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The Success of Reproductive Mechanisms in Solidago speciosa var. speciosa, A Threatened Pennsylvania Plant

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Anstract:

Solidago speciosa var. *speciosa* or "showy goldenrod" is a threatened Pennsylvania plant with only a handful of known populations. Little is known about the mechanisms this species employs to perpetuate a population. To understand the role that reproductive modes play in the limited distribution of this goldenrod and the relative importance of sexual reproduction and clonal growth, the genetic diversity, clonal structure and pollinator abundance of three *Solidago speciosa* var. *speciosa* populations from Montgomery County were investigated. To test the hypothesis that *S. speciosa* var. *speciosa,* like other goldenrods, is capable of clonal growth Inter-Simple Sequence Repeats were used. Ten primers have supported the importance of clonal growth in the FCL and FCU populations while a more complex reproductive history is needed to explain the BS population. Genetic variability is below .50 in all populations (Shannon's diversity index) but is highest (.42) in BS. A pollinator exclusion study has neither confirmed nor rejected that *S. speciosa* var. *speciosa* has the capacity for autonomous selfing. Analysis of pollinator abundance indicates that *Apis mellifera* and *Bombus impatiens* are the primary pollinators of *S. speciosa* var. *speciosa.* More research needs to be done to further understand the convoluted nature of *S. speciosa's* genetic variability across populations.

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Introduction

Solidago speciosa var. speciosa or "showy goldenrod" is a threatened Pennsylvania plant (DCNR 2007) with only a handful of known populations. The native goldenrod is characterized by showy inflorescences up to one foot long consisting of an erect panicle of small yellow flowers. The two- to six-foot plants are found in thickets, fields and roadsides, flowering from August to October (Rhoads & Block, 2007).

The perpetuation of a species is carried out via sexual and/or asexual reproduction. Understanding the reproductive mode of a species is critical in furthering understanding of genetic variability and genetic drift. This, in turn, is critical for the conservation of a species. While Solidago speciosa var. speciosa's morphology and growing conditions have been extensively published in the arena of horticulture there has been a great lack of research on the plant in general. My project aimed to test several reproductive questions: what pollinates it, is it self compatible, and what type of reproduction accounts for the main body of a population; sexual or asexual?

Methods and Materials

Populations. Three populations were studied from July through October, 2007 in Montgomery County. Each population is approximately 10 minutes outside of Sumneytown, PA; two are found in the Fulshaw-Craeg Nature Preserve (N40.33848, W075.42133) and another on Boy Scout property (N40.34861, W075.43131). Each population contained several hundred individuals as well as a number of other *Solidagos* and co-flowering species. The Fulshaw-Craeg populations were separated by a small creek; the population above the creek was denoted as Fulshaw-Craeg Upper (FCU) while the population below the creek was Fulshaw-Craeg Lower (FCL). The Boy Scout property is approximately one mile from Fulshaw-Craeg and is denoted as BS.

Insect sampling and visitation. The pollinators of *S. speciosa* were determined by catching and identifying any species that came into contact with its floral parts. Identified reoccurring pollinators were documented by a tally system. Random plots of 1m x 1m were observed for 15 minute intervals throughout the day for a total of 10 hours per population. Observations took place from 9am to 5pm to get the best representation of pollinator visitation.

Compatibility. Ten randomly chosen individuals per population were bagged in the bud stage with nylon mesh bags to determine if *S. speciosa* could produce seed in the absence of pollinators. The entire panicle was bagged due to the minuscule nature of the flowers. The bags remained on the individuals for the duration of the flowering season. To assess the percentage of female reproductive success/pollinator success, ten randomly chosen individuals were tagged and left untreated (un-bagged) per population. The control allowed for natural out-crossing to occur. Bagged and un-bagged (tagged) flowers were collected and seed set was defined through germination rates.

Germination. Seeds were removed from inflorescences and germinated. Two germination methods with three replicates each were employed to obtain the greatest germination

success. Treatments included direct sowing and cold stratification. For each treatment 25 seeds per individual were sown directly into a 65% peat moss, 20% perlite and 15% vermiculite mixture (Fafard #2). The direct sow flats were placed in the Greenhouse while the cold stratification flats were stored at 20°C for 4 months. After 4 months they were removed and placed in the greenhouse. The number of seeds that geminated was scored.

Tissue sampling. For each population new leaf samples (two per plant) were collected in 1m increments among adjacent plants. A total of 20 samples were taken from each population and stored at -80° in silica gel prior to DNA isolation (Dong et al. 2006).

