).

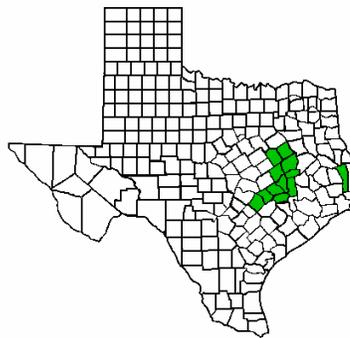


Fig. 7 Distribution of known populations of *Spiranthes parksii*. Populations are known for Brazos, Grimes, Burleson, Washington, Madison, Leon, Robertson, Limestone, Freestone, Bastrop, Lee and Jasper counties. Map modified from Texas A&M Bioinformatics Working Group.

METHODS

I. Use of microsatellite markers, along with field and laboratory observations to define the breeding system of *Spiranthes parksii*, and to identify the pattern and distribution of genetic diversity in *S. parksii* populations.

We will use molecular-genetic markers to examine the breeding system (including the levels of apomixis, inbreeding and outcrossing), population structure and reproductive dynamics (including the partitioning of genetic variation within-populations and among populations) of *S. parksii*. This approach will allow an estimation of effective population sizes,

gene flow between populations, and overall genetic diversity of the species, and will provide insight into the evolutionary origin(s) of S. parksii. For purposes of comparison, some of these analyses of S. parksii will be conducted in parallel with analyses in its more wide-spread, morphologically variable and ecologically diverse sister taxon S. cernua. This comparative approach will yield valuable insights into the reproductive and population-level processes in S. parksii, a rare and geographically and ecologically restricted taxon, and will identify possible negative trends in the population-genetic dynamics of S. parksii that may lead to extinction.

Toward this goal, we have developed a large suite of DNA microsatellite markers from S. parksii that is suitable for investigations in both S. parksii and S. cernua. Although the development of microsatellite markers requires a considerable investment of time and resources (see preliminary results, section V, below), they are simple to use, highly reproducible, and highly informative (due to their co-dominance and high allelic diversity). As described below, we have identified a suite of 25-30 microsatellite loci that (based on DNA sequence) are structurally well suited for molecular-marker analysis. After preliminary testing of these loci on a small collection of geographically diverse individuals, we will select a smaller set of 10-12 robust and highly informative (having high allelic diversity) markers for large-scale analysis using fluorescently-labeled primers (Hex, Fam, Tet, Ned).

DNA was isolated from a comprehensive collection of *S. parksii* individuals, representing the global distribution of the species. DNAs from 298 S. parksii individuals have been collected thus far (see preliminary results, section V). Microsatellite loci from these samples were amplified using fluorescently-labeled primers and the genotyped using the ABI3100 automated capillary DNA sequencer. Fragment sizes from each individual will were analyzed

and compiled using the Genotyper software suite (Applied Biosystems Inc.). Additional microscopic and molecular-genetic work was performed to detect apomixis (as described in Section II below).

Estimation of current effective population sizes (N_e) as well as detection of recent changes in effective population sizes will be conducted using maximum likelihood, coalescent and Bayesian statistical methods (Lukart and England, 1999). Current and past patterns of intraspecific gene flow within S. parksii populations will be examined using related mathematical approaches (Lukart and England, 1999).

Levels of gene flow between populations will be examined with respect to distance between populations and possible barriers (such as urban development) between populations. We will use also use DNA-based molecular marker data to determine the levels of and distribution of genetic diversity among S. parksii populations. Partitioning of genetic diversity among meta-populations, populations, genetic neighborhoods, and individuals will be examined using the Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) and F_{st} (Slatkin, 1995) methods.

II. Grow accessions of S. parksii from seed to determine the extent of sexual reproduction, and to develop protocols for possible eventual reintroduction efforts.

Very little is known about the reproductive biology of S. parksii. In this project, seeds of S. parksii will be examined under the microscope for viable embryos, nonviable embryos and polyembryony — which is a putative indicator of apomixis. This will provide an initial estimate of % viable seed set and the % of seeds that are putatively apomictic in origin. Seeds

will be germinated and seedlings propagated using established orchid culture methods. Mature seedlings will be propagated in the green house. Plants will be observed for blooms, at which time fertilization experiments will be initiated. Microsatellite markers will provide a "genetic fingerprint" to verify that the polyembryonic seeds are produced by apomixis. This will be done by comparing the microsatellite profiles of the parents, polyembryonic seeds, and normal seeds from controlled crosses. The profiles of the polyembryonic seedlings will match the profiles of the maternal parent if they are the result of apomixis, while those produced by sexual reproduction will contain markers found in both parents. If it is verified that the polyembryonic seeds are apomictic and the normal seeds are the result of sexual reproduction, it will allow an easy survey to determine how reproductive efforts are partitioned (apomictic vs. sexual) in natural populations and if there is any correlation of the partitioning with population size, genetic diversity, and other population parameters.

In this set of experiments, we will also gather baseline data on effective methods for the collection, storage and utilization of seed resources that will be valuable if reintroductions are included in future recovery efforts.

III. Determine levels of gene flow between S. parksii and S. cernua

Hybridization with more common species is considered to be a cause of decline in 38% of endangered fish species world-wide (International Union for Conservation of Nature and Natural Resources). This number is likely to be far higher in plants where interspecific hybridization is much more common. Although experts consider S. parksii and its sister taxon S. cernua to be discrete species, they do share a close evolutionary relationship, and a small

number of plants have been characterized as possible hybrids of S. parksii and S. cernua. The genetic markers we are developing for S. parksii are co-dominant and hence well suited to test for hybridization. A comparison of microsatellite patterns in S. cernua populations with that of S. parksii populations will detect long-term patterns of gene flow between the two species. In addition, any plants that display a possible "hybrid" morphology can be tested directly through microsatellite fingerprinting. If the two species do hybridize, then proximity to S. cernua populations should be considered as a factor in selecting sites for preservation and/or reintroduction of S. parksii.

IV. Utilize critical biological information in the development of an updated Recovery Plan.

The determination of breeding system, minimum viable population size, population-genetic structure, patterns of gene flow and genetic diversity of S. parksii will allow us to assess the current preservation model, and to establish a conservation strategy that maximizes the long-term evolutionary potential of the species in a changing biotic and abiotic environment. These results will be incorporated into the work of the Recovery Team, presented at scientific meetings, published in peer reviewed journals, and placed on web pages to insure widespread dissemination of the information. The biological information obtained from this proposed research, combined with geographic (GIS) and other data, will facilitate the identification of target populations for conservation, determine if habitat fragmentation is a problem, develop effective management practices, and provide information and materials critical to the reintroduction of S. parksii, if that is deemed necessary to ensure its long-term survival.

RESULTS AND DISCUSSION

Sampling

Plants were sampled from populations at the locations indicated by the map below. Sampling focused on *S. parksii* but both peloric and woodland forms of *S. cernua* were also sampled when found in these locations.

