Esselman, E.J., T.A. Enders, M. Smith, and P.M. McKenzie. 2012. Examination of hybridization relationships between *Schoenoplectus hallii* and *S. saximontanus* (Cyperaceae) using ISSR markers. Phytoneuron 2012-36: 1–9. Published 23 April 2012. ISSN 2153 733X

EXAMINATION OF HYBRIDIZATION RELATIONSHIPS BETWEEN *SCHOENOPLECTUS HALLII* AND *S. SAXIMONTANUS* (CYPERACEAE) USING ISSR MARKERS

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ABSTRACT

Schoenoplectus hallii, commonly known as Hall's bulrush, is an annual species restricted to wetland habitats that experience fluctuating water levels. This rare annual has suffered significant population losses over the last 25 years and the survival and conservation of the taxon is a concern wherever it has been reported. Although habitat loss has been the main reason for the decline of *S. hallii*, hybridization appears to be a new threat to the survival of the species. Putative hybridization has been reported between *S. hallii* and *S. saximontanus* where the two species co-occur in some sites in Oklahoma. We examined individuals from both species and putative hybrids from the Wichita Mountains Wildlife Refuge and adjacent areas on the Fort Sill Military Reservation in Oklahoma using three ISSR primers. We identified six species-specific markers in *S. saximontanus* and nine in *S. hallii*, all of which were present in the putative hybrids plants. Our results support previous studies suggesting that the two species are hybridizing in Oklahoma and that hybridization could occur in other areas where populations overlap.

KEY WORDS: *Schoenoplectus*, hybridization, ISSR markers, conservation

Schoenoplectus hallii (A. Gray) S.G. Sm and *S. saximontanus* (Fernald) Raynal are sedge species that were once thought to be allopatric, with *S. hallii* occurring primarily in the Midwest and eastern USA and *S. saximontanus* largely confined to the western portions of the USA (Gleason & Cronquist 1991; Beatty et al. 2004). Both species belong to *Schoenoplectus* sect. *Supini* (Cherm.) J. Raynal. *Schoenoplectus hallii* has a global ranking of G2/G3 (imperiled/vulnerable) and is listed as "critically imperiled" in eight of the 11 states in which it occurs, "imperiled" in two, and "vulnerable" in one. Herbarium records indicate that it had been reported from Georgia and Massachusetts prior to 1981 (McKenzie et al. 2007), but those populations are likely extirpated (NatureServe 2011; McKenzie et al. 2007). *Schoenoplectus saximontanus* has a global ranking of G5 (secure) (NatureServe 2011), but it is listed as "critically imperiled" in British Columbia as well as in 7 of the 11 states where it occurs. It has been reported from two states in Mexico (Smith 2002). Throughout its range, *S. saximontanus* is considered to be an uncommon species whose distribution is scattered (Smith 2002).

Schoenoplectus hallii and *S. saximontanus* are obligate wetland species that have similar habitat requirements — most often sandy, rocky or gravelly soil, occasionally clay, around the margins of ponds, ditches and swales with fluctuating water levels, and a scarcity of other plants as competitors (Smith 2002; McKenzie et al. 2007). They most commonly complete their life cycle as annuals, but short-lived perennials have been reported from Texas (O'Kennon & McLemore 2004). Dispersal within and among sites is thought to be facilitated by migrating waterfowl and a variety of large mammals, including elk, cattle, bison, and feral pigs (Magrath 2002; McKenzie et al. 2007).

Both species have 2–3 small basal leaves and tufted stems about 4–40 cm long with small, inconspicuous rhizomes. The species are amphicarpic (having two distinct types of achenes), with numerous inflorescences on aerial stems containing perfect flowers, and occasional pistillate flowers enclosed in a leaf sheath at the base (Smith 2002). Although it is difficult to distinguish between the species vegetatively, the achenes of *Schoenoplectus hallii* are 2-sided and flowers have 2-lobed styles, whereas, achenes of *S. saximontanus* are 3-sided and flowers have 3-lobed styles (Smith 2002). Achenes of both species have transverse ridging, but Magrath (2002) and Smith and McKenzie (2011) reported that the ridges on *S. saximontanus* are "winged," while those on *S. hallii* are smooth.