DNA isolation. DNA extraction and isolation was carried out using a modified CTAB protocol (Doyle and Doyle 1990). An entire leaf was placed into a mortar with a pestle and covered with saran wrap and put in -80° for one hour. The leaf material was then ground into a fine powder and transferred into a 1.5mL Eppendorf tube. A volume of 650μl of extraction buffer, consisting of 2% CTAB, was added to the tube and incubated at 65°C for 45min. The suspension was then extracted with 650μl of chloroform-isoamyl alcohol (24:1) and centrifuged at 10,000 *g* for 10 minutes. The aqueous layer was transferred to a new 1.5mL tube. 1000μl of absolute alcohol was then added to the aqueous solution and the mixed solution was left at -20°C for an hour to precipitate the DNA. After centrifugation for 10 min, the precipitate was washed with chilled 75% alcohol and dissolved in 1000μl of H_2O. The DNA concentration of each sample was measured using and ultraviolet (UV) spectrophotometer.

ISSR analysis.   All 60 samples were tested using inter-simple repeat sequences (ISSR). Ten primers were screened (Dong et al. 2006) (Table 1). Polymerase chain reaction was carried out in 20μl containing 1μl of primer, 18μl of Taqcomplete-1.1X Master Mix with 2.0μl $MgCl_2$ (GeneChoice, Inc.) and 1μl DNA template. The program began with an initial denaturation at 94°C for 4 min, followed by 40 cycles of 45s at 94°C, 30s at an optimized temperature per primer (Table 1), 30s at 72°C and finally 10 min at 72°C. The PCR products were separated on 2% agarose containing 13μl of Cybersafe in a 1X TAE buffer with a voltage of 50V. The gels were then visualized and photographed under UV light. A Lambda DNA/EcoRI + HindIII marker was used as a molecular weight standard.

Table 1. Sequence and annealing temperature of the 10 inter-simple sequence repeat primers used for amplification of *Solidago speciosa* var. *speciosa* DNA fragments.

Code of Primer	Sequence	Temperature Used for PCR
807	AGA GAG AGA GAG AGA GT	52.3°C
818	CAC ACA CAC ACA CAC AG	43.1°C
825	ACA CAC ACA CAC ACA CT	43.1°C
827	ACA CAC ACA CAC ACA CG	49.6 °C
834	AGA GAG AGA GAG AGA GYT	49°C
841	GAG AGA GAG AGA GAG AYC	42°C
842	GAG AGA GAG AGA GAG AYG	52.3°C
856	ACA CAC ACA CAC ACA CYA	45.8 °C
864	ATG ATG ATG ATG ATG ATG	52.3°C
880	GGA GAG GAG AGG AGA	52.3°C

Data analysis. As a dominant marker, ISSR bands were hand scored as present (1) or absent (0). Data was run in PAUP* 4.0b10 (Swofford 1998), Structure 2.2 (Pritchard et al. 2007) and Popgene32 (Yeh et al. 1997).

The detection of a genetic stratification was performed with the Structure program using the admixture model. This model assumes that the genome of individuals is a mixture of genes originating from K unknown 'ancestral' populations that may have undergone introgression events. Under this model, the structure algorithm estimates the proportion of membership (genome ancestry) of each individual in each of the K ancestral populations. This model assumes that the unknown K ancestral populations are at Hardy–Weinberg and linkage equilibrium.

Phylogenetic analyses were performed with the PAUP* software. After a heuristic search, the equally most-parsimonious trees and one strict consensus tree were obtained. The maximum parsimony tree is presented here. Bootstrap analysis was not used as a supplemental assay due to lack of data (Weins 2006).

The Shannon index was calculated as $H_0 = -\Sigma\ p_i \log_2 p_i$ (Lewontin, 1972) in which p_i is the frequency of a given ISSR fragment. Popgene software was used to calculate the Shannon diversity index. The Simpson's diversity index (D) was used to estimate clonal variation ($D = 1-[\Sigma n_i(n_i-1)/N(N-1)]$, where n_i is the number of samples of the ith genotype and N is the total number of samples). The index D should range from 0 (a population is composed of a single clone) to 1 (each sample in a population is unique).

Non-DNA data analysis was carried out in Microsoft Office Excel 2007.

Results

Pollination. Eleven insect species were observed visiting all three populations with *Apis mellifera* being the most common (Fig. 1-3). The eleven pollinators noted were *Bombus impatiens, Vespa maculifrons, Osmia sp.*, *Xylcopa sp.*, *Apis mellifera, Ancistrocerus antilope, Eristalis transversa, Diabrotica undecimpuncata, Pierus rapae,* an unidentified Syrphid fly and an unidentified beetle.

A total of 1,129 pollinators was observed at the Boy Scout population (Fig 1). *Apis mellifera* was the most common pollinator comprising 63% of all the insects observed. *Apis mellifera* was most active during the hours of 10 am to 3 pm. The next most frequent pollinator was *Vespa maculifrons* at 15% visitation. *Coleoptera* was noted at 8% of BS visitation and *Bombus impatiens* was also observed at 6% pollinator occurrence (Table 1).