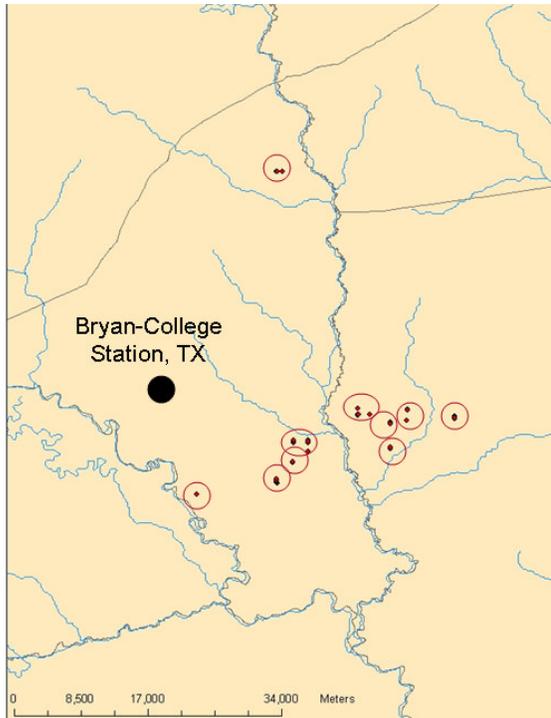


Fig. 8 Distribution of core populations of *Spiranthes parksii*. Plants were sampled from ten populations indicated on the map in Brazos and Grimes counties. Additional samples were obtained from Limestone and Bastrop counties which are not indicated on this map.

A simple field DNA extraction method was used that has produced genomic DNA of suitable quality and quantity for PCR-based applications (Pepper & Norwood 2001). Sampling was nondestructive and genomic DNA was obtained from tissue of only one or two cauline or floral bracts. DNA was ground in an extraction buffer (200mM Tris pH 8.0, 250 mM NaCl, 25mM EDTA pH 8, .5% SDS) until the extraction

was completed by a series of isopropanol precipitations in the lab. The final pellet was resuspended in 100ul .5X TE buffer and stored at -20°. DNA was extracted from 296 individuals of *S. parksii* as well as 209 individuals of *S. cernua* from ten populations in the core distribution in Brazos and Grimes counties, Texas during fall 2003. DNA was obtained from 19 plants identified as *S. parksii* and one as *S. cernua* (cf) in Limestone county fall 2004. Three plants identified as *S. parksii*, 3 as *S. cernua* (cf) and four as *S. cernua* (of) form and one non-resupinate individual of *S. cernua* were sampled at the Stengl Lost Pines Biological Station in Bastrop County in fall of 2004. DNA from a population of *S. magnicamporum* was collected in Blanco County, TX in fall of 2003. DNA from populations of *S. vernalis*, *S. lacera* var. *gracilis* (Raf.) Raf. var. *gracilis* (Bigelow) Luer, and *S. praecox* (Walt.) S. Wats were also obtained by the same procedure.

Chloroplast DNA Variation

Plastid DNA, including cpDNA, has been used in molecular systematics at various taxonomic levels. Several “universal primers” have been designed and tested on various taxa to determine which regions are most appropriate for studies at a given taxonomic level ([Taberlet et al. 1991](#)). The heavily used trnT-trnF region has been used within the Orchidaceae at the intraspecific (Trapnell et al. 2004), specific (Bellstedt et al. 2001), as well tribal levels (Salazar et al. 2003). Its utility in *Spiranthes* and more specifically within the *S. cernua* complex is not known.

Primers designed from the more conserved gene regions are indicated by a black box, whereas the non-coding regions amplified by these primers are indicated by a black line (Fig. 9). These primers were tested on *Spiranthes* in attempt to find regions most suitable for use in *S. parksii*.

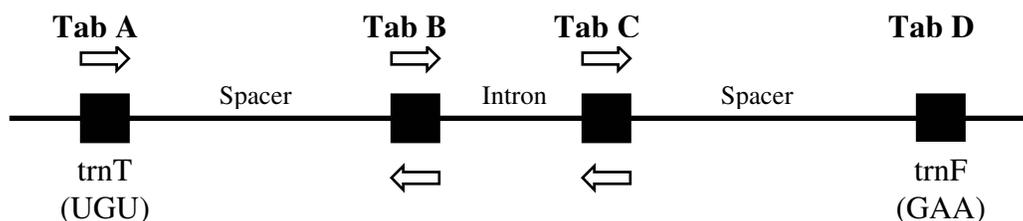


Fig. 9 TrnT-trnF spacer and intron regions of the chloroplast genome.

Table 1 cpDNA primer name and sequence. Primer name and sequence used for amplification and sequencing of trnT-trnF cpDNA region.

Primer Name	Sequence 5'-3'
trnT-F (tabA)	CATTACAAATGCGATGCTCT
trnT-F (alt Tab A)	CAAATGCGATGCTCTAACCT
trnT-F (alt Tab A interior)	AATATTACTGACTCCMTTTTKATTTTCKAG
trnT-F (TabB)	TCTACCGATTTTCGCCATATC
trnT-F (Tab C)	CGAAATCGGTAGACGCTACG
trnT-F (Tab D)	GGGATAGAGGGACTTGAAC
trnT-F (Tab E)	GGTTCAAGTCCCTCTATCCC
trnT-F (Tab F)	ATTTGAACTGGTGACACGAG

Results

Tab A&B

Amplification of Tab A was problematic in *Spiranthes*. This is not so surprising given that others using these same primers had amplification problems in various other taxa

(Taberlet *et al.* 1991). This region amplified with a weak product and sequencing efforts were not completely successful. However, approximately 400 bp of readable sequence among 5 individuals *S. parksii*, 3 of *S. cernua* (of) and one *S. cernua* (cf) was obtained for this region using the alternate primer, TrnA2 for amplification as well as for sequencing reactions. While *S. cernua* (of) was identical to *S. parksii* in all samples, *S. cernua* (cf) differed by a one base insertion of the base thymine.

Tab C&D

No nucleotide variation was detected in a 570bp region among 5 samples of *S. cernua* (of), 3 of *S. parksii* and 1 of *S. cernua* (cf). However, several indels as well as base substitutions were present between these samples and *Spiranthes lacera* var. *gracilis*.

Tab E&F

Four samples of *S. parksii*, two of *S. cernua* (of), and one of *S. cernua* (cf) were sequenced at the Tab E&F intergenic spacer region. The size of this segment was approximately 502bp for all seven samples, however, 392bp of readable sequence across all samples was obtained at which all samples were identical.

Discussion

The chloroplast regions sequenced show no variation between samples of *S. cernua* (of) and *S. parksii*. A one base pair insertion detected in the 1,362 base pairs examined in *S. cernua* (cf) may be indicative of some differentiation of this form compared to *S. cernua* (of) or *S. parksii*. This additional base pair was previously dismissed as a sequencing error, though an independent attempt to sequence the same sample for the same region in

another lab produced the same results (L. Dueck, personal communication). Though few samples were examined at these chloroplast regions, they appear to be invariant in the local members of the *S. cernua* as well as *S. parksii* and would not be informative in resolving relationships between the two. Chloroplast sequencing techniques are routinely used to address systematic questions at the specific level, but due to their slower mutation rates, they are not as informative at the population level or among closely related species in comparison to other techniques. The trnL intron region appears to be most informative of these regions examined for more distantly related species of *Spiranthes*, but uninformative for resolving relationships between *S. parksii* and *S. cernua*, thus no further efforts to sequence cpDNA segments were made.

Amplified Fragment Length Polymorphism

AFLPs (amplified fragment length polymorphism) are dominant markers used to examine DNA fragments which have been selectively amplified from a digested genome. Variation among individuals in fragments is created when a mutation occurs in the DNA region recognized by the enzyme used to digest the genome or by insertions or deletions in the amplification product itself. These mutations give fragments different mobility when run on an electrophoretic gel. AFLP markers are dominant and scored in terms of their presence or absence in a particular individual. Bands of similar mobility are assumed to be identical by descent, though this may not be the case as fragments of the same size may be generated from non-homologous regions of the genome. However,

this type of homoplasy is considered to be negligible with this technique. This technique is powerful in its ability to detect overall similarity or differences rapidly relative to other molecular techniques as no prior sequence knowledge of the species of interest is required. This feature makes AFLPs particularly useful in the study of non-model organisms. Most encouraging to us was the recently published finding that in *Spiranthes romanzoffiana* Cham. from the British Isles, 86 polymorphic AFLP fragments were detected, within and among populations, using just three primer combinations across 205 individuals sampled (Forrest *et. al.* 2004). In our study, we genotyped 209 samples of *S. parksii* from ten populations in Brazos and Grimes Counties, TX, using four AFLP primer combinations.

