

Growth and Development of Two Species of *Sisyrinchium* and their hybrids from
Southern Central Washington and Northern Central Oregon

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Figure 1. *S. sarmentosum*

Figure 2. *S. idahoense*

Sisyrinchium sarmentosum (Suksd. Ex. Greene) is a flowering plant in the Iridaceae, found in Southern Washington and Northern Oregon. It is considered threatened in the state of Washington [Washington DNR 2014, CPC 2011] and is being considered for inclusion on the endangered species list by the US Fish and Wildlife Service [USFWS 2014]. Threats to the species include grazing by ungulates, invasive plants, human activity, habitat encroachment and hybridization with a similar species, *Sisyrinchium idahoense*. *S. idahoense* occurs in a larger range, stretching from British Columbia to Northern California and as far east as Montana, Wyoming and Colorado, encompassing the area in which *S. sarmentosum* is known to occur [USDA 2014]. *S. idahoense* is divided into 4 subspecies across its range, all of which can be found in the state of Washington [USDA 2014, Knoke and Giblin 2014]. This study, like a few studies before [Raven 2006, Wilson et. al. 2000], examines the threat of hybridization and specifically focuses on areas where the ranges between the species overlap in Southern Washington and Northern Oregon.

Confusion surrounding the genus *Sisyrinchium* apparently preceded the writing of Bicknell's bulletin in 1895. In reference to herbarium specimens of *S. idahoense*; "I find the labels on

specimens of this plant variously inscribed with the names, *S. angustifolium*, *S. anceps*, *S. mucronatum* and *S. bellum*” [Bicknell 1895]. Despite differences identified between *S. sarmentosum* and *S. idahoense* in Bicknell’s paper, it is not mentioned in the paper where the specimens used for the descriptions of *S. idahoense* originated from. Vouchers could have been collected from anywhere in the range of *S. idahoense* where features of *S. idahoense* differ strongly from those of *S. sarmentosum*. The Vascular Plants of the Pacific Northwest echoes the confusion mentioned by Bicknell; both *S. sarmentosum* and *S. idahoense* are equated to *S. angustifolium* (Mill.). Even Hitchcock and Cronquist [1973] admit that “The classification of blue-flowered *Sisyrinchiums* is in a chaotic state.”

Douglass Henderson [1976] began the process of sorting out the genus *Sisyrinchium* in the Pacific Northwest in A Biosystematic Study of the Pacific Northwestern Blue-Eyed Grasses. Henderson identified two major problems with previous designations. First, descriptions were based on herbarium specimens, which for *Sisyrinchium*, do not tend to keep well when it comes to minute structural characteristics of the flowers. Second, not a large enough sample size was used for the descriptions to account for the variation within populations. To remedy these two problems, Henderson collected plants from 67 populations across the Pacific Northwest and Rocky Mountains to grow in a greenhouse and common garden. The plants were then used to study cytology, breeding systems, artificial hybridizations and phenotypic plasticity [Henderson 1976]. Henderson was able to distinguish 8 species of *Sisyrinchium*. In doing so, Henderson also determined the ploidy levels (chromosome counts) of all species identified in his paper, determining that *S. sarmentosum* is duodecaploid ($n=48$) while *S. idahoense* were found to have both octoploid ($n=32$) and duodecaploid individuals [Henderson 1976]. Henderson found that duodecaploid *S. idahoense* were compatible with *S. sarmentosum*. In examining artificial hybridization, Henderson found a high degree of fertility in hybrids [Henderson 1976]. Unfortunately, Henderson was only able to collect *S. sarmentosum* from two populations in Southern Washington, two populations that have not been relocated in following studies, and did not sample any *S. idahoense* populations in the vicinity.

To examine the role of rhizomatous growth and seed production in the population dynamics of *S. sarmentosum*, Wilson, Doede and Hipkins [2000] examined the genetic variation of 6 *S. sarmentosum* populations. Leaf samples were taken from 6 populations in the Gifford Pinchot

National Forest. Isozyme electrophoresis was used to examine enzyme band patterns which were then translated to phenotypic diversity measures as a representation of genetic variation.

Unfortunately, genetic interpretation was not possible due to a lack of band segregation, a result of the large range of alleles in the genome and the overcompensation of the Shannon-Weaver index used to analyze the data [Wilson et al. 2000]. One explanation, as the authors mentioned in their introduction, was the difficulty separating individual plants due to the theory of *S.*

sarmentosum's ability to spread rhizomatously; researchers may have had multiple samples from what were essentially clones of the same plant. On the other hand, Wilson et. al. [2000] noted that "*S. sarmentosum* seems to have much less enzyme variation seen in the average narrowly endemic taxon", supporting the hypothesis that *S. sarmentosum* is a recent divergence from *S. idahoense*.

In 2006, Raven conducted several studies to examine characteristics Henderson had used to distinguish *S. sarmentosum* from *S. idahoense*. Her project included a hybridization study looking at flower characteristics of the two species in the field and included data and results from an unpublished study by DeWoody and Hipkins [2015]. In the hybridization study, Raven [2006] measured flower color, tepal shape, tepal length and width, tepal reflexion, time of flower opening, style elongation, flower stem lengths, inner and outer bract length and even seed surface pitting. To study the reproductive biology, Raven [2006] measured the number of flower stems, number of fruits, number and size of seeds and conducted a germination study. In her study, each site was representative of a species for a total of five sites representing three species designations. Most measurements did not differ consistently between sites. For example, South Prairie (*S. sarmentosum*) plants had significantly shorter outer bracts than Little Crater Meadow plants (*S. sarmentosum*) and Little White Salmon plants (Unknown species) [Raven 2006]. On the other hand, style exertion, time of flower opening, seed color and seed texture did not significantly differ between sites [Raven 2006]. The results of the hybridization study draw a complex and confusing map of the characteristics used to distinguish between *Sisyrinchium* occurrences and do not seem to be a reliable way to distinguish between species.

In contrast to the hybridization and reproductive biology studies, a genetic study by DeWoody and Hipkins [2015] revealed strong genetic differences between the two populations of *S. sarmentosum* and one population of *S. idahoense*. Using Random Amplified Polymorphic DNA

(RAPD) genetic sampling techniques, researchers found more variation within populations for *S. sarmentosum* than previously found by Wilson et. al. [2000] but found seven alleles unique to *S. sarmentosum*. Raven [2006] attributed the different results to the use of RAPD instead of isozyme to analyze the genome, giving a genetic basis for differences between *S. sarmentosum* and *S. idahoense* and possibly a method for identifying hybrids of the two species.