A total of 479 pollinators was observed at the Fulshaw-Craeg Lower (FCL) population (Fig 2). *Apis mellifera* was the most common pollinator comprising 45% of all the insects observed. The honeybee was most active between the hours of 9am to 2pm. FCL also saw high

visitation from *Vespa maculifrons* (16%) followed by *Ancistrocerus antilope* (14%) and then *Bombus impatiens* (11%) (Table 1).

A total of 928 pollinators was observed at the Fulshaw-Craeg Upper population (Fig 3). *Apis mellifera* was the most common pollinator comprising 46% of all the insects observed. The honeybee was most active during the hours of 9am and 2pm (Fig. 3). *Bombus impatiens* (19%) and *Osmia sp* (18%) were the next most common pollinators (Table 1).

Table 2. Pollinator percentages at each population.

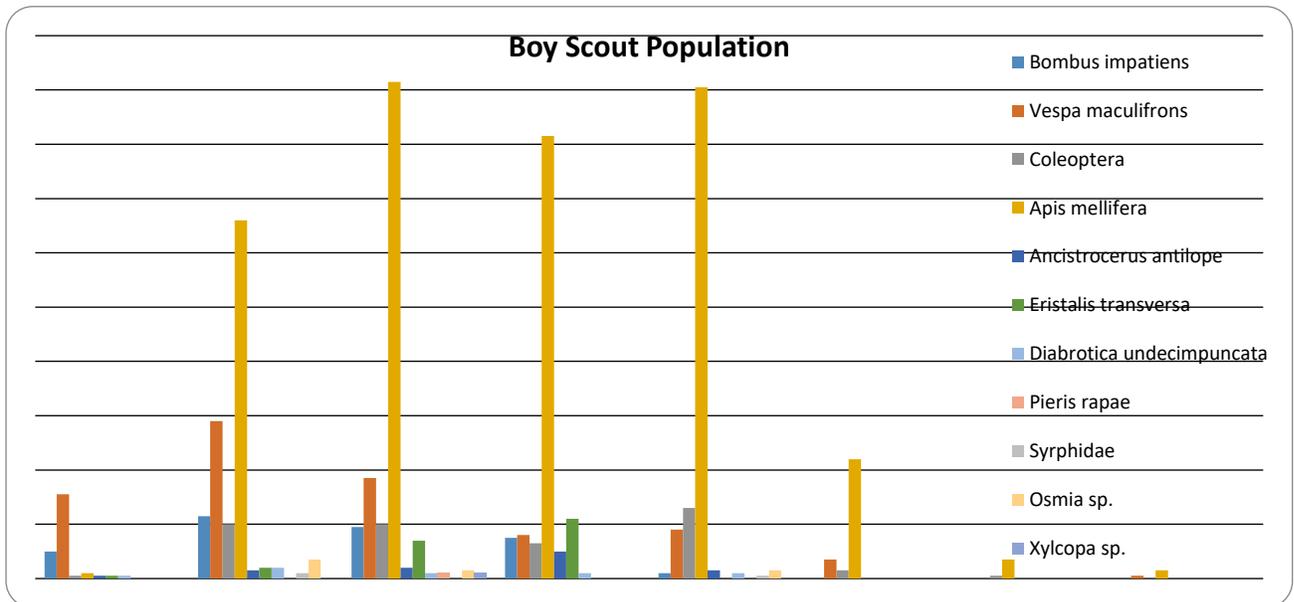| Pollinator	Boy Scout pollinator %	Fulshaw-Craeg Upper pollinator %	Fulshaw-Craeg Lower pollinator %
Bombus impatiens	6.1116	19.504	10.647
Vespa maculifrons	14.880	7.0043	16.492
Coleoptera	7.4402	1.6163	5.0104
Apis mellifera	63.330	45.689	45.093
Ancistrocerus antilope	1.8600	5.8189	13.569
Eristalis transversa	3.6315	0.9698	1.252
Diabrotica undecimpuncata	0.9743	1.6163	1.0438
Pierus rapae	0.1771	0	0
Syrphidae	0.2657	0	2.0876
Osmia sp.	1.1514	17.780	4.8019
Xylcopa sp.	0.177148	0	0