Results

In stark contrast to the previous work of Forrest *et al.* (2004) on *Spiranthes romanzoffiana* Cham., we found remarkably low levels of AFLP polymorphism within *S. parksii* and among *S. parksii* and the two morphological forms of *S. cernua*. We did uncover four polymorphic AFLP fragments, generated using three primer combinations (E-CAA/M-CAT, E-GAA/M-CAT and E-GGA/MCAT), that were present only in the *S. cernua* complex. A fourth primer combination tested, E-TGA/ M-CAT, produced no polymorphic fragments. As AFLPs are dominant markers, polymorphisms are scored as either the presence or absence of a particular DNA fragment. Three polymorphic AFLP fragments were present only in the closed flower form of *S. cernua*. One of these fragments was absent in two samples of the closed flower form of *S. cernua* indicating

intraspecific polymorphism for this marker. The fourth polymorphic AFLP fragment was present only in *S. parksii* and the open flower form of *S. cernua*.

For statistical analysis, data were scored at the four polymorphic markers as presence or absence of bands; Individuals that produced a given AFLP fragment were scored with a “1” while those without were scored with a “0”. A neighbor joining tree was constructed based on average genetic distances between pair-wise samples and a bootstrap analysis was performed with 1000 replicates (Fig. 10).

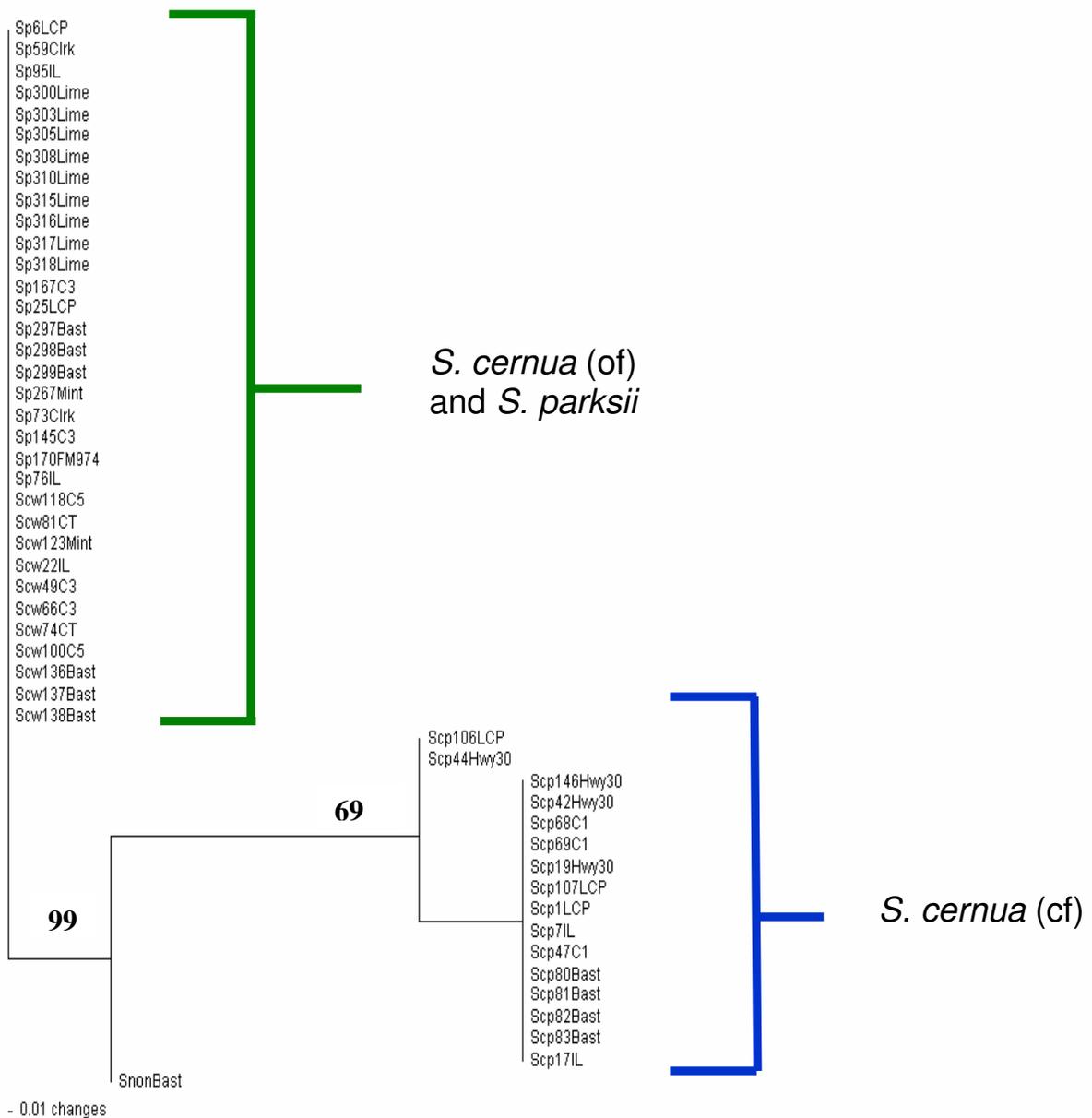


Fig. 10 NJ phylogram constructed from AFLP data. Samples labeled “sp” followed by a number represent *S. parksii* from 10 populations whereas samples labeled “scw” followed by a number represent *S. cernua* (of) from 6 populations. Samples labeled “scp” represent *S. cernua* (cf) from 5 populations. Population designations follow the sample ID. Percent bootstrap support values are indicated in bold.

Discussion

The resulting AFLP-based tree (Fig. 10) clearly shows a separation of the closed flower form from the open flower form of *S. cernua* as well as *S. parksii* with bootstrap support of 99% (Fig. 10). These results were surprising and seemed contrary to Sheviak's observation that floral form of *S. cernua* is an expression of environmental influences. The two forms of *S. cernua* were expected to cluster together and form a separate group from *S. parksii*. However, these results suggest that rather than forms, these are two genetically differentiated groups.

Generally, markers were either present in this form and absent *S. parksii* or *S. cernua* (of), though one marker was present in both *S. parksii* and *S. cernua* (of), yet absent in *S. cernua* (cf). Because none of the four polymorphic markers found were shared among the two, these results strongly suggest that the closed flower form of *S. cernua* is more distant from *S. cernua* (of) than previously thought. Two samples, scp44 and scp106, were more similar to *S. cernua* (of) or *S. parksii* because they did not have one marker present on the other individuals of the same form. Thus AFLP analysis detected some variation within the *S. cernua* closed form, and more markers may detect more differences among them.

Spiranthes parksii and *S. cernua* (of) are genetically indistinguishable from each other in this analysis. Further, within these two taxa no polymorphic AFLP fragments were detected using four primer combinations. Within each taxon, the lack of allelic variation could be due to severe inbreeding, genetic bottlenecks, founder effects or

genetic drift. Alternatively, low genetic variation could result from an apomictic reproductive mode, in which case at least some loci would likely show fixed heterozygous patterns, which would not be detected by AFLPs. A co-dominant marker system, such as microsatellites, can detect heterozygotes, and is needed to resolve this issue.

