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SEQUENCING RE-DEFINES *SPIRANTHES* RELATIONSHIPS, WITH IMPLICATIONS FOR RARE AND ENDANGERED TAXA

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Introduction

Species delimitation in the genus *Spiranthes* L.C. Richard (Spiranthinae, Cranichideae, Orchidoideae) has long been problematic, due mainly to morphological polymorphism confounded by hybridization and polyploidy, particularly in the *S. cernua* (L.) Rich. species complex (Correll 1950, Luer 1975, Sheviak 1982). Official records indicate that 462 taxa names have been used for *Spiranthes* historically (RBG-Kew 2006), but less than a tenth of those are recognized today, and there is still concern about the species status of some. Although *Spiranthes* is considered to have a worldwide distribution, only eight of these occur outside of temperate North America.

All 26 currently recognized *Spiranthes* taxa in the Flora of North America (Sheviak & Brown 2002) have some form of conservation listing in a U.S. state (except *S. casei* var. *novascotiae* Catling, found only in Canada), due mainly to tenuous occurrence at the edge of their range in those locations. The less-serious listing denominations include: Exploitably Vulnerable, Rare, Sensitive, and Special Concern. Two unrecognized taxa are other exceptions – *S. americana* (either extirpated or a synonym for *S. torta* [Thunb.] Garay & H.R.Sweet) is Proposed Endangered by Florida due to endemism and rarity, and newly described *S. sylvatica* P.M.Brown (Brown 2001a) has not been listed by any state.

Most of these *Spiranthes* taxa are also federally or state-listed as Threatened, Proposed Endangered, or Endangered (Table 1). However, some taxa, such as those endemic to or now limited to one or few locations, should be targeted for special protection. These include: *S. brevibrabris* Lindl., *S. delitescens* Sheviak, *S. eatonii* Ames ex P.M.Br., *S. floridana* (Wherry) Cory, *S. infernalis* Sheviak, *S. parksii* Correll, and *S. torta*. Federally threatened *S. diluvialis* Sheviak is not

endemic to one area, but rare throughout its range and unusual as an allopolyploid. Other taxa that can also be identified as genetically important as well as rare should be targeted for maintenance of biodiversity.

Molecular genetic techniques can provide a suite of markers from which to choose the scale of taxonomic discrimination required (Soltis & Soltis 1998, Soltis & Gitzendanner 1998, Avise 2004). Nucleotide sequencing, particularly of several genes in combination, is successfully used to address issues of phylogenetics and species delimitation, critical when conservation resources to protect threatened and endangered taxa must be focused. It is thus our goal in determining phylogenetic relationships among *Spiranthes* through sequence analysis to help identify these unique taxa and verify the taxonomic status of the endemic group members. Circumscribing the genetic individuality of these species of concern is a basic foundation on which to build further conservation efforts.

Methods

As part of a larger *Spiranthes* phylogeny project, all 27 taxa found in temperate North America have now been sampled except *S. casei* var. *novascotiae* and *S. ovalis* var. *ovalis*. Voucher specimens, when available, were deposited in the Clemson (SC) University herbarium (CLEMS). Three *Spiranthes* found in Europe and Asia (*S. aestivalis* [Poir.] Rich., *S. sinensis* [Pers.] Ames, *S. spiralis* [L.] Chevall.), as well as an outgroup taxon (*Sacoila lanceolata* var. *lanceolata* [Aubl.] Garay), were also included in the analyses.

DNA was extracted from the plant tissue, and four genes/regions representing all three genomes were sequenced according to protocols outlined in Dueck et al. (2005). The DNA segments analyzed include

TABLE 1. Critical conservation listings for *Spiranthes* in the U.S.