Figure 1. Pollinator occurrence at the Boy Scout property. The horizontal axis is the hourly increments while the vertical axis is the actual number of pollinators observed.
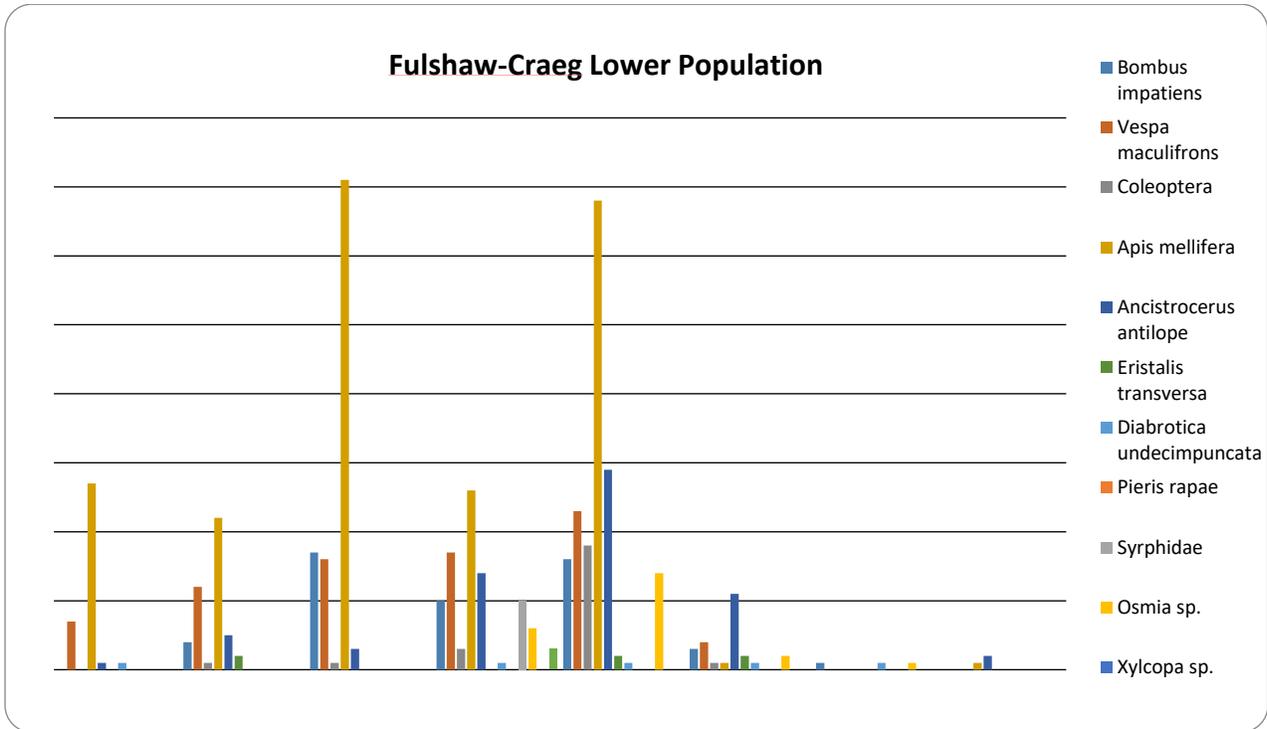

Figure 2. Pollinator occurrence at FCL. The horizontal axis is the hourly increments while the vertical axis is the actual number of pollinators observed.
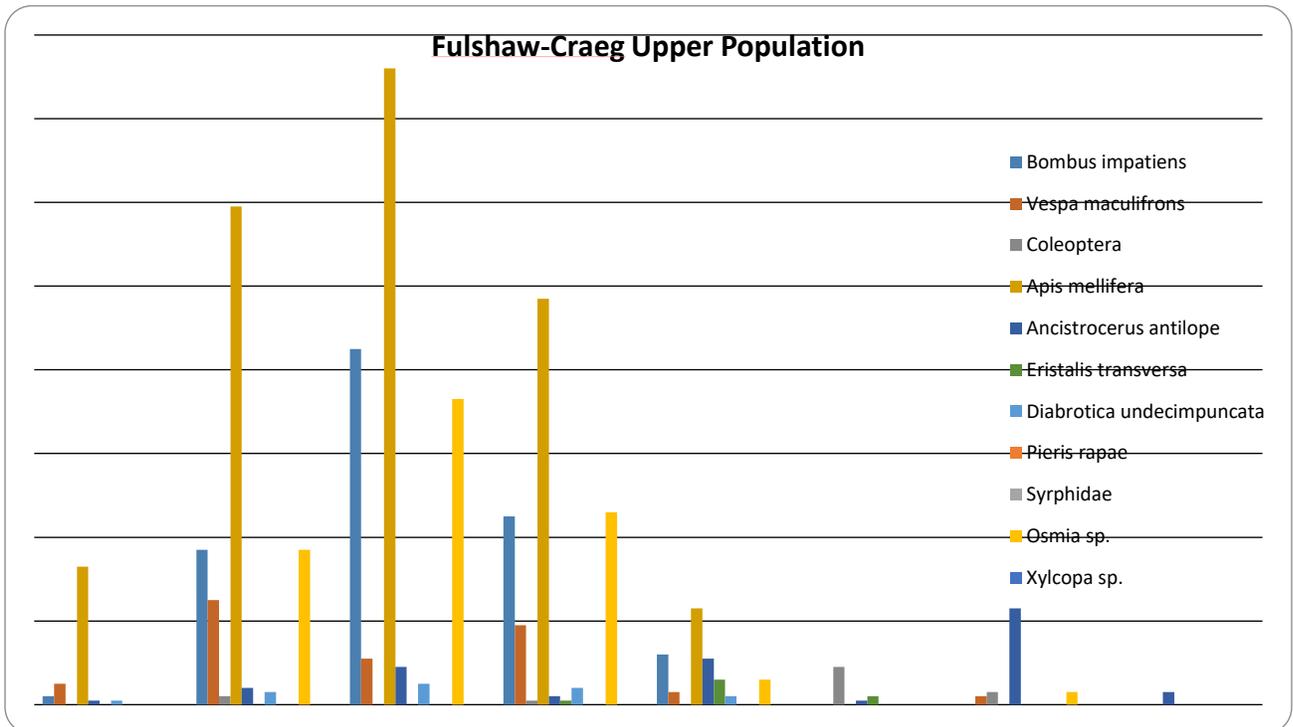
Figure 3. Pollinator occurrence at FCU. The horizontal axis is the hourly increments while the vertical axis is the actual number of pollinators observed.