Two recent studies [Chaveau et. al. 2011, Karst and Wilson 2012] examined the phylogeny of *Sisyrinchium* and several closely related genera. Using Polymerase Chain Reaction (PCR) techniques for DNA extraction and Bayesian and maximum likelihood to create a tree describing the relationships between many members of the genus, the researchers placed *S. sarmentosum* no more than 4 branches from *S. idahoense* in the analysis by Chaveau et. al. [2011] and no more than 2 branches from *S. idahoense* in the analysis by Karst and Wilson [2012]. Unfortunately, both studies did not capture the full spectrum of variability of the two species; Karst and Wilson [2012] used two genotypes of each species in their analysis while Chaveau et. al [2011] used 8 genotypes of each species in their analysis. It also isn't immediately apparent which populations these genotypes represent, whether or not any of the populations examined in this study are represented. Nonetheless, these two studies support the designation of *S. sarmentosum* and *S. idahoense* and support DeWoody and Hipkins [2015] findings of significant genetic differences between the two species, supporting a method for identifying hybrids of *S. sarmentosum* and *S. idahoense*.

Following one of the suggestions of Raven in her 2007 study, Washington Rare Plant Care and Conservation and the US Forest Service have begun the *Sisyrinchium* Common Garden project. The theory behind common garden experiments is that if differences between species do appear, it will be easy to rule out the effects of the environment since all plants should experience the same environmental factors. In the summer of 2013, volunteers collected measurements on flower morphology of 8 populations of both *S. sarmentosum* and *S. idahoense* and their suspected hybrids (each phenotype is represented in at least 3 populations). Volunteers measured characteristics similar to those used in Raven's 2006 study and subsequently collected seed from those plants. The following winter, seeds were characterized based on seed color and texture, as in Raven's 2007 study, and placed in an incubator to germinate. As part of the Common Garden Project, the plants will be grown to flowering at the Center for Urban Horticulture to reduce the

influence of the environment on flower characteristics. Flower morphologies between the species designations will be compared to differentiate between them.

Other common garden studies [Cordell et. al. 1997, Bower and Aitken 2008, Gallagher et. al. 1988, Vitasse et. al. 2009, Warren et. al. 2005] have used similar measures of growth to examine phenotypic and morphological differences in plants from different areas without the effect of the environment. My study aims to do the same although, rather than examining known differences in plant morphology, it intends to search for differences in traits. No morphological data have previously been recorded for these two species; most data referring to *S. sarmentosum* and *S. idahoense* have focused on floral characteristics. Two probable explanations for the lack of recent morphological data in the latest two studies of the plants are discussed here. For one, biomass data requires the entire plant be harvested which would not be an option for a wild plant designated as threatened. Secondly, seeds failed to germinate in Raven's study [2006] for unknown reasons, excluding the option of biomass sampling for greenhouse grown plants. Both genetic studies [DeWoody and Hipkins 2015, Wilson et. al. 2000] used plant material collected in the field which meant researchers harvested only what was required to run the genetic testing. Raven [2006] and Wilson et. al. [2000] suggest that differences may lie in rhizome formation by *S. sarmentosum*, but due to the destructive nature of analysis, as mentioned before, researchers were unable to confirm this trait. In a common garden study, this trait may appear as a higher below-ground to above-ground biomass ratio. Additionally, other characteristics that appear to be different can possibly be attributed to genetic differences described by DeWoody and Hipkins [2015] in their RAPD analysis. While it will be hard to say definitively that observed traits are due to specific alleles, my study will hopefully steer future studies in the right direction and narrow down the number of possible characteristics to assess when identifying *S. sarmentosum* and *S. idahoense*. Ideally, my study will show significant differences between species co-occurring within a site ($\tau_i \neq 0$ for all i). My study aims to answer the question: can measures of plant growth be used to assess differences between *S. sarmentosum* and *S. idahoense* and their suspected hybrids?

Methods

The current study utilized a three stage sampling design with individual plant ID numbers nested within species which were nested within sites. In the summer of 2013, floral characteristics of *S. sarmentosum*, *S. idahoense* and their suspected hybrids from 8 populations were measured.

Plants were assigned numbers at this point, hereafter referred to as the maternal ID, and tagged to



Figure 3. Bleaching *Sisyrrinchium*



Figure 4. Germinated *Sisyrrinchium* seeds

aid in identification. Volunteers returned later that summer to collect seed from labeled plants for cleaning and storage until the project was ready to begin. While the maternal parentage was identified using floral characteristics, pollen transfer was not monitored, meaning the species designation assigned to seed is specifically the maternal species designation. Seeds were selected solely based on the number of seeds per maternal ID; maternal ID's with between 15 and 20 seeds were selected for germination as a precaution against low sample sizes.

Seeds were germinated in the Center for Urban Horticulture's tissue culture lab in the spring of 2014. Raven [2006] reported difficulties germinating *S. sarmentosum* without treatment while earlier unpublished germination experiments determined that bleaching the seeds of *S. sarmentosum* increased germination, possibly due to the thinning of the seed coat.

Because of this, seeds were bleached (Fig. 3), placed onto 9cm filter paper wetted with de-ionized water, placed inside of a petri dish which was then placed inside of an incubator set for winter. The incubator was cycled through the four seasons three

times over 12 months at varying intervals each cycle. This was due to a lack of communication and the shortening of available time to complete the project; season length in the incubator decreased towards the end of the germination phase of the project. Plants used in this experiment germinated during late summer and winter when the incubator was set to 1st summer and the 2nd spring (Fig. 4). Plants were then grown in 8 x 128 cell trays in the hoopouses in the yard behind

the Douglas Research Conservatory until early spring (1st and 2nd data collection) and late spring (3rd data collection).

Measurements of plant height, number of shoots, leaf area, above-ground biomass and below-ground biomass were taken in the lab to gain an understanding of the basic growth habits of *S sarmentosum*, *S. idahoense* and hybrids. Measurements of these five characteristics would enable the study to show changes in growth as well as the ability to quantify differences in plant size and allocation strategies between species included in the study.

Plant height was measured using the longest leaf not exhibiting signs of senescence, measuring from the soil line to the apex of the leaf. The number of visible shoots the plant had produced were counted; by default, each plant had 1 adventitious shoot.