SPECIES	FEDERAL STATUS	GLOBAL RANKING	STATE STATUS	STATE RANKING	STATE	NOTES
<i>S. brevibrabis</i>		G1	E	S1	FL	1 extant FL site
<i>S. casei</i> v. <i>casei</i>		G4	E	S1	NH, PA	
<i>S. delitescens</i>	E	G1	HS	S1	AZ	endemic to 1 AZ site
<i>S. diluvialis</i>	T	G2	E T	S1 S2, S1	WA MT, NE	found in 8 states
<i>S. eatonii</i>			PE		FL	2 extant FL sites
<i>S. floridana</i>		G1	PE	S1	FL	1 extant FL site
<i>S. infernalis</i>		G1	T	S1	NV	endemic to 1 NV site
<i>S. lacera</i>			T		IA	
<i>S. lacera</i> v. <i>gracilis</i>		G5	X/PE		ME	
<i>S. laciniata</i>		G4G5	E T/E T	S1	NJ NC FL SC	
<i>S. longibrabis</i>		G3	T T/PE		NC FL	
<i>S. lucida</i>		G5	E T/E T	S1	IL, IA, MD NC KY, ME, NH, TN	
<i>S. magnicamporum</i>		G3G4	E T	S1	GA, IN, NM KY	
<i>S. ochroleuca</i>		G4G5	E T/E T	S2	MD, TN NC IN	
<i>S. odorata</i>		G5	X/E E		MD KY, TN	
<i>S. ovalis</i> v. <i>eros</i> .		G5	E T	S1	FL, PA IA, MI, seUS	
<i>S. ovalis</i> v. <i>ovalis</i>		G5	E		FL	
<i>S. parksii</i>	E	G3	E	S3	TX	endemic to east TX
<i>S. romanzoffiana</i>		G5	E E T	S1	IN MA, IL, PA IA, OH	
<i>S. torta</i>		G4	E	S1	FL	endemic to south FL
<i>S. tuberosa</i>			E T/E T		RI NJ FL	
<i>S. vernalis</i>		G5	E T		IL, NH, NY, PA MA, IA	

Main source: <http://plants.usda.gov/threat.html>

X = Extirpated/Historical
E = Endangered
PE = Proposed Endangered
T = Threatened
HS = Highly Safeguarded

G1 = critically imperiled globally
G2 = imperiled globally due to rarity/vulnerability
G3 = very rare throughout range or found restricted locally
G4 = apparently secure globally but rare in some places
G5 = demonstrably secure globally but rare in some places
S1 = critically imperiled in state; **S2** = imperiled in state

two plastid regions – a non-coding section of *trnS-fM* and the *trnL* intron, one non-coding region in mitochondrial gene *NAD7*, and the nuclear ribosomal ITS region including ITS 1, 5.8S, and 2. Resulting electropherograms were contiged and edited, and the four matrices of consensus sequences were aligned. These data sets were then analyzed using the parsimony criterion in PAUP*, and support values for relationships were calculated by performing jackknife analyses of 5000 replicates. Since the separate gene trees were concordant, data were also combined and analyzed together, but only samples complete for all four genes were included.

Because the main project is still in progress, however, the four-gene cladogram shown here (from June 2006) does not include many samples subsequently collected. These later samples have been sequenced, but their analyses not rigorously tested yet, so their results will be discussed as preliminary findings only.

Results

Of the 123 total samples analyzed by June 2006, 99 samples were complete with all four gene sequences and thus were used to produce the figure included. Two taxa subsequently sampled (*S. porrifolia* Lindl., *S. torta*) were not included in this analysis, but their placement is discussed below in light of more recent analyses. Other taxa not included due to unavailability are *S. casei* var. *novascotiae*, *S. lacera* var. *lacera* (Raf.) Raf., and *S. ovalis* var. *ovalis* Lindl. Over 3500 base pairs (bp) were used in the combined analysis, and statistically the ITS 1-2 segment had the most variable (26%) and informative (18%) sites. However, the *trnS-fM* segment had the best-resolved topology for a single-gene tree (not shown).

A strict consensus cladogram of the *Spiranthes* phylogeny based on the four-gene combined analysis is shown in Figure 1, with jackknife support values added above branches. There is strong support (99%) for a division of the genus into two major groups with some strong species clades within each. The lower group, a paraphyletic grade of taxa, contains distinct species *S. infernalis* and *S. lucida* (H.H.Eaton) Ames, and a weakly supported *S. romanzoffiana* Cham. clade in which strongly supported *S. delitescens* and unsupported but separate *S. diluvialis* reside. In single trees from maternally inherited plastid and mitochondrial genes, *S. diluvialis* was within the same clade as *S. romanzoffiana*, but in the tree based on nuclear ITS sequences, it was within the clade containing *S. mag-*

nicamporum Sheviak (not shown). A moderately supported division (81%) within the lower paraphyletic grade separates the latter five species from strong clades of combined *S. praecox* (Walter) S.Watson and *S. sylvatica* (no distinction between them shown from this analysis), all three Old World species, and *S. tuberosa* Raf. The upper group contains a moderately supported clade of closely related species, with *S. brevilabris* and *S. floridana* as distinct, but with *S. eatonii* and *S. lacera* as indistinguishable from each other. *S. vernalis* Engelm. & A.Gray, *S. laciniata* (Small) Ames, and *S. longilabris* Lindl. are monophyletic and thus strongly supported species. *S. ovalis* var. *erostellata* appears as a strongly supported subclade within a broad unsupported *S. magnicamporum-S. odorata* (Nutt.) Lindl. group. *S. odorata* is polyphyletic, appearing within no fewer than three different clades. The two remaining derived clades consist of moderately strong support (86%) for a *S. cernua-S. parksii* clade in which *S. parksii* is indistinguishable, and an unsupported clade with *S. casei* Catling & Cruise and *S. ochroleuca* (Rydb.) Rydb. separate but also unsupported. No distinct identity for *S. cernua* was supported from these samples and analysis.

