

## ORIGIN AND CONSERVATION GENETICS OF THE THREATENED UTE LADIES'-TRESSES, *SPIRANTHES* *DILUVIALIS* (ORCHIDACEAE)<sup>1</sup>

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The Ute ladies'-tresses, *Spiranthes diluvialis*, is listed as a threatened orchid in west-central United States by the Federal government. Information on its origin and patterns of genetic variation is needed to develop effective conservation strategies for this species. DNA sequencing and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to evaluate genetic variation and structure of 23 populations of *S. diluvialis*. In addition, four congeneric species were analyzed to determine possible origins of the putative allotetraploid *S. diluvialis*. DNA sequencing and PCR-RFLP analysis of the nuclear ribosomal internal transcribed spacer (ITS) and mitochondrial and chloroplast DNA noncoding regions revealed no genetic variation within or among populations of *S. diluvialis*. DNA sequencing revealed that *S. diluvialis* has rDNA of both *S. magnicamporum* and *S. romanzoffiana*, supporting the proposed origin of the allotetraploid. Parsimony and maximum likelihood analyses of cpDNA and mtDNA sequences revealed that these *S. diluvialis* organellar sequences were most closely related to those of *S. romanzoffiana*, providing evidence that the latter species is the maternal parent of *S. diluvialis*. The lack of genetic diversity is significant for the development of a long-term conservation strategy for *S. diluvialis*.

**Key words:** phylogenetics; population genetics; Orchidaceae; *Spiranthes*; threatened species.

The Ute ladies'-tresses (*Spiranthes diluvialis* Sheviak) (Orchidaceae) is a terrestrial, insect-pollinated orchid (Sipes and Tepedino, 1995), with scattered populations throughout west-central United States (Dressler, 1981; Heidel, 1997; Hildebrand, 1998). Sheviak (1984) suggested that, based on morphological and cytological data, *S. diluvialis* ( $2n = 74$ ) originated as the allopolyploid offspring of a cross between *S. magnicamporum* Sheviak ( $2n = 30$ ) and *S. romanzoffiana* Chamisso ( $2n = 44$ ). In 1992, *S. diluvialis* was listed as a federally threatened species because of relatively low population numbers, fluctuations in monitored population size, loss of the species' riparian habitat through urban development, stream channelization, recreational development, and exotic species invasion (U.S. Fish and Wildlife Service, 1992).

Comparative population studies using both biochemical (allozyme) and molecular genetic methods are needed to obtain information on the levels and patterns of genetic diversity in wild orchids (Wong and Sun, 1999). Several aspects of conservation biology, such as determining the extent of genetic diversity in conservation programs and restoration of threatened populations, can only be addressed by detailed population genetic studies (Hamrick and Godt, 1995).

A previous allozyme study by Arft and Ranker (1998) revealed high levels of genetic variation within but low levels of genetic differentiation among 12 populations of *S. diluvialis*

from Utah and Colorado. In recent years, several new and widely distributed Ute ladies'-tresses populations have been found that have not been subjected to genetic analyses. The objective of the present study was to determine the extent and distribution of genetic variation within and among populations of *S. diluvialis* representing its entire geographical range. This information may be valuable for conservation and recovery planning efforts for the species (U.S. Fish and Wildlife, 1995). DNA sequence variation analysis of nuclear and maternally inherited chloroplast and mitochondrial DNA was conducted to test the hypothesis that *S. magnicamporum* and *S. romanzoffiana* are the parental species of *S. diluvialis*. We employed DNA sequencing and polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLP) of nuclear and maternally inherited noncoding DNA regions for this study. PCR-RFLP is a relatively simple, reliable, repeatable, and inexpensive technique that uses dried specimens unlike allozyme analysis and provides discreet character states that can be used for phylogenetic analyses and diagnostics (Taylor and Szalanski, 1999).