The plant was then rinsed using tap water and above-ground and below-ground biomass were separated at the highest root on the crown of the plant. The leaves were then separated from each other and were carefully arranged on quartered printer paper. Plants were photographed using a 8 mega pixel auto focus camera (Samsung Galaxy S3) to be analyzed for leaf area and were then folded into the printer paper to be placed into a drying oven.

Plants were dried at 200°F for 58 hours in the drying oven. The plants were then removed and the above-ground and below-ground masses were measured using an analytical balance.



Figure 5. Leaf area raw input to



Figure 6. Measured input in ImageJ

The process of measuring leaf area using traditional methods seemed impractical and inaccurate for such a small plant. Measurement using a ruler and the geometric formula for a generalized shape seemed too inaccurate while the transparent belt conveyor method seemed impractical and more fit for use on larger plants. It was decided that image analysis using a computer program

would be the most practical given the number of samples and the size of the plants. In addition, the measurements could be conducted again using more accurate methods if needed. To use this method, photos were first optimized for brightness, contrast, shadows and highlights in Adobe Lightroom 4.4 (Fig. 5). The images were then renamed based on the designation assigned in the separation step. Images were analyzed using ImageJ, following the process outlined in the Plant Ecophysiology lab manual [Kim et. al. 2014]. Particles less than 0.01 cm^2 were excluded in the final step of measurement (Fig. 6), since particles that small were probably not part of the leaves. Measurements were copied to a Microsoft Excel spreadsheet. Below-ground to above-ground biomass allocation and specific leaf area were calculated in Excel to obtain 7 response variables. Once all measurements were obtained, data were analyzed using R (version 3.1.2) and R Studio. Measurements were not independent of each other, so a principal components analysis was conducted to obtain independent response variables to analyze all measurements at once, rather than doing a two factor ANOVA on each measured variable. The principal component analysis also indicated which response variables were more closely related than others and also indicated how much variance each variable accounted for. Once the principal component analysis was complete, a two factor ANOVA with one random variable was conducted on the principal component scores for the first two principal components, as they accounted for the most variability. Population and species were fixed factors whereas maternal ID was considered a random factor since selection of maternal ID in the germination stage was based on one characteristic that did not reflect plant health or growth. Moreover, an uneven number of individuals was represented in the experiment, making representation random as well since selection was not based on population or species. Adding the random factor allowed the study to account for variation found between plants within the same maternal group. A Tukey HSD approximation was run on factors where p values were less than 0.05 to determine where the largest differences were and where possible.

The current study focused on populations where *Sisyrinchium* hybrids were identified, since the distinctions between *S. sarmentosum* and *S. idahoense* are less clear at those populations. Plants of 4 possible species from 5 possible populations were measured for the characteristics described above, although each population did not necessarily include all species. An inventory of the plants revealed varying numbers of representatives for each combination of population and

species, resulting in at least 2 replications for each combination of population and species for plants aged 3 months and 5 months old. Population and species representation was limited further in plants ages 10 months through 7 months old, mainly determined by germination rates earlier in the study. In the 3 month and 5 month age group, the goal in increasing the number of replications of *S. idahoense* at Peterson Prairie was to bring the distribution of species across all populations as close to equal as possible, since the focus of the study was to distinguish between species and not so much between populations.

The following tables (Tables 1a through 1d) show the numbers of samples for each site and species combination for each age class, illustrating the limitations in representation.

3 mo	FCHC	PP	SP	CM	LCM	Total
SISA	12	4	6	2	16	40
SIID	0	32	0	0	0	32
SIHY	16	8	0	2	15	41
Unknown	8	2	17	10	4	41
Total	36	46	23	14	35	154

Table 1a. Number of samples taken for all factors for the 3 month age class

5 mo	FCHC	PP	SP	CM	LCM	Total
SISA	27	9	8	6	29	79
SIID	0	41	0	0	0	41
SIHY	28	15	0	12	26	81
Unknown	15	4	26	13	10	68
Total	70	69	34	31	65	269

Table 1b. Number of samples taken for all factors for the 5 month age class

7 & 8	FCHC	PP	SP	CM	LCM	Total
SISA	19	21	1	0	23	64
SIID	0	0	0	0	0	0
SIHY	33	6	0	1	32	72
Unknown	7	2	3	1	7	20
Total	59	29	4	2	62	156

Table 1c. Number of samples taken for all factors for the 7 & 8 month age class

9 & 10	FCHC	PP	SP	CM	LCM	Total
SISA	1	3	0	0	0	4
SIID	0	0	0	0	0	0
SIHY	3	2	0	0	1	6
Unknown	0	1	0	0	0	1
Total	4	6	0	0	1	11

Table 1d. Number of samples taken for all factors for the 9 & 10 month age class

Results - Three Month Age Class

In the initial analysis of plants in the 3 month age class, most plants in the 3 month age class had one shoot (the main shoot) and only a few fast growing individuals grew more than one shoot. The number of shoots did not seem to accurately describe growth and was removed, resulting in a more balanced analysis. The first principal component was highly correlated with characteristics associated with plant size (Fig. 7 and Table 2). Principal component two is negatively correlated with above-ground to below-ground biomass ratio and the above-ground area (Fig. 7 and Table 5) which seem to loosely describe allocation strategies.