Five states (Kansas, Missouri, Nebraska, Oklahoma, and Texas) have populations of both species (NatureServe 2011); however, only Oklahoma (Magrath 2002), Kansas (Craig Freeman pers. comm. 2006) and Texas (Bob O'Kennon, pers. comm. 2007) have sites with mixed populations. Although *Schoenoplectus saximontanus* occurs in eight counties in Oklahoma, it co-occurs with *S. hallii* in only Comanche County (Oklahoma Vascular Plant Database 2012). In 2000, 134 sites at the Wichita Mountains Wildlife Refuge (WMWR) in Comanche County were surveyed for *S. hallii* and *S. saximontanus* (Magrath 2002). At that time no evidence of hybrids was reported (Magrath 2002; McKenzie et al. 2007). Young (2002) examined plants from two sites on the WMWR using amplified fragment length polymorphism (AFLP) data. She indicated there was no evidence for gene flow or hybridization between the taxa but acknowledged the possibility of hybridization.

In August 2001, Smith re-examined the population sites surveyed by Magrath in 2000 and noted plants that she identified as potential hybrids. In 2002, Smith and McKenzie returned to the site and collected voucher specimens and individuals for a laboratory study. Some plants had achenes that appeared to be 2-sided like those of *Schoenoplectus hallii*, except the usually flat or convex side contained a conspicuous bulge and the achene ridges often had the "winged" appearance reported by Magrath (2002). Other individuals had both 2- and 3-lobed styles with corresponding 2- or 3-sided achenes, with winged ridges present on some but lacking on others. Other plants produced only a few viable-looking achenes, with the majority of inflorescences bearing a preponderance of aborted achenes. Based on these observations, Smith et al. (2004) reported the first recorded observation of the putative hybrid, *Schoenoplectus hallii* x *S. saximontanus*.

In subsequent years (2007–2010), Smith and McKenzie conducted surveys and collected specimens of *Schoenoplectus* from selected sites on the WMWR and on adjacent areas of the Fort Sill Military Reservation (FSMR) (Smith & McKenzie 2011). At all four sites where the species occurred in mixed populations, putative hybrids were present. From 2007 to 2010, the relative number of hybrids at mixed sites increased compared to *S. hallii* and *S. saximontanus*. At one population site, neither parent species was present in 2010, but the putative hybrid was abundant (Smith & McKenzie 2011).

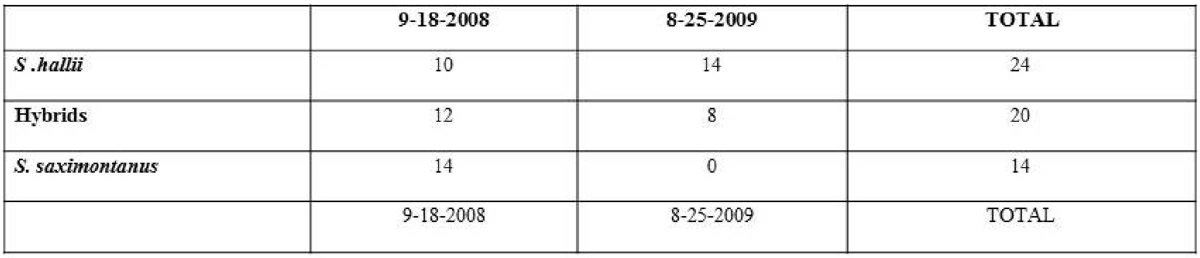
Molecular markers and ISSRs

A molecular marker that has proven useful in genetic diversity studies is inter-simple sequence repeats (ISSRs) (Esselman et al. 1999). Unlike some other molecular markers, ISSRs require no prior knowledge of the exact DNA sequence (Godwin et al. 1997), show more genetic diversity (Nagaoka and Ogihara 1997), and are an effective means for examining similarities or differences between species (Esselman et al. 1999). ISSR marker analyses have been used in conjunction with morphological characteristics to examine hybridization between the mustard family species *Physaria bellii* Mulligan and *P. vitulifera* Rydberg (Kothera et al. 2007).