### MATERIALS AND METHODS

**Plant materials**—Leaf samples were collected from 22 populations of *S. diluvialis* throughout its geographical range in full compliance with a permit issued to G.S. by the U.S. Fish and Wildlife Service (Table 1). Populations of *S. magnicamporum*, *S. romanzoffiana*, *S. cernua* (L.) L.C. Richard, and *S. vernalis* Engelman and Gray were also sampled (Table 1). Western prairie fringed orchid, *Platanthera praeclara* Sheviak and Bowles, was collected to act as the outgroup taxon. Individual leaf samples, 10 × 60 mm, were stored in plastic bags with silica gel prior to analysis. Whole-plant vouchers of each species were deposited in the Charles E. Bessey Herbarium, University of Nebraska State Museum, Lincoln, Nebraska, USA. Voucher leaf samples from each sampled orchid were deposited in the plant materials collection of the Nebraska Game and Parks Commission, Lincoln, Nebraska, USA.

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TABLE 1. Summary of the species sampled, their location, and the number of individuals analyzed from each population.

Species	Site (no.) of collection
<i>Spiranthes diluvialis</i> Sheviak	Boulder Co., Colorado (16); Jefferson Co., Colorado (5); Moffat Co., Colorado (4); Madison Co., Idaho (5); Jefferson Co., Idaho (5); Bonneville Co., Idaho (10); Beaverhead Co., Montana (5); Gallatin Co., Montana (4); Jefferson Co., Montana (5); Madison Co., Montana (5); Sioux Co., Nebraska (10); Utah Co., Utah (10) Unitah Co., Utah (4); Garfield Co., Utah (5); Okanogan Co., Washington (5); Converse Co., Wyoming (4); Niobrara Co., Wyoming (4); Goshen Co., Wyoming (5); Laramie Co., Wyoming (5)
<i>S. vernalis</i> Engelman and Gray	Lancaster Co., Nebraska (1); Pottawattamie Co., Iowa (1)
<i>S. magnicamporum</i> Sheviak	Pottawattamie Co., Iowa (2); Rock Co., Nebraska (3); Banner Co., Nebraska (3); Pierce Co., Nebraska (3)
<i>S. romanzoffiana</i> Chamisso	Boulder Co., Colorado (4); Bonneville Co., Idaho (1); Albany Co., Wyoming (4)
<i>S. cernua</i> (L.)	Sheridan Co., Nebraska (4); Garfield Co., Nebraska (1)
<i>Platanthera praeclara</i> Sheviak and Bowles	Diecken Prairie, Nebraska (2)

**DNA analysis**—DNA was isolated from individual 5 × 30 mm leaf tissue samples using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, Minnesota, USA). The nuclear ribosomal DNA ITS1 and ITS2 regions were amplified using the primers ITS5 and ITS4 (Baldwin, 1992). The cpDNA tRNA-Ser to tRNA-fMET noncoding region was amplified with the universal primers *trnS* and *trnM* (Demesure, Sodzi, and Petit, 1995). A portion of the mtDNA NAD 7 gene noncoding region was amplified with the universal primers NAD7-1 and NAD7-2 (Demesure, Sodzi, and Petit, 1995). PCR was conducted using 1.0 µL of isolated DNA per Szalanski et al. (2000) with the thermocycler conditions outlined by Baldwin (1992) for the ITS marker and Demesure, Sodzi, and Petit (1995) for the cpDNA and mtDNA markers.

For DNA sequencing of the rDNA, mtDNA, and cpDNA markers, amplified DNA from five *S. diluvialis* (Boulder County, Colorado; Madison County, Idaho; Jefferson County, Montana; Unitah County, Utah; and Sioux County, Nebraska), one *S. vernalis* (Lancaster County, Nebraska), one *S. magnicamporum* (Pottawattamie County, Iowa), one *S. romanzoffiana* (Boulder County, Colorado), one *S. cernua* (Garfield County, Nebraska) and one *P. Praeclara* was purified following Szalanski et al. (2000) and sent to the DNA Sequencing Facility, Iowa State University (Ames, Iowa, USA) for direct sequencing in both directions. Consensus sequences for each individual were derived using GCG (Genetics Computer Group, Madison, Wisconsin, USA) GAP program. The GenBank accession numbers for each sequence are GBAN-AF243329–AF243333, GBAN-AF301440–AF301445, and GBAN-AF301464–AF301469.