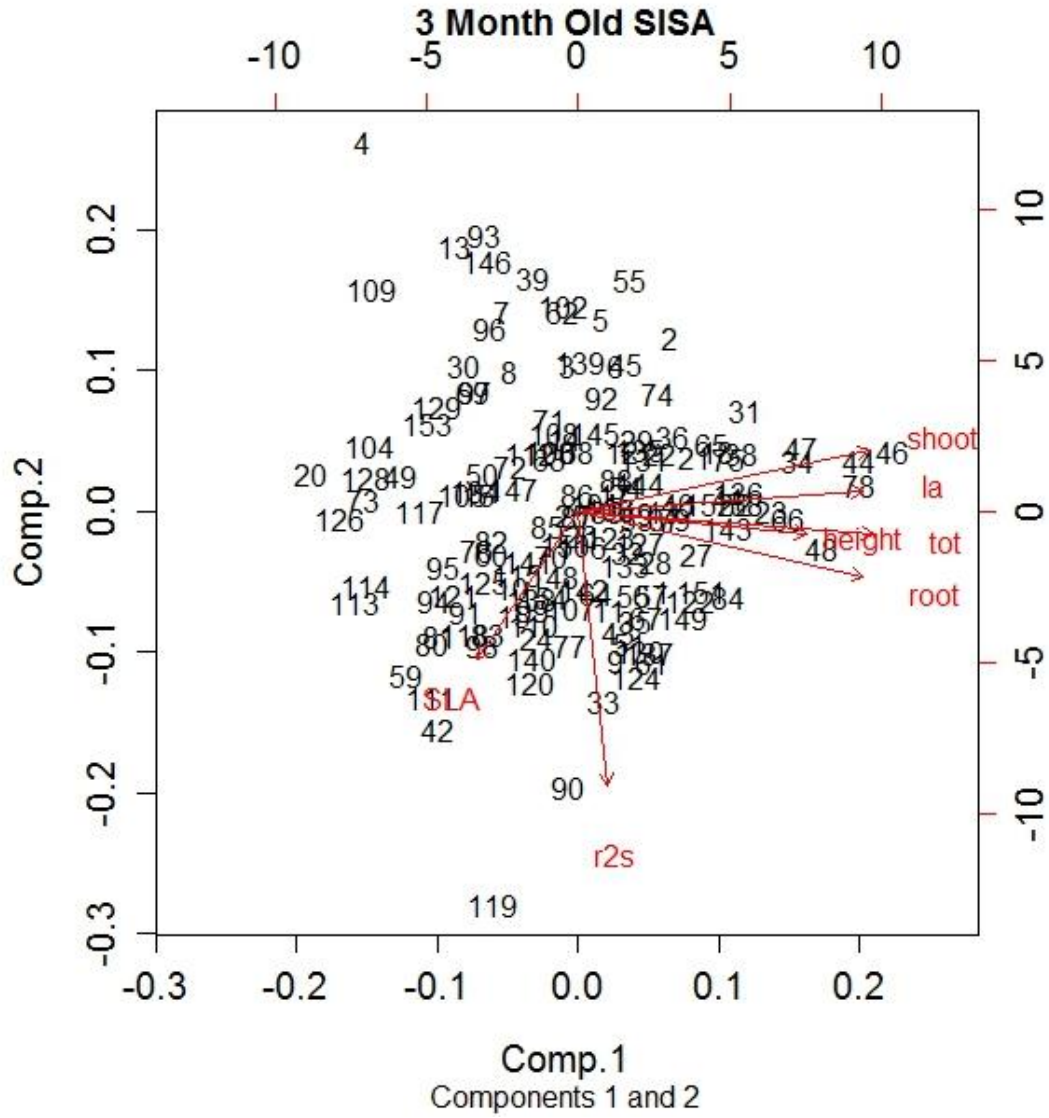


Figure 7. Biplot of adjusted loadings and components after removal of shoot count

3 mo	Comp.1	Comp.2	Comp.3
height	0.36145	-0.0677	-0.42474
root	0.45313	-0.19862	0.168095
shoot	0.46206	0.185889	-0.01403
tot	0.46896	-0.07123	0.109839
r2s	0.04690	-0.84038	0.375693
1a	0.45029	0.063877	-0.23829
SLA	-0.16004	-0.45389	-0.76233

Table 2. Adjusted loadings for 3 month age class

3 mo	Prop of Var
Comp.1	0.62898005
Comp.2	0.16915052
Comp.3	0.1464797
Comp.4	0.04899401
Comp.5	0.00524911
Comp.6	0.00114661

Table 3. Proportion of variance for components of 3 month age class

Table 3 describes the relative variance each component contributes to the overall variance of the data. Component 1 (Plant size) accounts for over half of the total variance while component 2 accounts for close to one fifth of the variance (Table 3). Combined, both factors account for close to 80% of the overall variance.

A two way ANOVA with one random effect of the 3 month old plants (Table 4), using component 1 as the response variable, yielded the following.

	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
population	15.00436	3.751089	4	61.04723	1.179161	0.328937
species	8.99751	2.999172	3	54.18949	0.942794	0.426442
pop:species	23.32431	3.332044	7	60.85812	1.047433	0.408233

Table 4. ANOVA results for component 1 of the 3 month age class

From these results, the test fails to reject all three null hypothesis ($\tau_i = 0$ for all i) for component 1 at $\alpha = 0.05$ level of significance, concluding that there are no significant differences for any of the factors when examining component 1.

	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
population	7.150413	1.787603	4	55.23184	2.486054	0.053899
species	8.531653	2.843884	3	50.93704	3.955044	0.013048
pop:species	12.06806	1.724008	7	55.10075	2.397611	0.032432