The objective of this report is to present DNA evidence to confirm the existence of hybridization between *Schoenoplectus hallii* and *S. saximontanus*. We used the identification of ISSR markers for *S. hallii* and *S. saximontanus* present in putative hybrids as a criterion for confirming hybridization in plants collected from sites in the WMWR and FSMR in Oklahoma where the parental species co-occur (Smith et al. 2004; McKenzie et al. 2007; Smith & McKenzie 2011).

MATERIALS AND METHODS

Parent and hybrid plant material was collected from the WMWR site in 2008 and 2009. Ten additional hybrid plants collected from the FSMR site in 2010 were included in our analysis. Smith and McKenzie (2011) noted that while the parental species had been present in past years at the FSMR site, only hybrid plants were present in 2010. For this reason, only hybrid plants are included in our analysis from this site.



	9-18-2008	8-25-2009	TOTAL
S .hallii	10	14	24
Hybrids	12	8	20
S. saximontanus	14	0	14
	9-18-2008	8-25-2009	TOTAL

Table 1. Number of plants examined with ISSR markers from the Wichita Mountains Wildlife Refuge (WMWR), Oklahoma.

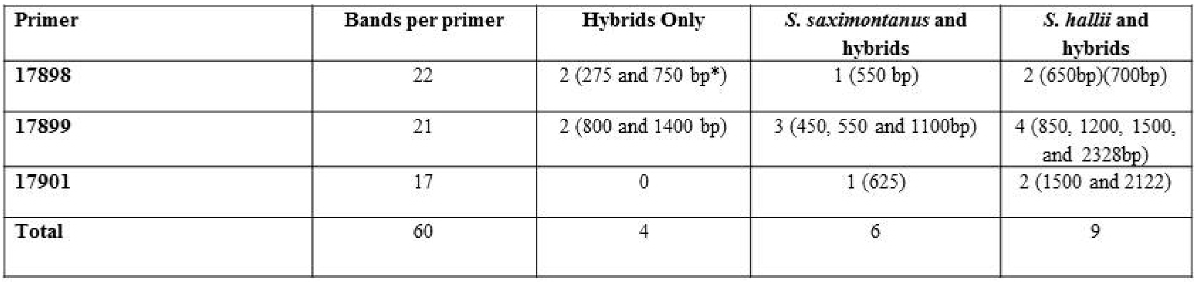
Species and putative hybrid identification was made in the field using a hand lens to examine achene morphology. All voucher specimens are deposited at the Missouri Botanical Garden (MO) with duplicates distributed to other herbaria. The number of plants collected per year and the total number of plants examined is as listed in Table 1.

Culms were dried in silica gel at the collection sites and processed in the laboratory of Dr. Esselman at Southern Illinois University Edwardsville. They were frozen with liquid nitrogen and stored at -80 C until DNA was extracted using Dneasy miniprep kits from Qiagen. The ISSR reactions protocols follow Esselman et al. (1999). The primer designations and compositions [17898 (CA) 6RY, 17899 (CA) 6RG, and 17901 (GT) 6YR] were obtained from GenoSys. Bands amplified by PCR were characterized on 1.5% agarose gels in 1x Tris-borate-EDTA buffer. Gels were stained with ethidium bromide. A 100 bp ladder (Gibco/BRL) was run with the PCR generated fragments to determine band sizes. All samples were run at least twice for every primer to ensure band reproducibility.

The gels were read and analyzed using the Bio-Rad Gel Doc XR Molecular Imager. Band presence values were entered into a program written by Vera Ford, UC-Davis to generate average pairwise similarity values.

RESULTS

A total of 60 bands were scored. Primer 17898 revealed a total of 22 bands, Primer 17899 revealed 21 bands, and Primer 17901 had seventeen bands scored (Table 2). We found six bands unique to *Schoenoplectus saximontanus* and nine unique to *S. hallii* (Table 2). These bands were found to be reliable species markers in that they were consistently present in the parental plants. Both sets of bands were found in the hybrid plants from WMWR and FSMR populations.