AFLPs are well-suited for finding levels of variation and overall similarity between samples as well as identifying clones and even potentially hybrids. However, they are limited in that they are dominant markers and do not provide any information regarding heterozygosity, both of which are critical components for examining breeding systems and for conducting informed population management practices. In most cases, the high numbers of polymorphic markers, which can be obtained with relative speed and ease, partially compensate for its limitations. In our study the overall levels of AFLP polymorphism were too low to make any conclusions about breeding systems (including apomixes) or population structure.

Microsatellites

Microsatellites are highly repetitive regions of the genome found in all eukaryotic organisms and are characterized by a nucleotide repeat unit of two to six nucleotides that is repeated tandemly of up to 100 times. They are assumed to be evenly distributed over genomes (Dietrich *et al.* 1996), but rare within coding regions (Wang *et al.* 1994). Mutations at microsatellite loci occur primarily due to slip-strand mis-pairing during

DNA replication, which results in an increase or decrease in the number of repeat units. Thus, variation is scored in terms of length of the number of repeats at a locus. These markers have several advantages over other marker types. Microsatellites are PCR based and require only small amounts of tissue for genomic DNA preparation and subsequent. Further, variation has been found at microsatellite loci in populations with low levels of isozyme variation..Additionally, alleles are co-dominant and can be unambiguously sized at single-nucleotide resolution using capillary electrophoresis instruments. Due to their co-dominance, hypervariability and presumed selective neutrality, microsatellites are increasingly becoming the tool of choice in population genetic studies.

Molecular markers have been particularly useful in resolving relationships among closely related taxa particularly among difficult and morphologically variable groups (Avice 1994). The use of molecular genetics in population biology dates back to the 1960s when protein electrophoresis revealed extensive variation at alleles for allozymes (Lewontin & Hubby 1966; Harris 1966). However, protein electrophoresis is thought to underestimate variation due to both undetectable differences in amino acids at the 3rd codon position as well as the slowly evolving nature of proteins. Previous analysis of isozymes detected no patterns of genetic partitioning in populations of *S. parksii* and *S. cernua* (H. Wilson, unpublished data). With highly variable loci such as microsatellites, the potential to resolve differences between closely related groups is much higher. This powerful tool is used not only to survey genetic variation, but also to investigate historical demographic patterns such as detection of previous population

expansions or contractions and bottlenecks. These markers are potentially useful in *Spiranthes* in addressing whether low genetic variation among individuals detected by AFLPs is due to non-selective genetic processes, such as inbreeding, drift and bottlenecks, or due to apomixis.

Results

Table 3. Characterization of microsatellite loci. Locus name is listed with the fluorescent dye used in parentheses. Forward and reverse sequences are listed 5'-3', followed by repeat motif and approximate amplicon size.

Locus	Primer sequence	Repeat Motif	Amplicon Size
3A (FAM)	F- AATGACTGATGACAGTCGAAG R- GCTATGCCATCACTCACGTG	(CTT) ₇	64
8D2 (HEX)	F- CATTATCGTCGGTCACCGTT R-CATGAGCCTAGCGCGATCTT	(CT) ₁₄	92
sp2-11H (HEX)	F AAACTAGGGTCAATAAACTCCG R-ACATTAGGATGAATATTCTTTGGC	(GA) ₂₅	222
sp2-12C (FAM)	F- TCTCCGTAGATATCACACTCG R- CAATAGTGCAACTTTGATGTCCG	(GA) ₁₄ (A) ₇	139
sp3-9A (FAM)	F-AAGATATTTAAGATGCACAATCGC R-AACCCTTGTAGGATTTTATTGG	(CT) ₂₅	192
sp3-11B (TET)	F- TTCTCTCAATCCCATAGCTGG R- TTCTCGGTGTTTCATCATCTCG	(CT)?(CTT)?	305
sp3-3A (HEX)	F- AT GCA ATA CAT AGC AGC CGC R- GCTGTATAAGATTTTCGGTCTCC	(GA) ₉ (GA) ₃	261
sp4-9C (Hex)	F- AGAATGTCCACAGTAACAGCG R- ATTCATAAATAGCAGTTGTTCCAG	(CT) ₁₈ (T) ₉	173
sp4-1A (FAM)	F- TGGGAATGACTCATCACAGTCG R- TGCCACGCCATCACTCACG	(GAA) ₉	71
sp4-5E (TET)	F- AGGTGGAATTCTGTACACTG R- CAACTCGAACTGATCTTCTGG	(GAA) ₇	97

A set of 96 samples were amplified with primers for 10 loci. Of the nine loci which cross-amplified in *S. magnicamporum*, eight were polymorphic. At locus 3A, samples of *S. cernua* and *S. parksii* were monomorphic with an allele size of 64. However, *S.*

vernalis had an allele size of 61bp for this locus. Locus sp4-1A was also monomorphic across all samples of *S. cernua* and *S. parksii* analyzed, thus these particular loci were not informative and excluded from further analysis. Sp3-11B amplified with three peaks in *S. cernua* (of) and *S. parksii* and due to difficulties in interpretation, this locus was discarded as well, leaving seven polymorphic loci which were used in the analysis. Locus sp2-12C did not cross amplify in *S. magnicamporum*. Data from the ABI 3100 were scored manually in terms of allele size in base pairs from electropherogram peak traces (Fig 11).

Spiranthes vernalis did not cross amplify with many primers and was not useful for comparative purposes for *S. parksii*. When amplification was successful, some individuals of *S. vernalis* were difficult to interpret because they contained four peaks. These four peak profiles suggest polyploidy may also occur in this species. However, chromosome counts of *S. vernalis* indicate it is a diploid species and polyploidy as well as polyembryony are not known for this species. These peak traces might be a PCR artifact due to non-specific amplification. Regardless, the inclusion of *S. vernalis* did not help clarify relationships within the *S. cernua* complex in this study.

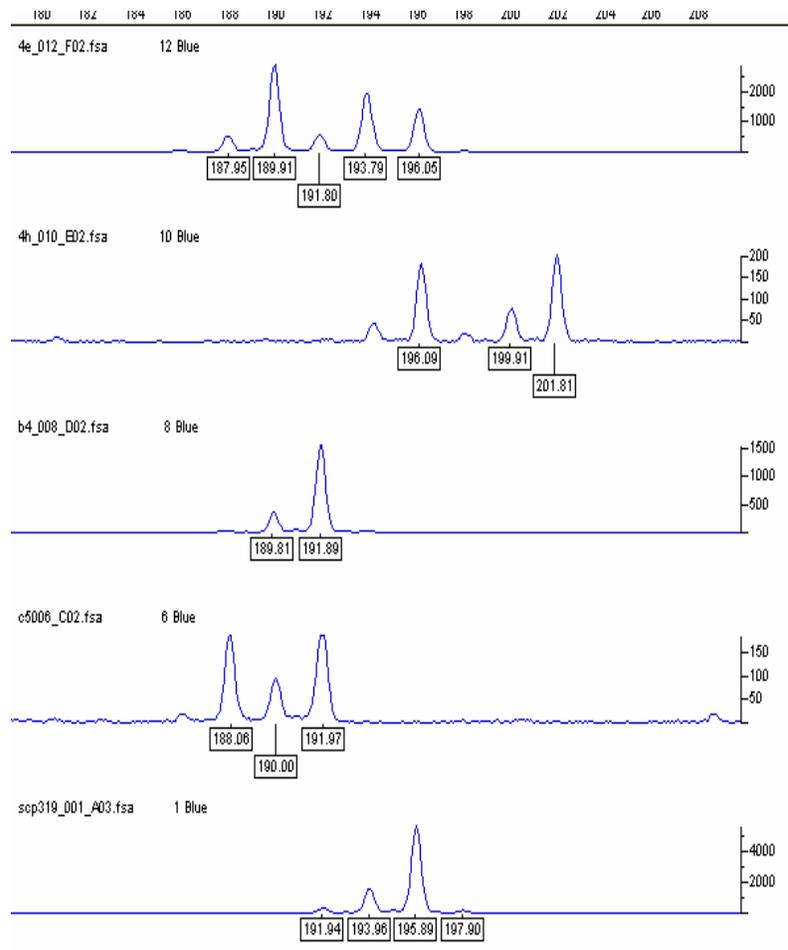


Fig. 11 Electropherogram of microsatellite traces. Individuals of *S. cernua* from eastern locations shown occur in both homozygous (third row) and heterozygous (first and second row) for different alleles at locus sp3-9A. The fifth row represents a homozygous individual of *S. cernua* (cf).