Table 5. ANOVA results for component 2 of the 3 month age class

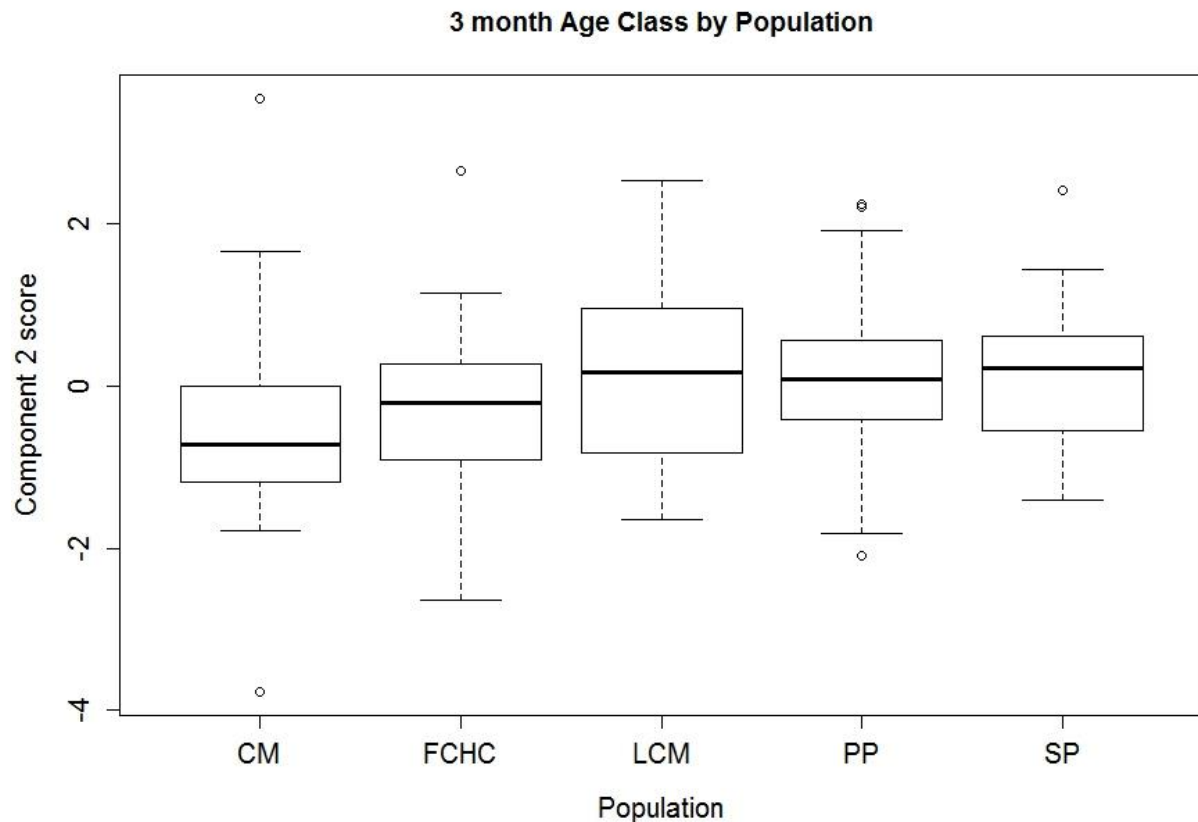


Figure 8. Component 2 scores divided by population for the 3 month age class

From the second ANOVA on component 2 scores (Table 5), there are two significant effects at $\alpha = 0.05$ (The effects of species and the interaction effects) and one moderately significant effect (Effects of population). Due to the unbalanced nature of the distribution of plants among populations and species, further statistical analysis of the main effects was not possible. Instead, a boxplot of sites shows that *S. idahoense* plants had a narrower range of component 2 scores at the higher end of overall scores (Fig. 8). A boxplot of species and component 2 scores showed plants from Clackamas meadow had lower scores overall, meaning plants had higher above-ground to below-ground ratios (Fig. 9).

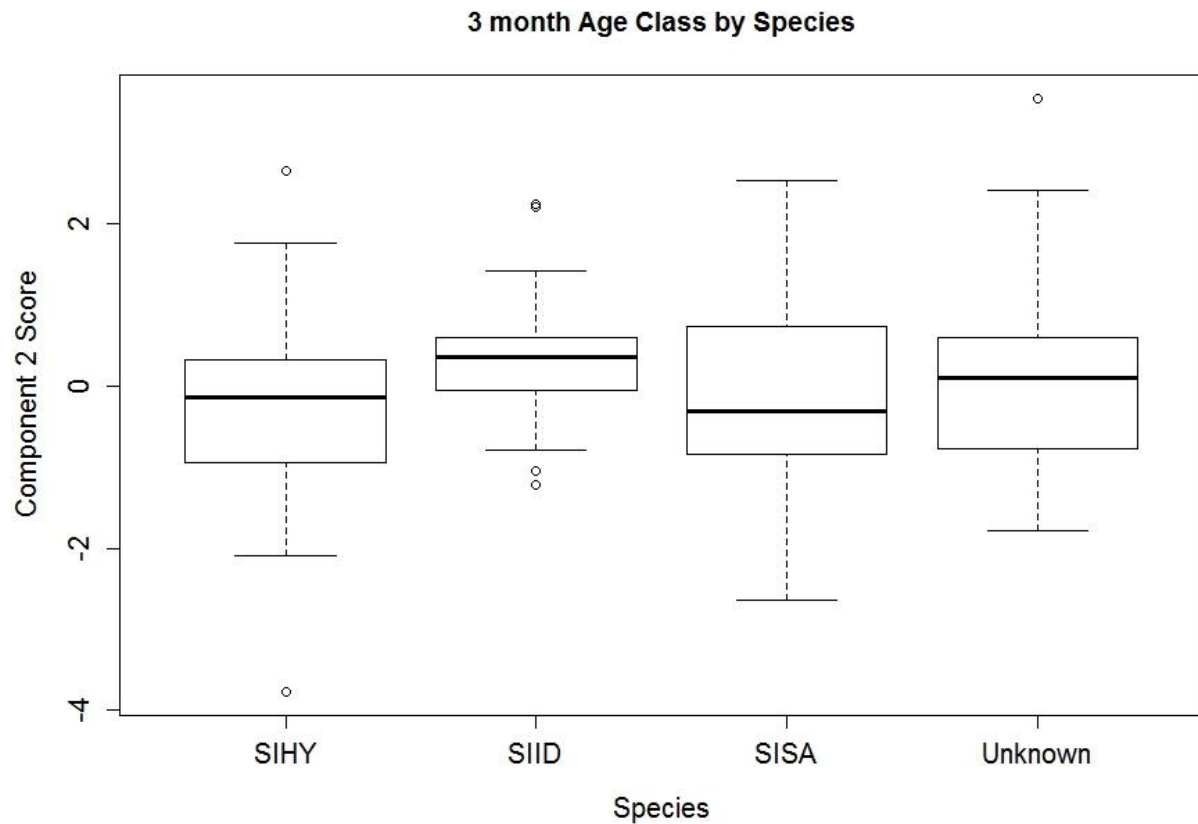


Figure 9. Component 2 scores divided by species for the 3 month age class
 Looking at the interaction effects, Clackamas Meadow hybrids were the only plants that were slightly significant from any other population x species combination (Table 6 and Fig. 10).

contrast	estimate	SE	df	t.ratio	p.value
CM, SIHY - PP, SIID	-2.79439	0.871	55.58	-3.208	0.1746
CM, SIHY - LCM, SISA	-3.15949	0.88233	58.8	-3.581	0.0704
CM, SIHY - SP, Unknown	-2.84475	0.87863	59.1	-3.238	0.1615

*Pairwise comparisons with a p.value higher than 0.20 or NA omitted for brevity.