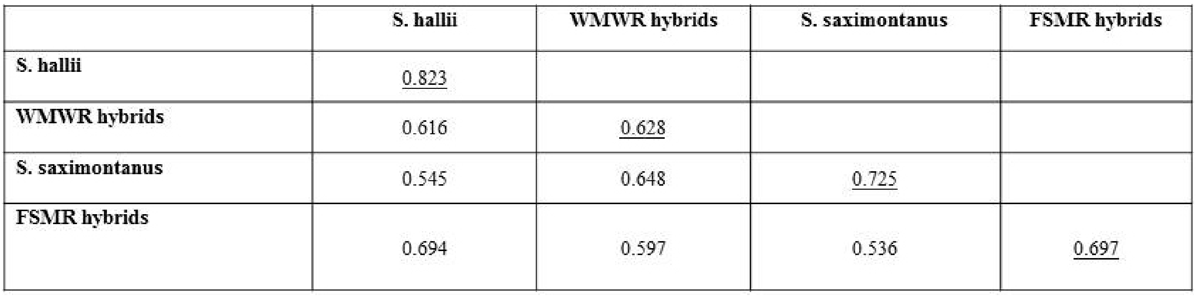


Primer	Bands per primer	Hybrids Only	*S. saximontanus* and hybrids	*S. hallii* and hybrids
17898	22	2 (275 and 750 bp*)	1 (550 bp)	2 (650bp)(700bp)
17899	21	2 (800 and 1400 bp)	3 (450, 550 and 1100bp)	4 (850, 1200, 1500, and 2328bp)
17901	17	0	1 (625)	2 (1500 and 2122)
Total	60	4	6	9

Table 2. Total number of bands per primer, numbers of unique bands per species and number of the unique bands present in the hybrid plants. *Band unique to FSMR.

Three bands were found to be unique to the hybrids at both sample sites. The FSMR plants contained one additional unique band (17898, 750bp). We did not see complete additivity in any of the hybrids examined from either site.

The distance values for all populations analyzed using ISSR markers are presented in Table 3. A distance value of one indicates complete ISSR marker similarity among compared individuals. The highest similarities occur in the within taxa comparisons. Individuals of *Schoenoplectus hallii* are the most genetically similar to other *S. hallii* plants (0.823), as also is the case with *S. saximontanus* (0.725). The FSMR hybrid plants have next highest within group similarity (0.697), followed closely in similarity to the *S. hallii* from the WMWR (0.694). The highest within group diversity was observed in the WMWR hybrids (0.628).



	S. hallii	**WMWR hybrids**	**S. saximontanus**	**FSMR hybrids**
S. hallii	0.823			
WMWR hybrids	0.616	0.628		
S. saximontanus	0.545	0.648	0.725	
FSMR hybrids	0.694	0.597	0.536	0.697

Table 3. Average pairwise similarity values in comparison within and among taxa. A similarity value of 1 indicates complete similarity, while a value of 0 indicates no similarity. Within group similarity values are underlined.

In among-group comparisons, the most similar were the FSMR hybrids and *Schoenoplectus hallii* (0.694), followed by the WMWR hybrids and *S. saximontanus* (0.648). The lowest similarity values were seen when comparing *S. saximontanus* with the FSMR (0.536) hybrids and the next least similar taxa were *S. saximontanus* and *S. hallii* (0.545).

DISCUSSION

Species specific markers

Support for hybridization between the two taxa was evidenced by species specific ISSR markers present in parental and hybrid plants. We were able to find markers specific for each species: *Schoenoplectus hallii* (9 markers) and *S. saximontanus* (6 markers) (Table 2).

Archdale et al. (2010) and Stapay et al. (2011) previously demonstrated that identical ISSR markers were present in other populations of *Schoenoplectus hallii* and *S. saximontanus*. Species specific markers were consistent in *S. hallii* plants examined from Missouri and Illinois and other populations in WMWR where the hybrids have not yet been documented. The species specific markers were also consistent in all *S. saximontanus* plants examined from a population in Texas and a non-hybrid site in WMWR.

Young's (2002) AFLP marker results indicated that *Schoenoplectus hallii* and *S. saximontanus* are distinct with species specific markers. Our ISSR marker data are consistent with this result. Based on the presence of unique species markers (Table 2) and the higher within population similarity values compared to the between species comparisons (Table 3), these taxa are distinct and not exhibiting introgression or extensive gene flow.