For PCR-RFLP analysis of the specimens that were not subjected to DNA sequencing, restriction sites were predicted from the DNA sequence data using Webcutter 2.0 (Heiman, 1997). Amplified DNA was digested according to manufacturer's (New England Biolabs, Beverly, Massachusetts) recommendations per Cherry et al. (1997) using the restriction enzymes *Hha* I, *Hinf* I, *Msp* I, and *ScrF* I for the ITS amplicon, *Alu* I, *Apo* I, and *Dpn* II for the cpDNA amplicon, and *Alu* I, *Rsa* I, and *ScrF* I for the mtDNA amplicon. Fragments were separated by vertical polyacrylamide gel electrophoresis (PAGE) following Taylor et al. (1997).

**Data analysis**—Ribosomal, chloroplast, and mitochondrial DNA sequences were aligned with GCG PILEUP program (with a gap weight of 5.0 and a gap length weight of 1.0) and adjusted manually using *Platanthera praeclara* as the outgroup taxon. Maximum likelihood and unweighted parsimony analysis on the alignments was conducted with PAUP\* 4.0b2 (Swofford, 1999). Gaps were treated as missing characters for all analysis. The reliability of trees was tested with a bootstrap test (Felsenstein, 1985), which included 1000 resamplings. For the maximum likelihood analysis, rates were assumed to follow a gamma distribution with shape parameter estimated via maximum likelihood based on the general-time-reversible model (GTR) (Yang, 1994). A total of 73 distinct data patterns were used under this model for the rDNA ITS1 data set, 150 for the mtDNA data set, and 31 for the

cpDNA data set. The starting branch lengths were obtained using the Rogers-Swofford approximation method.

## RESULTS AND DISCUSSION

The ITS amplicon was 746 bp in size for *S. diluvialis*, *S. magnicamporum*, and *S. romanzoffiana*. The cpDNA amplicon was 947 bp in size for *S. diluvialis* and ranged from 921 to 965 bp among the other four *Spiranthes* species. The mtDNA amplicon was 1147 bp for all of the *S. diluvialis* and varied from 1085 to 1153 among the other *Spiranthes* taxa. PCR-RFLP analysis of the rDNA ITS marker from 116 *S. diluvialis* using four restriction enzymes detected 22 sites and screened 98 bp or 13% of the 746 bp amplicon. Analysis of the cpDNA marker detected 12 sites and screened 6% of the amplicon and PCR-RFLP of the mtDNA marker detected 15 sites and screened 67 bp. No genetic variation was observed among the 116 *S. diluvialis* subjected to RFLP analysis. PCR-RFLP did not detect any intraspecific genetic variation in the other sampled *Spiranthes* taxa using the restriction enzymes used for *S. diluvialis*, but did differentiate the five *Spiranthes* taxa.

DNA sequencing of five *S. diluvialis* revealed no genetic variation in the rDNA, mtDNA, and cpDNA amplicons. The rDNA sequences of the five *Spiranthes* species were aligned using *Platanthera praeclara* as the outgroup taxon. The alignment of the rDNA sequences, resulted in a total of 781 characters, including gaps. Of these characters, 265 (34%) were variable and 18 (2%) were informative. The rDNA data set had only one most parsimonious tree (length = 294, CI = 0.98, CI excluding uninformative sites = 0.76) as documented using the Exhaustive search algorithm of PAUP\*, which examined all possible trees of this data set. Bootstrap analysis of the aligned *Spiranthes* species and *P. praeclara* rDNA ITS1 sequences revealed that *S. vernalis*, and *S. cernua* formed a distinct clade and *S. romanzoffiana* was the most divergent *Spiranthes* taxon (Fig. 1). Regardless of whether the starting tree was the most parsimonious tree or was obtained via stepwise addition, the maximum likelihood search found only one tree. This maximum likelihood tree,  $-\ln$  likelihood = 2164.37635, was similar to the most parsimonious tree. Phylogenetic analysis of the ITS data matrix reveals *S. diluvialis* and *S. magnicamporum* as the sister to the *S. vernalis*–*S. cernua* clade. *Spiranthes romanzoffiana* is supported as the sister