Allelic data were scored manually from peak traces and converted into a binary data matrix in which individuals either had a particular allele and were scored as “1” or did not have an allele for a given locus, and were scored as “0”. There were a total of 55 alleles from the seven loci. From these data, average pair wise distances were calculated.

Using *S. magnicamporum* as the outgroup, a neighbor joining tree was constructed in PAUP 4.02b (Fig. 12) and a bootstrap analysis was performed with 1,000 replicates (Fig. 13).

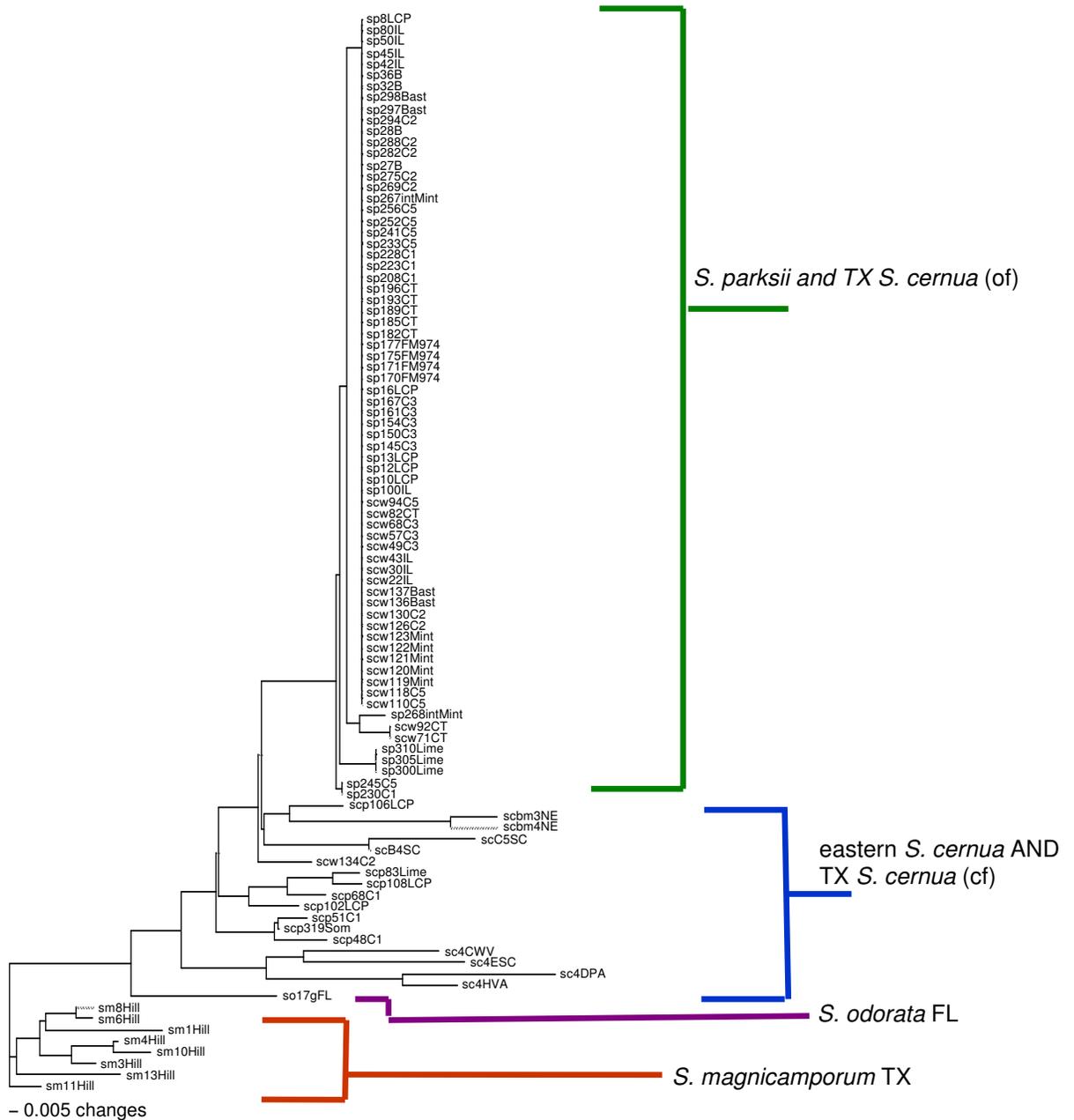


Fig. 12 NJ phylogram constructed from microsatellite data. Population designations are indicated at the end of the sample ID. sm= *S. magnicamporum*; sp= *S. parksii*; scw=*S. cernua* (of); scp= *S. cernua* (cf). so= *S. odorata*. Samples from PA, WV, SC, VA, NE and FL are indicated on the tree.