Table 6. Pairwise contrasts of site x species combinations with Tukey approximations for p-values for the 3 month age class

3 Month Age Class

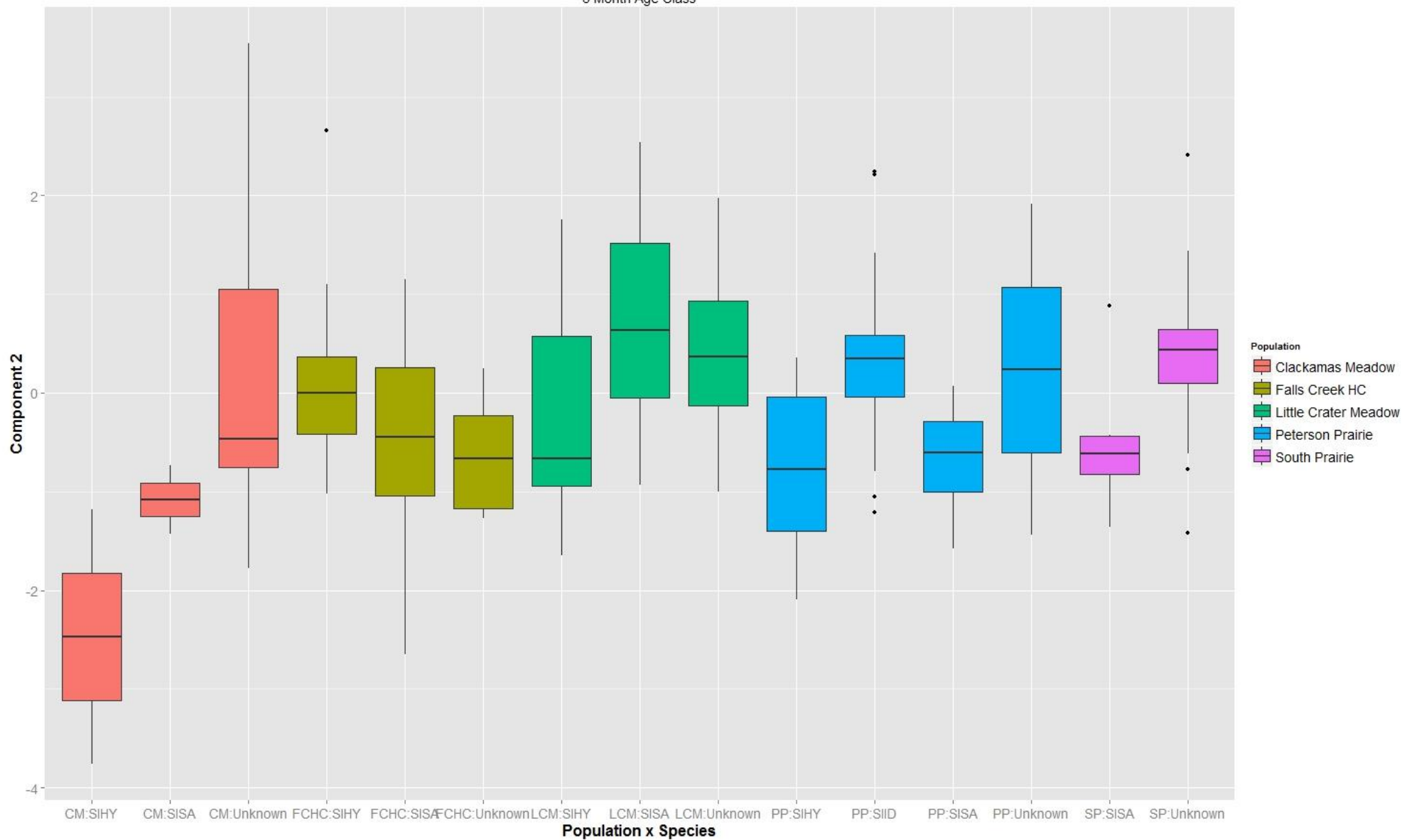


Figure 10. Component 2 scores divided by population x species combinations for the 3 month age class

Results - Five Month Age Class

Plants sampled in the 5 month age class were analyzed similarly to the 3 month age class, with the number of adventitious shoots included in the analysis. The dataset contained more samples than either the 3 month age or 8 month age classes (Table 1b). There was a considerable amount of variation in all factors, even within maternal ID groups. The principal component analysis weighted and related the variables similarly with the previous principal component analysis of the 3 month age class.

	Comp. 1	Comp. 2	Comp. 3
ht	-0.27363	0.54484	-0.14152
shoots	-0.33352	-0.20533	0.03874
r	-0.41487	-0.29589	-0.15777
s	-0.45761	0.06323	0.03755
tot	-0.45428	-0.13619	-0.07076
r.s	0.08809	-0.62227	-0.54756
la	-0.45168	0.14462	-0.08294
sla	0.11926	0.37783	-0.80029

Table 7. Loadings for the 5 month age class

	Prop of Var
Comp. 1	0.575067
Comp. 2	0.175468
Comp. 3	0.121463
Comp. 4	0.086858
Comp. 5	0.029814
Comp. 6	0.010485
Comp. 7	0.000846
Comp. 8	5.99E-17