     Germination. While in the field I observed pollinators collecting and foraging for pollen through the mesh bags. Due to tampering I had to abandon my initial question of whether or not *S. speciosa* could set seed in the absence of pollinators. However, I proceeded to germinate the controls to see whether *S. speciosa* could set viable seed in the presence of pollinators. 36.4% of FCL's controls and 32% of BS's controls germinated.

DNA.
Across Populations
Structure analysis of the data set revealed that the individuals were split into three distinct groups that corresponded to their ancestral populations (Fig 4).

     Each group contained individuals whose genome overlapped with the other populations. Figure 4 shows each individual represented by a vertical line. Each line is partitioned into segments that represent the individual's membership fractions in each ancestral population. For example, the segment for BS 9 reads that it is 0.70 green (BS) and 0.30 red (FCL).   FCL is largely comprised of individuals with 100% membership in its distinct group. FCL 13 is the only individual to have slight similarities with BS. BS displays an interesting pattern with the presence of all three population genomes. BS individuals sharing genomic structure with other groups include BS 9, 12 and BS 17-20. BS 1-2, 4-6, 10 and 13 also display minuscule elements from the Fulshaw-Craeg populations (barely visible from the top of the bars). Only two FCU individuals have common genomic characteristics with the other populations. FCU 13 shares genomic makeup with BS while FCU 15 shares structure with FCL and BS.