Support for hybridization

Young (2002) found no evidence for hybridization between sympatric populations of *Schoenoplectus hallii* and *S. saximontanus* on the WMWR. In contrast, our results confirm the initial suppositions of hybridization between *S. hallii* and *S. saximontanus* on the WMWR and FSMR based on morphological observations as reported by Smith et al. (2004), McKenzie et al. (2007) and Smith and McKenzie (2011). Plants previously identified by morphological characters as hybrids from both sites contained combinations of 9 species marker bands from *S. hallii* and 6 species marker bands from *S. saximontanus* (Table 2).

We did not find complete additivity in any of the hybrids. This was expected because we do not know what generations of hybrids were involved. They are most likely later generations than an F1 because the parents are now absent from some sites and the species is an annual. The inability to detect either parent during the 2010 visit strongly suggests that backcrossing and F2 generation plants were involved.

In the comparisons among taxa, the hybrids do not appear to be consistently more similar to one parent over the other (Table 3). The WMWR hybrids appear to be more similar to *Schoenoplectus saximontanus* from the site and the FSMR plants appear to be more similar to *S. hallii* collected from WMWR. More genetic similarities with one parent versus the other may reflect differences in abundance of *S. hallii* and *S. saximontanus* at the different study sites. There were no parental plants observed at the FSMR in 2010, so genetic comparisons with parents from that site are currently not possible.

Although *Schoenoplectus hallii* and *S. saximontanus* are currently distinct species, hybridization at the WMWR and FSMR in Oklahoma may threaten their existence at these sites. As reported by Smith and McKenzie (2011), no parental species were found at the FSMR site in 2010 where parents and hybrids were abundant in 2009. They also reported declines of both parental species at the WMWR site since 2007, and in 2010, *S. saximontanus* was absent from the site. The decline in the parental taxa and the increasing number of hybrids, and areas with them, suggest that the hybrids likely outcompete either of the parental species. If mixing of the two parental species continue, eventually it is possible that only the hybrids will occur on the WMWR and FSMR and other areas in the USA where *S. hallii* and *S. saximontanus* are now sympatric.

Why the number of hybrids is apparently increasing is unknown. Although the chromosome numbers (*Schoenoplectus hallii* n=11, *S. saximontanus* n=25) of the parental taxa differ, and many hybrid achenes examined during observations made between 2001 and 2010 appeared to be abortive (Smith et al. 2004; Smith & McKenzie 2011), viable seed was produced by others (Smith et al. 2004). Arnold et al. (2011) hypothesized that despite the low fertility of hybrid species, they are still able to produce viable gametes for successive generations. Despite the lack of information on the chromosome numbers of *S. hallii* x *S. saximontanus* hybrids, it is possible that changes in chromosome number or chromosomal substitutions or rearrangements could result in the increased fertility and success of these plants over time (Chester et al. 2012; Soltis & Soltis 2000). Future work should include examination of seed set and chromosome numbers of the hybrids. This information may provide insight as to why the hybrids are increasing in number and displacing the parents at some locations.

There are additional records of possible hybrids between closely related congeners of the *Supini* group of *Schoenoplectus* species. Galen Smith (2002) reported a suspected cross between *Schoenoplectus hallii* and *S. erectus* in Georgia where the two species are sympatric. There is a second specimen of *S. saximontanus* from Texas (Lundell and Lundell 1075, housed at MICH with a duplicate at BRIT) that is likely a hybrid between *S. hallii* and *S. erectus* (Poiret) Palla ex J. Raynal subsp. *raynalii* (Schuyler) Lye. The duplicate was annotated initially by A. E. Schuyler as a hybrid between *S. bergonsii* Schuyler (now *S. saximontanus*) and *S. wilkensii* Schuyler (now *S. erectus*). It was subsequently annotated by Galen Smith as *S. saximontanus* in 1993 as an atypical specimen of *S. saximontanus*, but with abortive and immature achenes (pers. comm. 2010). Dr. Anton Reznicek of the University of Michigan Herbarium compared the specimen with a duplicate of a hybrid collection of *S. hallii* x *S. saximontanus* (McKenzie 2317) and agreed that the Lundell and Lundell collection was of hybrid origin (pers. comm. 2010). Given that *S. hallii* is not known from the location, but *S. saximontanus* and *S. erectus* are, it is likely that hybridization involved these two species. Hybridization is also possible at two sites where *S. saximontanus* and *S. hallii* co-occur (Harper Co., Kansas, Freeman, pers. comm. 2005; and Wise Co., Texas, O'Kennon, pers. comm. 2004). Both *Schoenoplectus hallii* and *S. saximontanus* have been reported in Missouri and Nebraska, but the ranges of the two species currently do not overlap. Nonetheless, migrating waterfowl could bring both species in contact with one another at a future date.