Preliminary results from “work-in-progress” analyses (not shown) indicate some important findings for species of interest to conservation biologists. Differentiation of *S. eatonii* from *S. lacera* was not resolved by additional *S. lacera* samples. *S. porrifolia* is very closely related to *S. infernalis* and in fact may be hybridizing in the southwestern limits of its range, but the clade containing both species are distinct from and sister to *S. romanzoffiana*. Our samples of *S. torta* are sister to *S. laciniata* (and both to *S. brevilabris*) in the maternal genomes, but to *S. floridana* when the nuclear genome is included. And although inclusion of more samples of *S. cernua* is providing a better species identity for it along a northeastern Appalachian swath, *S. parksii* remains imbedded within the clade of southern *S. cernua*.

Discussion

For the first time, our phylogeny of *Spiranthes*, based on molecular data from all three genomes, is revealing the relationships among taxa found in the United States and abroad. Whereas several other aspects of the study are interesting, our goal here is to focus on known target species (endemic/endangered) and define any new species (unique/rare) of conservation concern.

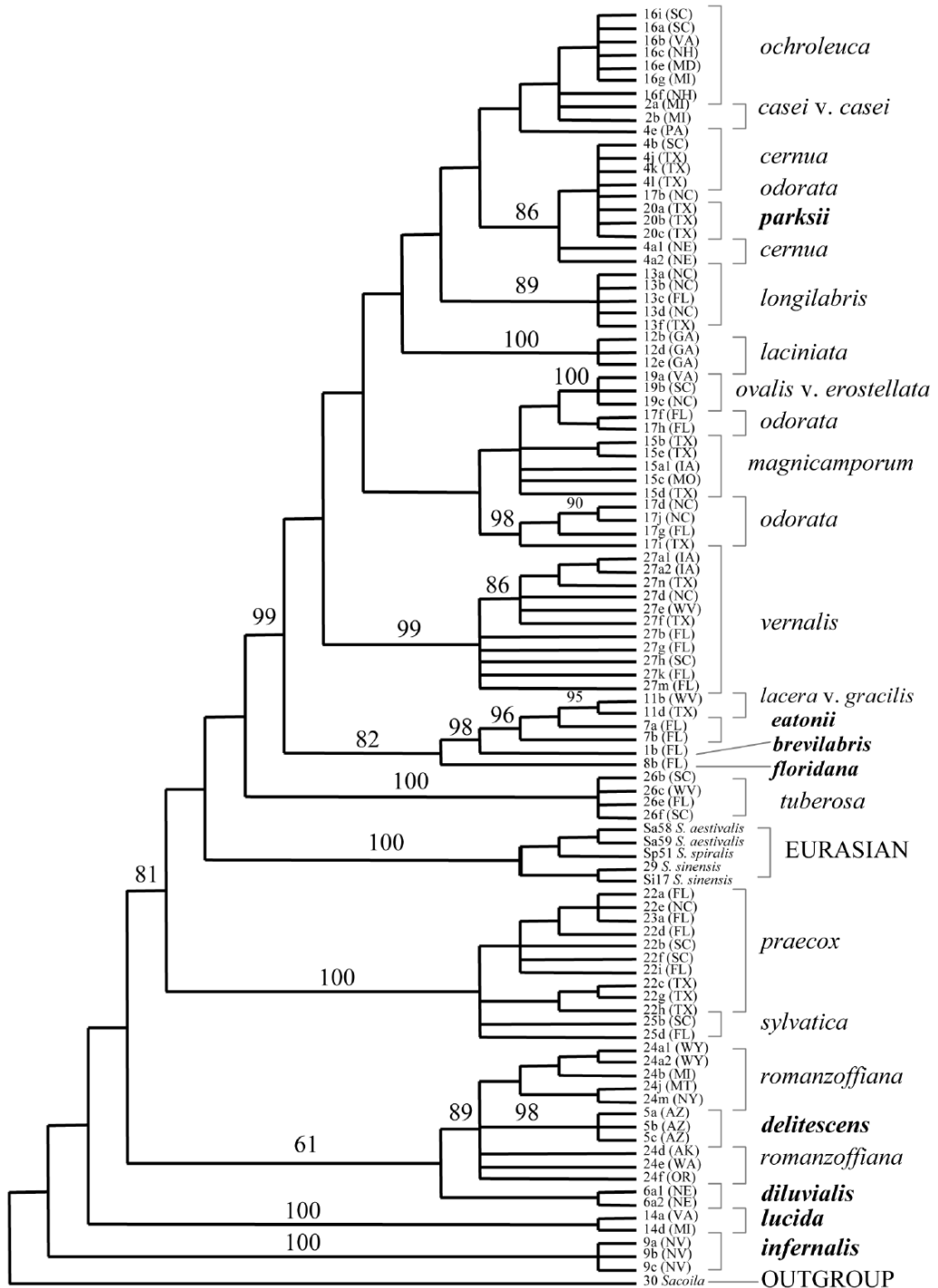


FIGURE 1. Strict consensus cladogram for taxa in *Spiranthes* genus, based on combined analysis of four genes/regions, from 489 trees (June 2006); jackknife support values >80 shown above branches; target species for conservation in **bold**. Two species subsequently sampled and analyzed but not included here are *S. porrifolia* and *S. torta*, both also of conservation concern. Sample identification numbers and U.S. state of collection shown at branch tips.

Most importantly, federally listed *S. parksii*, endangered and endemic to east-central Texas, may simply be an aberrant form of the more widespread *S. cernua*, contrary to accepted taxonomy. Cladograms from more than 3500 characters show that individuals of southern *S. cernua* form a monophyletic clade that includes *S. parksii* within it. Thus, based on our data and the phylogenetic species concept, *S. parksii* may not warrant species status, and its protection may be questioned. The tetraploid chromosome number ($2n=60$) of *S. parksii* and inclusion firmly within the *S. cernua* complex (Sheviak & Brown 2002) suggests that polyploidy may play a role in its ambiguity.

In contrast, species status was supported by our data for the two other federally listed species, *S. diluvialis* (threatened) and especially *S. delitescens* (endangered), both members of a broad *S. romanzoffiana* clade. The previously documented allopolyploid origin of *S. diluvialis* was confirmed by these data, with *S. magnicamporum* verified as its paternal ancestor. Allopolyploidy has also been suggested for *S. delitescens* by Sheviak & Brown (2002) based on the same chromosome number configuration ($2n=74$), but affinity with other than *S. romanzoffiana* was not confirmed by our data.