     The Neighbor-Joining tree (Fig 6) constructed with 55 individual's highlights the relationships between individuals. All populations form distinct clusters from one another with the exception of some individuals. All FCU individuals fell out with one another but several BS individuals (13, 16-20) and FCL7 nestled their way into the cluster. The FCL cluster formed a monophyletic group with the addition of BS9 and 12. The BS group formed a solid cluster of only BS individuals at the bottom of the tree.
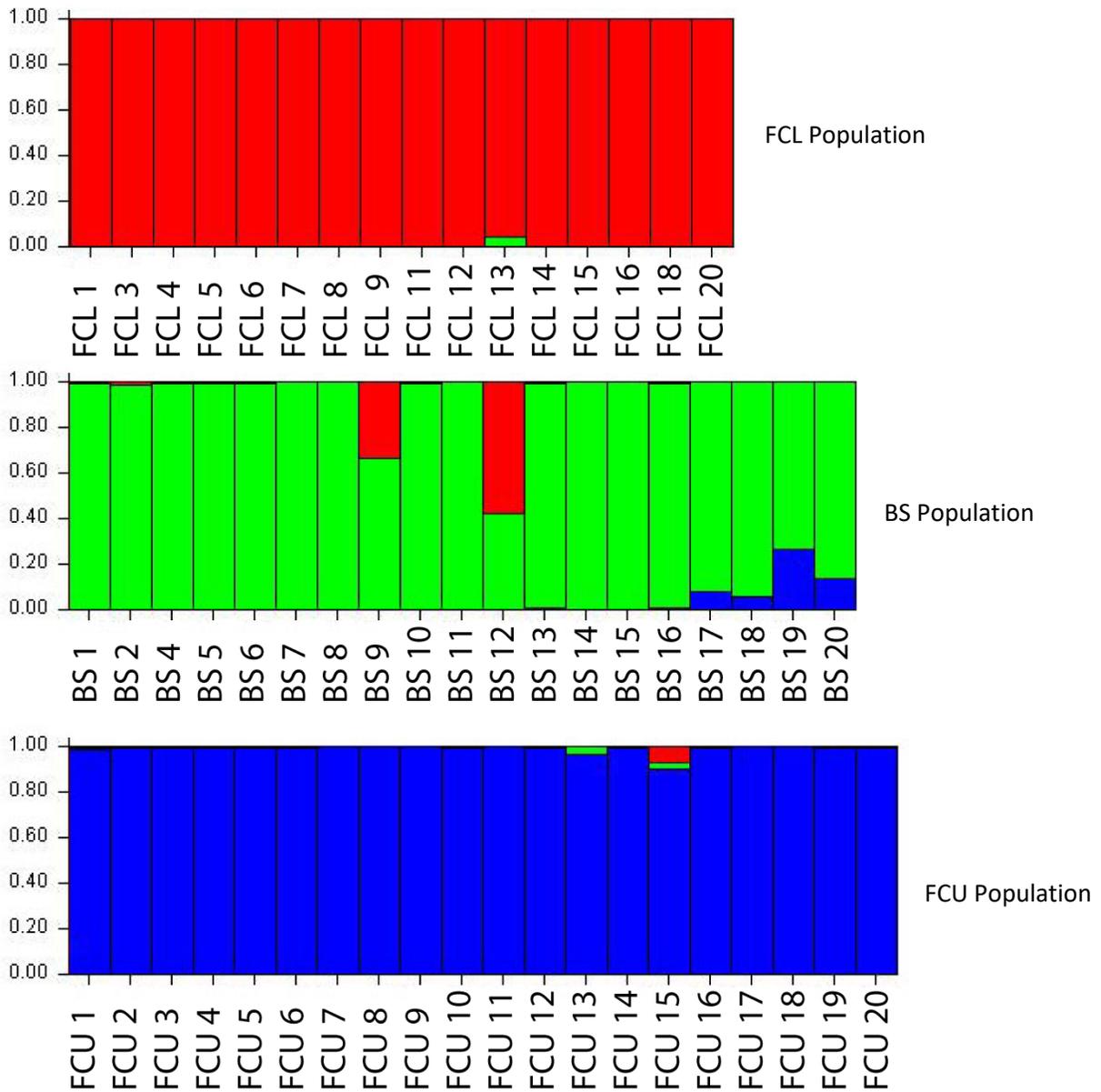
Figure 4. Bar plot of the proportional membership of individual *Solidago speciosa var. speciosa* accessions within each of the 3 inferred groups. Each individual is represented by a vertical colored line, which is partitioned into K segments that represents the individual's membership fractions in K clusters. Different colors indicated different populations. Individual lengths are proportional to each of the K inferred clusters. FCL is represented with red bars, BS with green bars and FCU with blue bars.
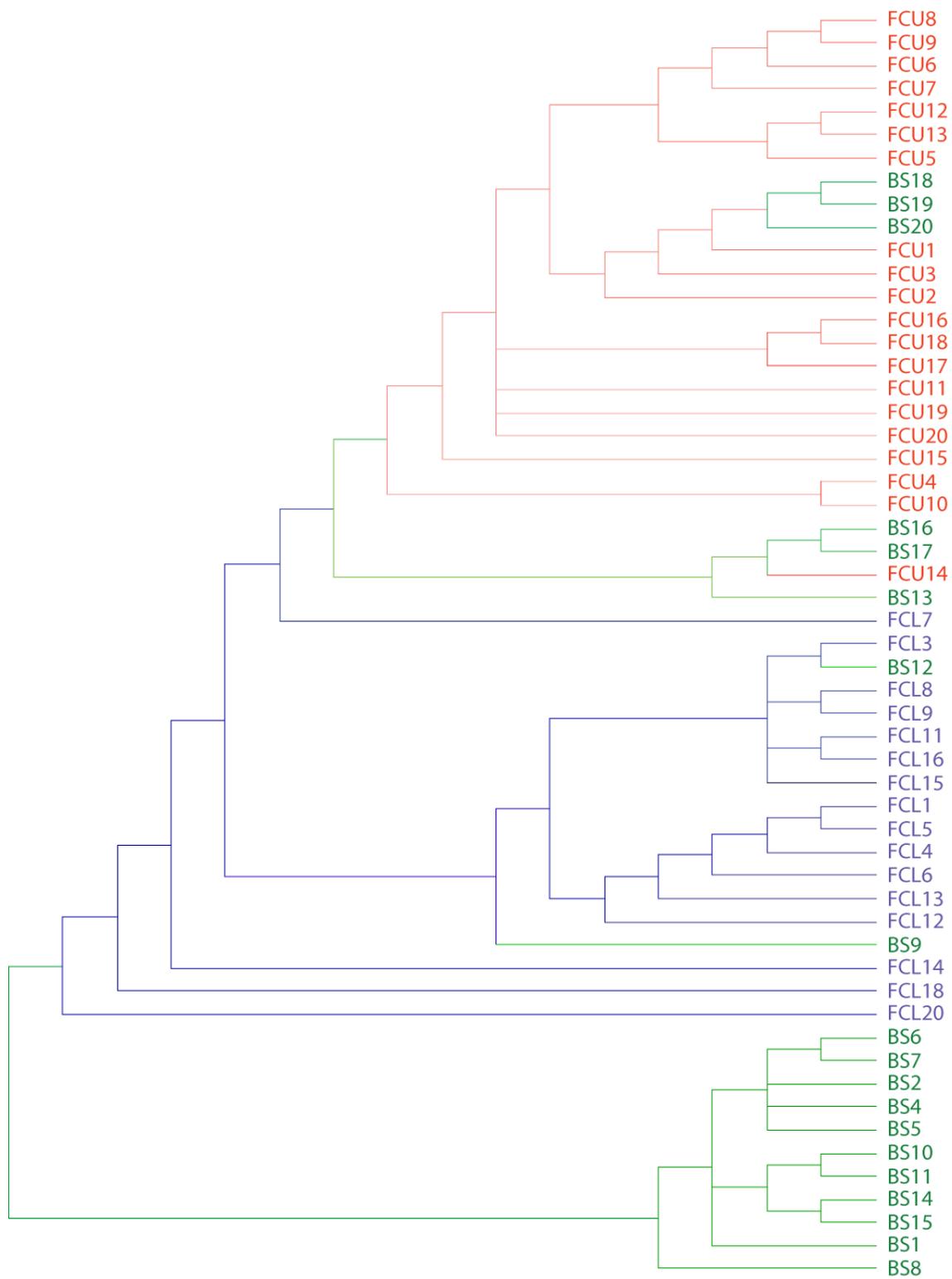
Figure 5. Neighbor joining tree highlighting the relationships between individuals. If populations were completely inbred they would all fall out in distinct clusters. Note that many individuals from the BS population are interspersed throughout the tree.