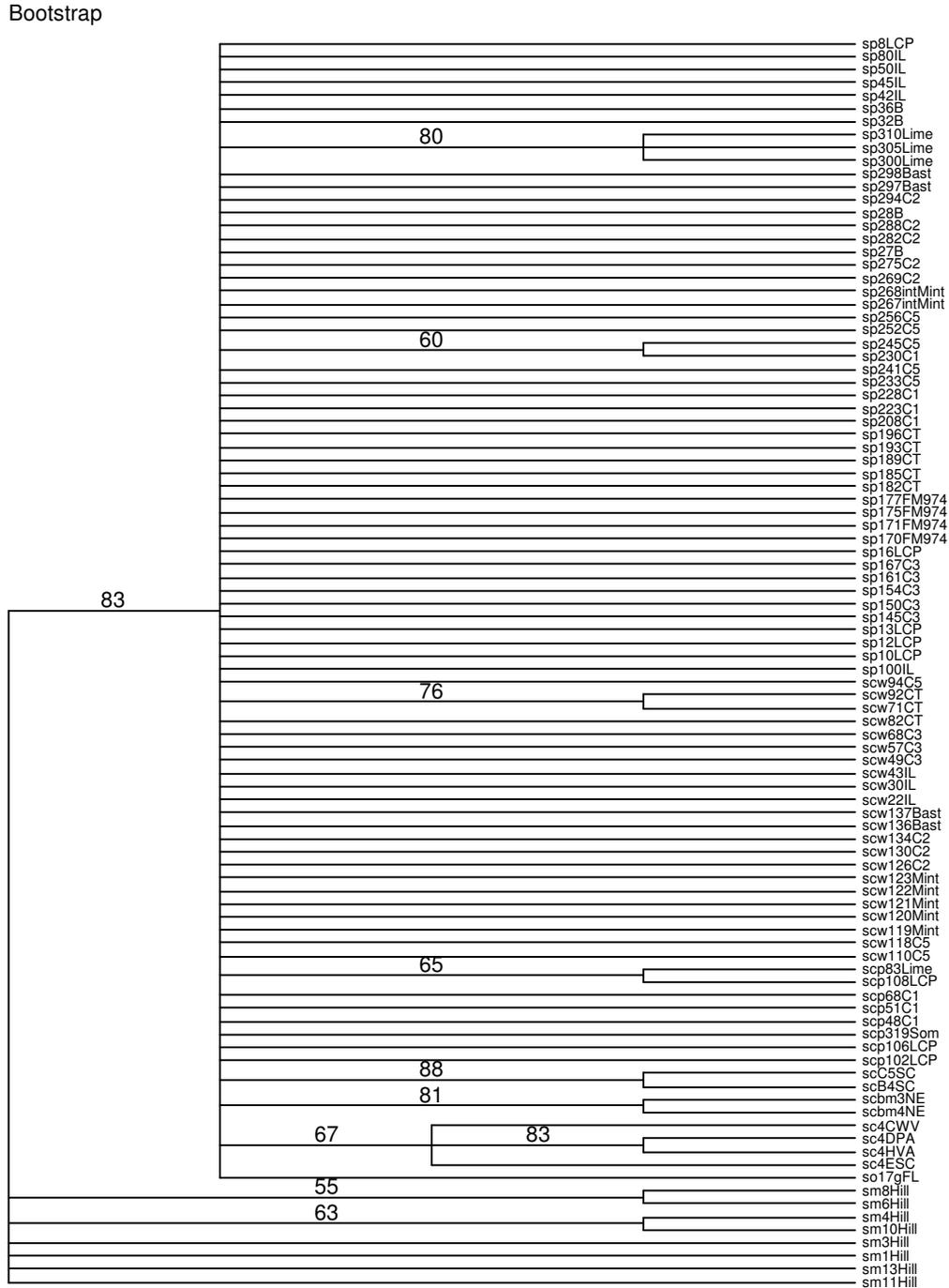


Fig. 13 Bootstrap consensus tree from microsatellite data. Population designations are located at the end of the sample ID. sm= *S. magnicamporum*; sp= *S. parksii*; scw=*S. cernua* (of); scp= *S. cernua* (cf); so = *S. odorata*. Samples from outside of TX are followed by their respective state abbreviations.

Few polymorphisms were detected among samples of *S. parksii* and *S. cernua* (of). Two samples of *S. parksii* and one *S. cernua* (of) (sp245, sp230 and scw134) from three different populations were homozygous at a locus where others were heterozygous. Another individual (sp268int), which was a morphological intermediate between *S. parksii* and *S. cernua* (of) contained an allele also found in *S. cernua* (cf). Two samples of *S. cernua* (of) (scw92 and scw71) from different populations in Brazos county also contained an allele that was also found in *S. cernua* (cf). Finally, three samples of *S. parksii* from Limestone County (sp300, sp305, sp310) contained a unique allele at locus 8D2 that was not present elsewhere in this dataset.

Individuals from Limestone County grouped together with bootstrap support of 80% due a unique allele at a single locus. Many of the eastern samples of *S. cernua* form groups with strong bootstrap support of 67% and higher. The topology of the tree otherwise is largely unstructured among samples of local *S. cernua* (of) and *S. parksii*, with the exception of a few samples which contain few informative polymorphisms. *Spiranthes parksii* and *S. cernua* (of) are nested within the *S. cernua* group, which also includes *S. cernua* (cf) and *S. cernua* from other states.

Observed heterozygosity was calculated for each taxon across loci (Table 4). Heterozygosity appeared to be fixed in four of the seven loci examined in both *S. parksii* and *S. cernua* (of) (locus sp2-11h, sp2-12c, sp4-9C and 8D2). A majority of these

samples were fixed for alleles 173bp and 177bp at locus sp4-9C. However, two individuals of *S. parksii* and one individual of *S. cernua* (of) were homozygous for allele 173bp. *Spiranthes magnicamporum* contained the lowest H_{obs} with an average value of .32.

The numbers of alleles per locus as well as average number of alleles across loci were calculated for each taxon (Table 5). *Spiranthes cernua* contained the highest average number of alleles per locus, while *S. parksii* and *S. cernua* (of) contained the lowest. The value for *S. cernua* (of) may be inflated due to sample “scw134”, which contains alleles that other samples of *S. cernua* (of) and *S. parksii* do not and is genetically more similar to *S. cernua* (of). *Spiranthes Magnicamporum*, as well as *S. cernua* (cf) showed intermediate values for this parameter. These values should be interpreted with caution as *S. magnicamporum* samples were from a single population, whereas *S. cernua* from eastern locations were from several, more geographically disjunct populations. Clearly, samples from several populations throughout the range would necessary to obtain unbiased estimates of these two parameters.

Table 4 Observed heterozygosity among taxa. H_{obs} is shown for each locus (columns 3-9) and then averaged across loci (AVE H_{obs}). Taxon (and form if applicable) is indicated on the left followed by samples size (N). NA indicates amplification was not successful.

Taxon	(N)	sp2-11h	sp2-12C	8D2	sp3-3a	sp3-9A	sp4-5E	sp4-9C	AVE H_{obs}
<i>S. parksii</i>	49	1.00	1.00	1.00	0.00	0.00	0.00	0.96	0.57
<i>S. cernua</i> (of)	22	1.00	1.00	1.00	0.00	0.00	0.00	0.95	0.56
<i>S. cernua</i> (cf)	8	0.88	0.38	0.50	0.00	0.25	0.75	0.13	0.41
<i>S. magnicamporum</i>	8	0.25	NA	0.00	0.29	0.38	1.00	0.00	0.32
<i>S. cernua</i> Eastern	10	0.50	0.57	0.50	0.50	0.60	0.60	0.71	0.57

Table 5 Number of alleles per locus. Average number of alleles is indicated by column “A”. Taxon (and form if applicable) is indicated on the left followed by samples size (N). NA indicates amplification was not successful.

Taxon	(N)	sp211h	sp212C	8D2	sp3 3a	sp39A	sp45E	sp4 9C	A
<i>S. parksii</i>	49	2.00	2.00	3.00	1.00	2.00	1.00	2.00	1.86
<i>S. cernua</i> (of)	22	2.00	2.00	2.00	2.00	3.00	1.00	2.00	2.00
<i>S. cernua</i> (cf)	8	3.00	4.00	2.00	2.00	4.00	2.00	3.00	2.86
<i>S. magnicamporum</i>	8	3.00	NA	1.00	4.00	2.00	2.00	4.00	2.67
<i>S. cernua</i> Eastern	10	4.00	7.00	3.00	7.00	8.00	3.00	6.00	5.43

Discussion

Breeding system plays a critical role in determining patterns of genetic diversity, and the limited genetic variation detected in *S. parksii* or between *S. cernua* (of) or *S. parksii* are likely due to high levels of asexual reproduction. The over-representation of specific genotypes suggests high levels of apomixis occur in both species and is in contrast with the previously held view that local *S. cernua* (of) was likely reproducing sexually with more frequency than *S. parksii*. The occurrence of fixed heterozygous profiles at several microsatellite loci are further support for an asexual reproductive mode and indicative that they are not suffering from inbreeding depression. The resulting tree (Fig 12) reflects these relationships and shows that *S. parksii* and *S. cernua* (of) together form a polytomy, which occupies much of the tree space.