Table 8. Proportion of variance for components of the 5 month age class

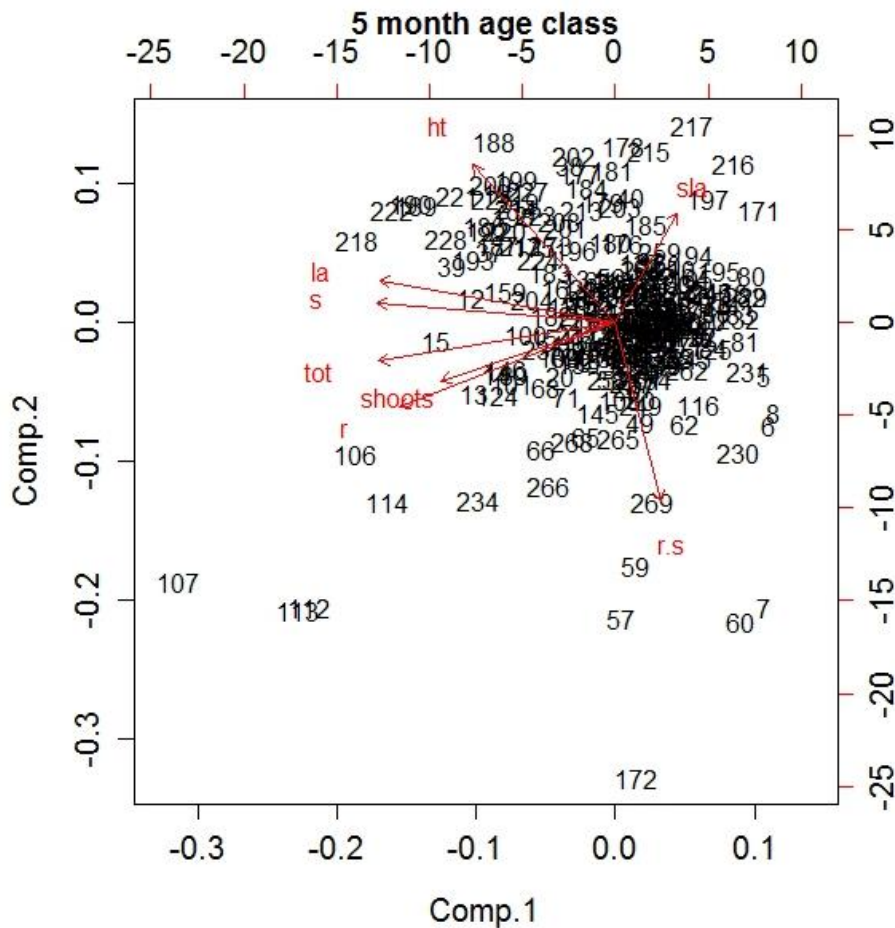


Figure 11. (Left) Biplot of loadings and components of the 5 month age class

In this case though, component 1 is weighted less by plant height, possibly affected by the inclusion of the number of adventitious shoots (Table 7 and Fig. 11). Component 1 also accounts for slightly less of the overall variance found in the data (Table 8). When combined with component 2, both account for just under 75% of the variance of the dataset (Table 8).

	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
population	14.45861	3.614652	4	63.71835	1.497725	0.213456
species	16.07515	5.358383	3	60.94482	2.220237	0.094836
pop:species	18.34836	2.621194	7	63.60303	1.086087	0.382808

Table 9. ANOVA results for component 1 of the 5 month age class

The ANOVA revealed no significant differences at $\alpha = 0.05$ for component 1 (Table 9). Species designation seems to be somewhat significant in this test (Table 9). As with the 3 month age class ANOVA, it was not possible to analyze the differences in R due to missing population x species combinations. Despite this, a boxplot of Component 1 scores (Fig. 12) reveals that *S. idahoense* plants scored significantly lower, meaning that plant size was larger for *S. idahoense* plants.



Figure 12. Component 1 scores divided by species for the 5 month age class

	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
population	1.880824	0.470206	4	56.94545	1.184335	0.327494
species	4.861407	1.620469	3	55.33951	4.081571	0.010895
pop:species	6.031585	0.861655	7	56.42287	2.170301	0.050659

Table 10. ANOVA results for component 2 of the 5 month age class

For component 2, species is a significant effect at $\alpha = 0.05$ and the interaction is also somewhat significant (Table 10) with the ANOVA rejecting the null hypothesis ($\tau_i = 0$ for all i) by a slight margin. A boxplot again shows that *S. idahoense* plants received a higher component 2 score (Fig. 13), meaning that plants had longer leaves and allocated more biomass above-ground.

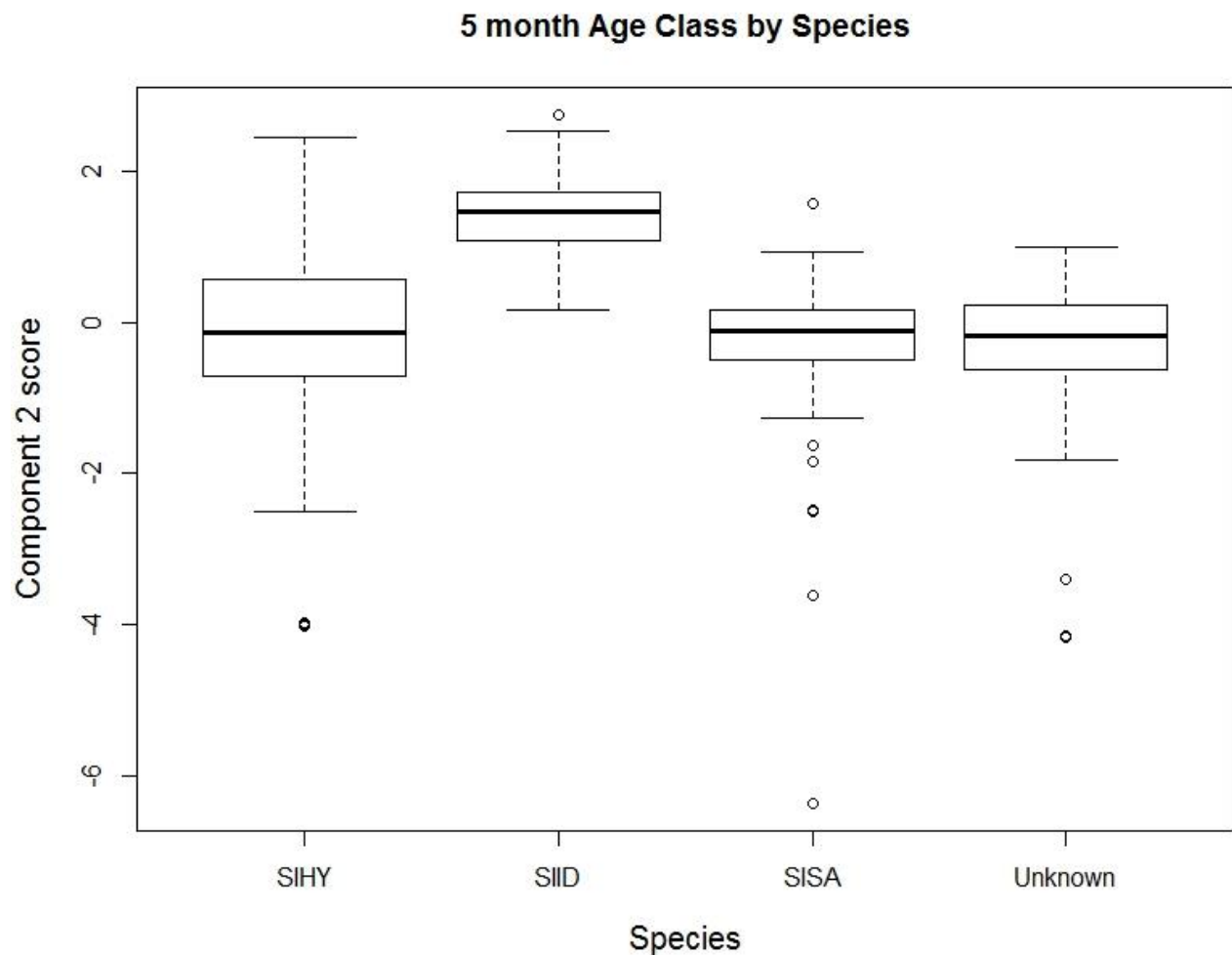


Figure 13. Component 2 scores divided by species for the 5 month age class

Analyzing the interaction effects produced the following results (Table 11).