Within populations
The percentage of polymorphic loci (P) was as high as 87.50% in FCU and as low as 50.83% in FCL. BS had a P of 84.17%. Shannon's diversity index for FCL was 0.2391, BS was 0.4239 and FCU was 0.3893. The 'I' value for a number of species from other studies obtained using ISSR only (Dong et al 2006) (Table 4) was compared with that of *S. speciosa* because it is difficult to compare diversity indices obtained using different molecular markers.

Table 3. Genetic and variability within populations of Solidago speciosa var. speciosa as revealed by ISSR analysis. N, sample size; P, percentage of polymorphic loci; I, Shannon's diversity index

Population	N	P (%)	I
FCL	16	50.83	0.2391
BS	19	84.17	0.4239
FCU	20	87.50	0.3893

Table 4. Comparison of Shannon's diversity index within *Solidago speciosa* var. *speciosa* populations with that obtained for other species using ISSR

Species	Ho†	Reproduction (S/A/SA)‡	References
Psammochloa villosa	0.05	A	Li and Ge (2001)
Chromolaena odorata	0.02	SA	Ye *et al.* (2004)
Oryza granulata	0.13	SA	Wu *et al.* (2004)
Changium smyrnioides	0.17	SA	Qiu *et al.* (2004)
Rubus arcticus	0.26	SA	Lindqvist-Kreuze *et al.* (2003)
Carthamus lanatus	0.28	SA	Ash *et al.* (2003)
Psathyrostachys huashanica	0.29	SA	Hang *et al.* (2004)
Solidago canadensis	0.39	SA	Dong et al 2006
Lilium nepalense	0.43	SA	He *et al.* (2003)
Tetraena mongolica	0.29	S	Ge *et al.* (2003)
Alliaria petiolata	0.96	S	Meekins *et al.* (2001)

Ho†: Mean of the Shannon's diversity index within populations. ‡: The mode of reproduction. S, sexual; A, asexual. SA, both sexual and asexual.