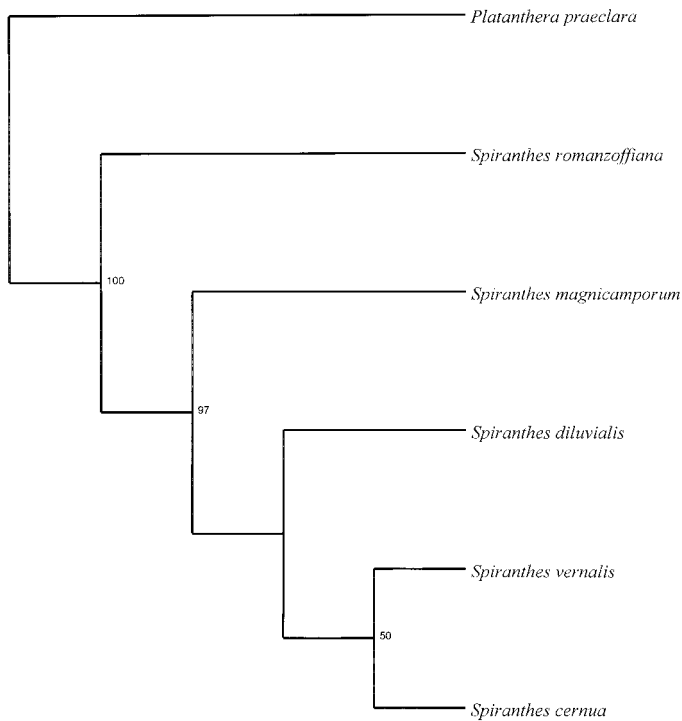


Fig. 1. Maximum parsimony cladogram generated by PHYLIP from five *Spiranthes* spp. ITS1 and ITS2 amplicon sequences. *Platanthera praecleara* is the outgroup taxon. Bootstrap values from 1000 data sets are provided.

clade to all of the other species of *Spiranthes*. The lack of a distinct relationship of *S. diluvialis* with *S. romanzoffiana* or *S. magnicamporum* may be due to heterogeneity at nine nucleotide sites within the *S. diluvialis* sequences. Analysis of the DNA sequence data in an electropherogram format confirmed that the heterogeneity was from nucleotide polymorphism in *S. romanzoffiana* and *S. magnicamporum* being present at the same nucleotide site in *S. diluvialis*.

The alignment of the mtDNA sequences resulted in a total of 1275 characters, including gaps. Of these characters, 157 (12%) were variable and 24 (2%) were parsimony informative. Both the parsimony analysis and maximum likelihood analysis ( $-\ln$  likelihood = 2712.77886) found only one tree (Fig. 2). *Spiranthes diluvialis* formed a sister group with *S. romanzoffiana*, and *S. magnicamporum*, *S. vernalis*, and *S. cernua* formed a distinct clade. All of the phylogenetic relationships were supported by >50% of the bootstraps. Because of a large number of tandem repeats in the 3' end of the cpDNA sequences, the sequences were truncated. The alignment of the truncated cpDNA sequences resulted in a total of 512 characters, including gaps. Of the 512 characters, 39 (8%) were variable and 3 (1%) were parsimony informative. The maximum likelihood tree ( $-\ln$  likelihood = 888.01117) found only one tree. Results from the cpDNA data set were similar to those from the mtDNA dataset (Fig. 2).