Four loci (sp2-11H, sp2-21C, 8d2 and sp4-9C) were particularly interesting in that most individuals of *S. cernua* (of) and *S. parksii* contained two identical peaks and showed a fixed heterozygous profile. Individuals at one of these loci, “sp4-9C” (Fig 15), had alleles at 173bp and 177bp. Two explanations for these patterns are possible: The

first is that *S. parksii* and sympatric *S. cernua* (of) are of allopolyploid origin and each contributing parent was homozygous at these loci. If inheritance is disomatic (genomes are divergent enough that only two chromosome pairings are possible during meiosis), these alleles would not segregate into gametes even if sexual reproduction does occur, or they will be perpetuated by the apparently high levels of apomixis. In this scenario, an increase in ploidy level is expected based on the presence of two genomes and seems unlikely based on previous chromosome counts, which indicate no difference in ploidy levels and that both *S. cernua* and *S. parksii* are tetraploids, as are most other members of the *S. cernua* complex.

The other possibility is that these individuals show a true fixed heterozygosity due to heterozygous individuals passing their genotype on to progeny through apomixis. This pattern could be broken by recombination events, which would eventually segregate alleles, and result in individuals that are homozygous as well as heterozygous for these particular loci. This is the current favored model for *S. parksii* because three individuals, two *S. parksii* and one *S. cernua* (of) from three populations, were homozygous at this locus for allele 173, indicating that plants are capable of reproducing sexually, though at low frequencies.

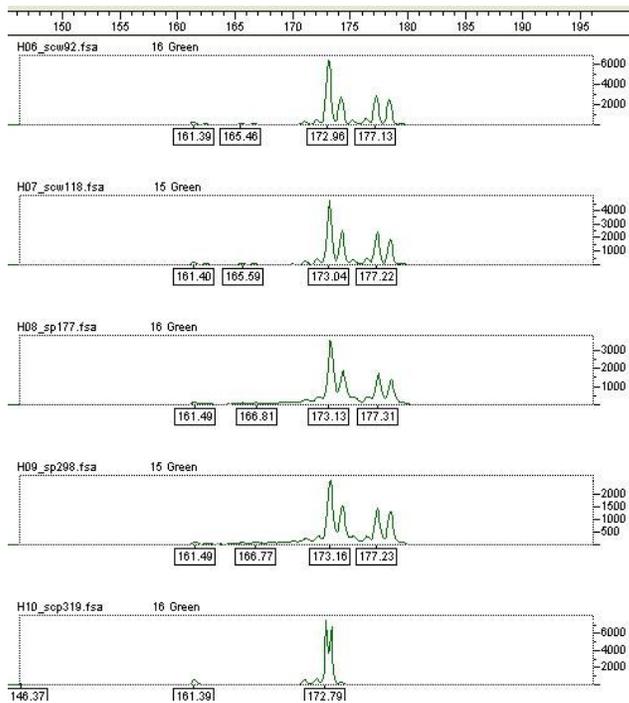


Fig 14. Individuals with a fixed heterozygous profile. Individuals of *S. cernua* (of) (rows one and two) and *S. parksii* (rows three and four) which have fixed heterozygous profiles at locus sp4-9C are shown. The bottom individual is an individual of *S. cernua* (cf) homozygous at the same locus.

Spiranthes cernua (cf) was expected to be mostly clonal through apomictic reproduction and to show less genetic variation than either *S. parksii* or *S. cernua* (of). It may seem counterintuitive that this form, recognized largely on the basis of having closed flowers, contained more genetic variation than similar taxa with open flowers. These results may be explained by this form's ability to produce open flowers as well as its preference for a more open habitat, providing better access to pollinators compared to individuals with open flowers in a woodland habitat, which is not the preferred habitat for the typical pollinators of *Spiranthes*.

It follows that a morphological discrimination of the peloric, closed flower form that occurs with open flowers from the open flower, woodland *S. cernua* may be problematic. One particular sample, “scw134”, illustrates this quandary as it was originally identified as the open flower form of *S. cernua*, but genetically it grouped with the closed flower samples of *S. cernua* (Fig. 12). Though they may be genetically differentiated, their overlapping habitat as well as similar morphology would make field identification messy and impractical among individuals with open flowers. From this perspective, Sheviak’s treatment of the two as forms seems justified.

This form may be reproducing apomictically, though it probably does not rely on asexual reproduction to the extent of either *S. parksii* or sympatric, woodland, *S. cernua* (of). There is some evidence that “races” which are intermediate in their reproductive mode, or capable of both apomixis and sexual reproduction, contain up to three embryos per seed but no more (Swamy 1948). An examination of the number of embryos per seed may offer additional support for the idea that the closed flower form is intermediate in terms of its ability to reproduce sexually as well as asexually. Based on the genetic data, this form is expected to contain fewer embryos per seed than either *S. cernua* (of) or *S. parksii*, both of which appear to be highly clonal and reproducing sexually only on rare occasions. Due to the current lack of understanding of the relationship between polyembryony and asexual reproduction, polyembryony should be used in conjunction with data from other sources (genetic or developmental) as this is an indirect character used to indicate asexual reproduction. However, the correlation between polyembryony and asexual reproduction is probably not strict.

Due to the controversy surrounding the use of microsatellite loci in non-focal taxa, a final note relating to cross amplification of microsatellite loci is warranted. Many caution against using microsatellites in non-focal taxa such as *S. cernua* and *S. magnicamporum* (those more distantly related to the one used to obtain microsatellite sequence data) to avoid ascertainment bias. Ascertainment bias results in an apparent, but biased reduction in genetic diversity due to the fact that regions with longer repeats are more variable and are typically selected for during the marker development phase. Compared to either *S. cernua* (of) or *S. parksii*, *S. magnicamporum*, a diploid taxon, contains more genetic variation in terms of number of alleles per locus (Table 5). It is not clear if ascertainment bias is affecting these results. The higher levels of genotypic as well as allelic diversity found in *S. magnicamporum* are unusual, particularly considering samples used in the analysis were from a single population. This result is likely a consequence of its sexual reproductive mode. *Spiranthes magnicamporum*, a diploid relative of *S. parksii*, did show slightly lower levels of average H_{obs} across loci (Table 4), which is consistent with results from other studies that show diploid species commonly harbor less heterozygosity than their polyploid relatives.

SUMMARY

Genetic Variability and Evolutionary Relationships of *S. parksii*

There has been much speculation on the origins of *S. parksii*, ranging from interspecific hybridization to founder events. However, there is no evidence that *S. parksii* contains alleles that are unique to any two potential progenitors. Also, though a founder event would result in a similar lack of genetic variation, it would result in the

more widespread, sympatric *S. cernua* showing much higher levels of diversity than its putative derivative, *S. parksii*. The founder hypothesis is not consistent with the observation of fixed heterozygosity at many of the microsatellite loci or with the similar average H_{obs} among the two (Table 4). Another explanation for the lack of diversity between the two species is that *S. parksii* and *S. cernua* (of) are recently derived species and have evolved few genetic differences, which have thus far gone undetected.

Though specific details of its origin (or origins) are still unknown, it seems that *S. parksii* and *S. cernua* (of) are both derivatives of the more widely distributed *S. cernua* complex. *Spiranthes parksii* and sympatric *S. cernua* (of) share alleles and contain less allelic variation than either *S. cernua* (cf) or *S. cernua* from other locations, which indicates they are likely very closely related.

These two taxa occur along the edge of the distribution of *S. cernua*. The apparent high level of apomixis in them may be an example of an adaptation to limited pollinator recruitment, which is often encountered along the periphery of a population. This idea has some anecdotal support from those who monitor populations, reporting little to no pollinator activity in *S. parksii*. Yet, others who have studied *S. cernua* in other geographic areas report many pollinators visiting flowers, which suggest a sexual reproductive mode may be more common for *S. cernua* from these other locations. A follow up on sexual populations of the complex from other geographic locations might provide additional support for this theory as populations with observed pollinator activity would be expected to harbor much higher levels of genetic variation. A previous study detected isozyme variation within populations of *S. cernua* in Nebraska suggesting

sexual reproduction is more common among individuals from this particular location (Unpublished, J. Schmidt 1992).