Discussion

Pollination. The two most common pollinators I observed visiting *S. speciosa* were *Apis mellifera* and *Bombus impatiens*. Large hymenopterans, including honeybees and bumblebees, have been reported to visit flowers and goldenrods (Clements and Long 1923; Robertson 1928; Hayden 1930; Pammel and King 1930; Morse 1977; Heinrich 1976, 1979; Parrish and Bazzaz 1979; Ginsberg 1983; Gross and Werner 1983; Harder 1985; Evans 1986; Johnson 1986). While bumblebees were the second most common in visitation occurrence it was still very low compared to *Apis mellifera*. In North America many native bee and wasp species are important pollinators. Most pollinators observed on *S. speciosa* are native to the Neartic region. *Apis mellifera* however is a native species of Africa and Europe now found worldwide mainly due to the practice of beekeeping.

Germination. Although I had to abandon my initial compatibility query I was able to ascertain that *S. speciosa* produces viable seed in the presence of a pollinator. Viable seed is an important mechanism in maintaining genetic diversity within a population.

DNA.
Within populations. Asexually reproducing plant species have low levels of genetic variation. *S. speciosa*'s result for the Shannon Diversity index ('I') was a mean value of 0.35 with all values below 0.50 (Table 3). Comparison of 'I' to a number of other studies shows that genetic diversity in *S. speciosa* was analogous to the mean value (0.25) of species than can reproduce both sexually and asexually (Table 4). These results imply that *S. speciosa* does not rely solely on ramet production and helps explain why *S. speciosa* sets viable seed.

Across population. The results found within populations are mirrored in the results across populations; *S. speciosa* relies on both sexual and asexual reproduction. The distance-based clustering method (Fig. 5) revealed interesting results concerning individuals across populations. If individuals resulted solely from asexual reproduction, they should only cluster with individuals of the same population. All descendants of a common ancestor are represented by a node belonging to the same clade defined by that node. While three monophyletic groups can be made out they are not exclusively comprised of their own individuals. Within the main clusters of FCL and FCU are interspersed individuals of BS. Six of the nineteen BS individuals are more closely related with members of FCL or FCU. BS forms a clade at the bottom of the tree but the nature of the population suggests a more complex history. FCL7 is the only Fulshaw-Craeg individual to be found in a clade outside of its own, being most closely related to members of FCU.

Structure analysis (Fig. 4) supports the findings of the neighbor-joining tree. The Fulshaw-Craeg individuals are almost completely independent of one another across populations. BS individuals share genomic structure with FCU and FCL with the pattern arising only in BS.

All data suggests that BS is the most genetically variable population while the FC populations rely heavily on asexual reproduction. FCL and FCU are separated by a small creek and are less than .5 miles from one another while BS is approximately a mile away. Logic would suggest that the FC populations would be more apt to partake in gene flow while the BS

population would rely more on asexual reproduction due to its isolated nature. The study's findings are contrary hence raising questions on the reproductive history of BS. With so many unknowns it is almost impossible to explain the genetic diversity seen. There are speculations however.

The study sites were once completely forested; unconducive *S. speciosa* habitat. Less than 200 years ago they were completely deforested and then later turned to agriculture fields. The deforestation may have allowed for the establishment of populations. It is at this time that the three populations may have been bigger and closer with ample gene flow. Due to recent habitat fragmentation the populations may have gone through a bottleneck leaving FCU and FCL to their own devices. The Fulshaw-Craeg populations have since then winnowed out most genetic variability and are now heavily inbred. The population at BS may be explained by disturbance. The plants may have arisen from a seed bank released during PECO land management trials. The use of herbicide may have may have cleared enough ground cover for seeds to germinate. Hence, the genetic variability seen today may have been commonplace when gene flow was more widespread in these populations. Over time and under the stress of fragmentation the species has decreased in size hence decreasing the species genomic diversity.

Today the sites are managed meadows with the possibility that populations never existed there before. Mowing of the sites allowed seeds that were dispersed to the area by chance find a niche. The Fulshaw-Craeg sites may have arose from only one seed that has mainly perpetuated via clonal growth while the Boy Scout population has relied more heavily on sexual reproduction. The reasons behind populations relying on sexual or asexual reproduction are not understood.

Conclusion

Solidago speciosa var. *speciosa's* reproductive mode does not prove to be as clear as one may hope. The species relies on pollination to set viable seed which is not required for population perpetuation. Continuation of the sites is being held in slightly inbred clusters and gene flow is relatively non-existent between populations. One population, BS, has arisen with notable genetic variability due to either intrinsic or extrinsic factors.

The BS population is the biggest most robust population of the three. Conservation efforts should be directed towards this population due to its substantial genetic variability. Protecting this population, one that is not protected at the moment, may guarantee genetic variability of the species for much longer.

More studies need to be done to crack the case of reproduction in *S. speciosa*. A tamper proof pollinator exclusion study can definitively solve whether or not *S. speciosa* can self. The DNA analysis can be taken a step further and done on more populations to see if the genomic structure of BS is seen in other surviving PA populations. A better understanding of the species genome in PA can facilitate a better understanding of its limited eastern range.

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