The genetic identity of all other state-listed *Spiranthes* species in the endemic/endangered group except one was confirmed. Although Wherry separated *S. floridana* from *S. brevilabris* in 1931 (as *Ibidium floridanum*, later changed to *S. floridana* by Cory and emended by Brown [2001b]), the epithet *S. brevilabris* var. *floridana* has persisted. Our study shows these rare taxa to be distinct species, each worthy of separate recovery plans. However, from the few samples of *S. eatonii* sequenced, we were not able to distinguish it genetically from *S. lacera*. Extremely rare *S. torta* does appear to be a distinct species but closely related to the above four taxa, although there is some evidence for its hybridization with *S. laciniata*, which could be confirmed by further studies.

Three taxa have also been identified as genetically isolated by our study. Extremely rare *S. infernalis*, also a member of the endemic/endangered group, is a distinct subclade within a broader *S. porrifolia* complex near the ancestral root of the topology. While *S. porrifolia*'s range covers the West coast states, its occurrence is sporadic, so linkage with rarer *S. infernalis* enables more concern for its conservation, also. And *S. lucida*, listed by eight states, is perhaps the most unique taxon genetically as sister to all other

Spiranthes in our recent preliminary work, and thus worthy of concerted conservation effort. We therefore suggest consideration of *S. infernalis* and *S. lucida* for federal listing status based on their basal position in the *Spiranthes* phylogeny, genetic uniqueness within the genus, and rarity.

These data demonstrate that molecular technologies have the power to elucidate genetic identity, focusing funding eligibility and conservation efforts such as *ex situ* propagation on the most appropriate subjects for maintenance of biodiversity.

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Lucy Dueck, M.Sc., has been a Research Professional in molecular ecology at SREL, a field outpost for the University of Georgia, for over seven years. Her interest in *Spiranthes* phylogeny developed from producing a booklet on the wild orchids of South Carolina for outreach purposes. She obtained a grant from the AOS to pursue a thorough phylogenetic survey after completing a pilot study on selected *Spiranthes* in South Carolina.

Ken Cameron, Ph.D., is Director of the Lewis B. and Dorothy Cullman Program for Molecular Systematic Studies and an Associate Curator at The New York Botanical Garden. He has published extensively on the molecular systematics of various plant families, but maintains a primary research focus on orchids. In particular, he has applied DNA sequencing to questions of phylogeny for *Vanilla* and its relatives, as well as studies of Orchidaceae as a whole.