Alternatively, *Spiranthes* species occurring in woodland habitat may be specifically adapted to their habitat, which naturally has a low occurrence of potential pollinators, due to unique combinations of genes from sexual *S. cernua*. Asexual reproduction is possibly selected for in a woodland habitat for this reason and if reproductive mode is an expression of genetic rather than environmental factors, an increase in pollinators will not increase sexual reproduction.

There is some speculation that polyploid, asexual species accumulate “mutational junk” due to the buffering capacity conferred from the presence of an additional genome. It is feasible that sexual reproduction becomes selected against due to the potentially lethal alleles occurring in a homozygous state in sexually produced offspring. Thus, the asexual reproductive mode and polyploidy become selected for as well as tightly linked. However, empirical evidence to support this is lacking. The genetic controls of apomixis for this genus are a black box and without more detailed genetic and developmental studies, the amount of plasticity in the reproductive mode of *Spiranthes* will remain unknown.

Accomplished Objectives

All three molecular-genetic techniques employed in this study, chloroplast sequence from the non-coding trnT-trnF region, AFLPs and microsatellite markers were congruent in that they detected little or no genetic variation within and between *S. parksii* and the sympatric open flower (or “woodland”) form of *S. cernua*. The AFLP and microsatellite markers did detect

genetic differentiation of as well as genetic variation in the closed flower form of *S. cernua*, which suggest higher levels of sexual reproduction may occur in this group. Data from the microsatellite loci revealed the most detail concerning the breeding system of *S. parksii* as well as genetic relationships within the *S. cernua* complex. *Spiranthes parksii* and *S. cernua* (of) had basically identical genetic profiles with *S. cernua* (cf) and other representatives of the *S. cernua* complex from the eastern U.S. divergent from *S. parksii* and *S. cernua* (of). The only consistent difference observed in *S. parksii* and *S. cernua* (of) was in several *S. parksii* plants from Limestone County, where all three plants screened had a unique allele. One of the markers was heterozygous in most individuals of *S. parksii* and *S. cernua* (of) with only a few exceptions, which indicates that their reproductive mode is mostly apomictic, with occasional instances of sexual reproduction. Finally, the alleles present in both *S. parksii* and sympatric *S. cernua* (of) are also found in the more widely distributed closed flower form of *S. cernua* indicating their origin(s) are from this group. These data suggest *S. parksii* and sympatric *S. cernua* (of) share a very recent common ancestor. These results address Objective I in Methods. Results from this study offer support for a predominantly asexual mode of reproduction with the likelihood of occasional sexual reproduction, addressing Objective II in part. Unfortunately, the high genetic similarities between these two taxa prevents the use of the genetic markers described in this investigation to determine if gene flow is occurring between them (Objective III). The lack of genetic diversity in available microsatellite markers in *S. parksii* prevented their use in preserve design and in selecting germplasm for conservation and reintroduction efforts.

It should be clearly stated that in our studies, we found no positive evidence to conclude that *S. parksii* should be combined with *S. cernua*, or reclassified in any way. However, it was surprising that we did not observe differences in the molecular genetic markers between *S. parksii* and *S. cernua*, given their clear morphological and habitat preference differences. It should be pointed out that microsatellite markers are generally considered to be neutral markers and represent only a tiny fraction of the whole genome. There clearly are morphological differences between *S. parksii* and *S. cernua* that resulted in the designation of *S. parksii* as a separate species and most, if not all of these differences undoubtedly have a genetic basis. Further, there is clear ecological and habitat differentiation, or more specifically habitat specialization in the case of *S. parksii*, which also undoubtedly has a genetic basis. Unfortunately, locating and characterizing the responsible genes is not possible using currently affordable technologies, particularly given the breeding system of *S. parksii*.

Recent studies from canids and related mammals (Fondon and Garner 2004) showed that microsatellite mutation rates vary significantly between taxonomic groups. The low levels of microsatellite variation observed in *S. parksii* and closely related taxa could simply be the result of a low endogenous microsatellite mutation rate. However, the level of observed microsatellite variation was much higher in the very closely related species of *S. ordorata*, *S. magnicamporum*, and in the isolates of *S. cernua* from the eastern U.S. Further a low level of genetic differentiation was also observed using AFLPs and non-coding chloroplast sequences, which mutate by different sets of mechanisms. More likely, our combined data reflect a very recent divergence of *S. parksii* from the *S. cernua* complex, combined with a largely apomictic

breeding system with occasional sexual reproduction. Indeed apomixis can itself lead to rapid speciation through the nearly instantaneous fixation of genetic biotypes.

Further attempts at finding molecular genetic markers that show variability within *S. parksii* and among the related taxa should focus on the development of markers from loci with larger number of microsatellite repeat units than the ones used in this investigation. Microsatellite mutation rates and observed variability in populations are both proportional to the number of repeat units —the length of the microsatellite repeat (Innan et al. 1997). We recently developed an improved procedure for consistently isolating microsatellite markers with longer repeat structures than those used in this investigation. In the present study, the average number of dinucleotide repeats 17.5 units and the average number of trinucleotide repeats was 7.7 units. Very recently we have captured and sequenced microsatellites from *S. parksii* with trinucleotide repeats of 35 units and dinucleotide repeats of 61 units. We recently found that similar markers, with very long repeat lengths, uncovered significant allelic variability in the asexual clonally-reproducing species *Arundo donax*. Because of their very recent acquisition, we have not yet had the opportunity to test these new markers for variability in *S. parksii* populations. However, we feel that the employment of these new markers has significant potential for uncovering the extent and partitioning of genetic variability in extant *S. parksii* populations. At present, in the absence of genetic marker data to assist in the selection of appropriate germplasm for reintroduction, we recommend the collection of seeds from plants from the nearest known locality where *S. parksii* occurs naturally. Further, we recommend that preserve acquisition be targeted to the largest populations with lowest threat of future disturbance or environmental alteration in the surrounding area; such populations will have the

greatest chance of long-term viability. These results and recommendations will be incorporated into the new Recovery Plan (Objective IV).

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SIGNIFICANT DEVIATIONS

Objective II (**Grow accessions of S. parksii from seed to determine the extent of sexual reproduction, and to develop protocols for possible eventual reintroduction efforts.**) was accomplished in part. The extent of sexual reproduction was estimated to be very low, based on the very low levels of recombination in the microsatellite marker profiles. The year in which we planned to collect seeds, 2005, was a very poor year for the orchids and we decided that it would not be in the best interests of conserving the plant to remove germplasm from natural populations under those conditions. One of the goals in collecting seed from a range of populations was to sample as much genetic diversity as possible, using the molecular genetic markers as a guide. Since we could find no consistent differences in the markers, with one exception (Limestone County), different criteria need to be developed to guide germplasm collection. This could not be accomplished during the duration of the project.

Objective III (**Determine levels of gene flow between S. parksii and S. cernua**) could not be accomplished. Microsatellite markers are generally considered to be the most variable and most informative of molecular genetic markers for studies of gene flow. We screened several hundred microsatellite loci, but could find no consistent differences between these two taxa for any of the markers. This lack of variation in genetic markers prevented us from determining levels of gene flow between *S. parksii* and *S. cernua*. It may be possible to do this part of the research in a limited way due to presence of a different *S. parksii* genotype in Limestone County, assuming that *S. cernua* is present in that area. However, this information

did not become available until late in the project and we were not able to follow it up in the time allotted to the project.