Because *S. diluvialis* is thought to be an allotetraploid between *S. romanzoffiana* and *S. magnicamporum* (Sheviak, 1984; Arft and Ranker, 1998), the most parsimonious explanation of the rDNA heterogeneity of *S. diluvialis* involves additivity of the rDNA repeat of *S. romanzoffiana* with those of *S. magnicamporum*. This additivity in allotetraploids has been

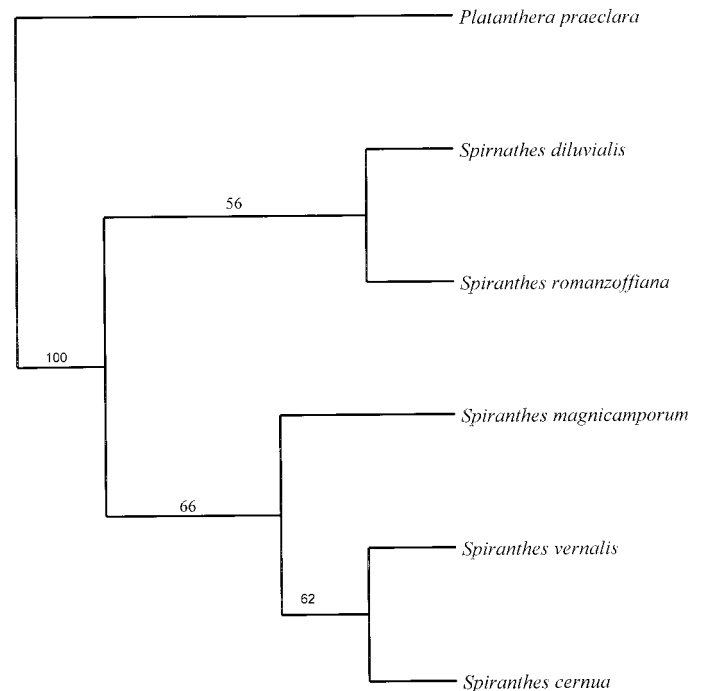


Fig. 2. Maximum parsimony cladogram generated by PHYLIP from the cpDNA amplicon sequences of five *Spiranthes* spp. *Platanthera praecleara* is the outgroup taxon. Bootstrap values from 1000 data sets are provided.

observed in other plant species including *Tragopogon mirus* Ownbey (Soltis and Soltis, 1991; Soltis et al., 1995).

Phylogenetic analysis of mtDNA and cpDNA, which is maternally inherited, revealed that *S. diluvialis* was more closely related to *S. romanzoffiana* than to *S. magnicamporum* providing evidence that *S. romanzoffiana* is the maternal parent of *S. diluvialis*. Our results support previous biochemical (Arft and Ranker, 1998) and morphological evidence (Sheviak, 1984) that *S. romanzoffiana* and *S. magnicamporum* are the parental species of *S. diluvialis*. In addition, based on cpDNA and mtDNA sequences, *S. romanzoffiana* appears to be the maternal parent of *S. diluvialis*.

There is no evidence for phylogeographic differentiation in *S. diluvialis* based on DNA sequencing and PCR-RFLP analysis of 116 individuals from 23 populations. This finding is supported by a previous allozyme study. Arft and Ranker (1998) concluded that the high level of allozyme diversity observed within populations of *S. diluvialis* appeared to be the result of its allopolyploid condition. This is supported by high levels of fixed, or nearly fixed, heterozygosity at several loci. In addition, allozyme divergence among *S. diluvialis* populations was low relative to other animal-pollinated, outcrossed, diploid species (Arft and Ranker, 1998; Hamrick et al., 1991). Low levels of genetic variation have been observed in *Spiranthes hongkongensis* Hu & Barr. (Sun, 1997).

The ultimate goal of conservation is to ensure the continuous survival of populations and to maintain their evolutionary potential (Hamrick and Godt, 1995). Priority for genetic conservation is based on the level of genetic diversity and the degree of gene differentiation between populations (Coates and Sokolowski, 1992).

The present study revealed no genetic differentiation among or within populations representing the known geographical

range of *S. diluvialis* using ITS, cpDNA, and mtDNA non-coding markers. Based on this, no populations of *S. diluvialis* are presently known that would be conservation priorities because of their genetic uniqueness.

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