There is possible evidence that hybridization between the two species near Lawton, Oklahoma, may be of recent origin and that hybrid plants may be gradually eliminating both parents. Although putative hybrids were discovered at the WMWR in 2002 (Smith et al. 2004), Smith and McKenzie examined many of the specimens collected by Larry Magrath on the WMWR in 2000 (Magrath 2002) and did not discover any evidence of hybridization. This was somewhat surprising because Magrath collected both species at some of the same sites visited by Smith and McKenzie in 2001 and 2002; however, not all of Magrath's 2000 collections were available, so it is possible that hybrid individuals were present in others that were not examined.

Possible impact of hybridization on conservation efforts and future research needs

According to conservation geneticists, potential effects of hybridization are numerous and pose a serious threat to the survival of a rare species that hybridizes with a closely related congener (Levin et al. 1996). Such may be the case in the interaction of the two species considered here.

The confirmation of hybridization between *Schoenoplectus hallii* and *S. saximontanus* at the WMWR and adjacent areas of the FSMR indicates that there is a threat to the persistence of *S. hallii* in Oklahoma, and possibly the overall population status of the species in North America. Documentation of hybridization between species of *Schoenoplectus* within sect. *Supini* strongly suggests that the genetic integrity of all members of this group could be compromised wherever sympatry occurs. Because waterfowl are very likely dispersal agents, there are few control measures practical or feasible that could be implemented to prevent such interactions. Nonetheless, monitoring of extant populations should be undertaken to assess changes in population status of each species.

Although the extinction of rare species typically is attributed to systematic environmental change that renders the habitat unsuitable (Harrison 1991; National Research Council 1995), hybridization may have a profound effect on the persistence of a species (Rieseberg 1991; Ellstrand 1992; Levin 2002; Rieseberg & Linder 1999). Wolf et al. (2001) noted that hybridization could result in extinction of rare plant species in five or fewer generations, and they viewed hybridization as perhaps the most rapidly acting genetic threat to endangered species.

Hybridization may reduce a population's growth rate by adversely affecting its reproductive effectiveness, its competitive status, and its interactions with herbivores (Levin et al. 1996). The numerical disadvantage of a rare species is compounded by the proliferation of fertile hybrids. The addition of these plants to a population containing two related plants decreases the proportional representation of the rare species. In time, this backcrossing can result in the assimilation of the rare species whose genetic identity will become extinct (Rhymer & Simberloff 1996). We see evidence of these processes in the interaction between *Schoenoplectus hallii* and *S. saximontanus*.

Additional surveys of *Schoenoplectus hallii* and *S. saximontanus* in Kansas and Texas where the two species are sympatric are warranted. Because *S. saximontanus* and *S. erectus* are sympatric in some areas of southern Texas, possible hybridization should be evaluated, especially at sites where both species have been documented. Genetic analyses of *S. erectus* subsp. *raynalii* are needed because there is some possibility that the species may be of hybrid origin. We have observed achenes of *S. hallii* x *S. saximontanus* hybrids that are similar in shape to those described by Smith (2002) for *S. erectus* subsp. *raynalii*, and variations in achenes of *S. erectus* contributed to the description of two species that are now synonymous with *S. erectus* (i.e. *S. wilkensii* and *S. erismanae* Schuyler). The same is the case for *S. bergonsii* that is now placed in synonym with *S. saximontanus*. In addition, the reproductive potential of *S. hallii* x *S. saximontanus* hybrids, estimates of pollen viability, seed set and ploidy level should be investigated.

ACKNOWLEDGEMENTS

We would like to thank the USFWS and the SIUE URCA program for supporting this project. We also thank Jeri Boone and Susan Farmington for assistance with plant collections.

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