contrast	estimat	SE	df	t.ratio	p.value
FCHC,SIHY - PP,SIID	-1.924	0.4699	58.09	-4.095	0.0167
PP,SIID - PP,SISA	2.7702	0.6544	63.51	4.233	0.0102
PP,SIID - LCM,Unknown	2.3669	0.5932	64.66	3.99	0.0213
PP,SIID - CM,SISA	2.5875	0.6788	72.97	3.811	0.034
PP,SIID - FCHC,SISA	1.6154	0.4686	66.33	3.447	0.0953
CM,SIHY - PP,SIID	-2.0167	0.5798	59.99	-3.478	0.0909
PP,SIID - LCM,SISA	1.5792	0.4594	59.18	3.437	0.101
PP,SIHY - PP,SISA	2.5249	0.7369	63.34	3.426	0.1017

P value adjustment: tukey method for comparing a family of 20 estimates

*Pairwise comparisons with a p.value higher than 0.20 or NA omitted for brevity.

Table 11. Pairwise contrasts of population x species combinations with Tukey approximations for p-values for the 5 month age class

We see that *S. idahoense* plants from Peterson Prairie were involved in all significant interactions at $\alpha = 0.05$ and $\alpha = 0.10$ (Table 11 and Fig. 14).

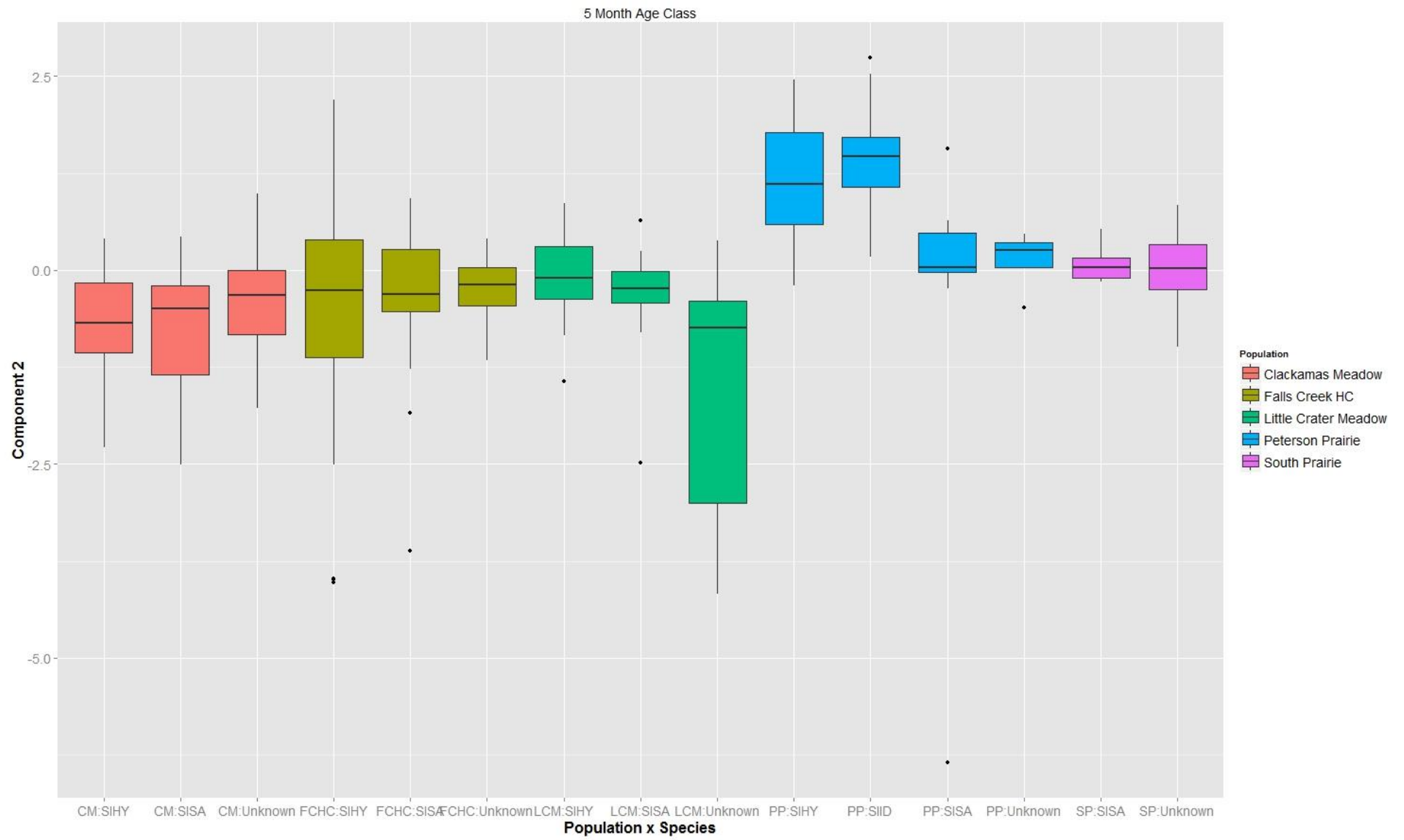


Figure 14. Component 2 scores divided by population x species combinations for the 5 month age class

Results – Seven and Eight Month Age Class

Due to the small sample size of combinations in the September data, I combined data from plants in the 7 and 8 month age class before analysis (Table 1c).

7 & 8 mo	Comp. 1	Comp. 2	Comp. 3
ht	-0.36589	0.07354	-0.01827
shoots	-0.35801	0.02989	0.00633
r	-0.41785	-0.24672	-0.12814
s	-0.43278	0.04510	0.14252
tot	-0.4332	-0.18798	-0.07077
r2s	0.08157	-0.69481	-0.61741
area	-0.42363	0.23409	-0.13909
SLA	0.04755	0.59824	-0.74655

Table 12. Loadings for the 7 & 8 month age class

& 8 mo	Prop. of Var.
Comp. 1	0.613552
Comp. 2	0.145106
Comp. 3	0.127580
Comp. 4	0.066265
Comp. 5	0.031194
Comp. 6	0.012994
Comp. 7	0.003309
Comp. 8	2.79E-17

Table 13. Proportion of variance for components of the 5 month age class

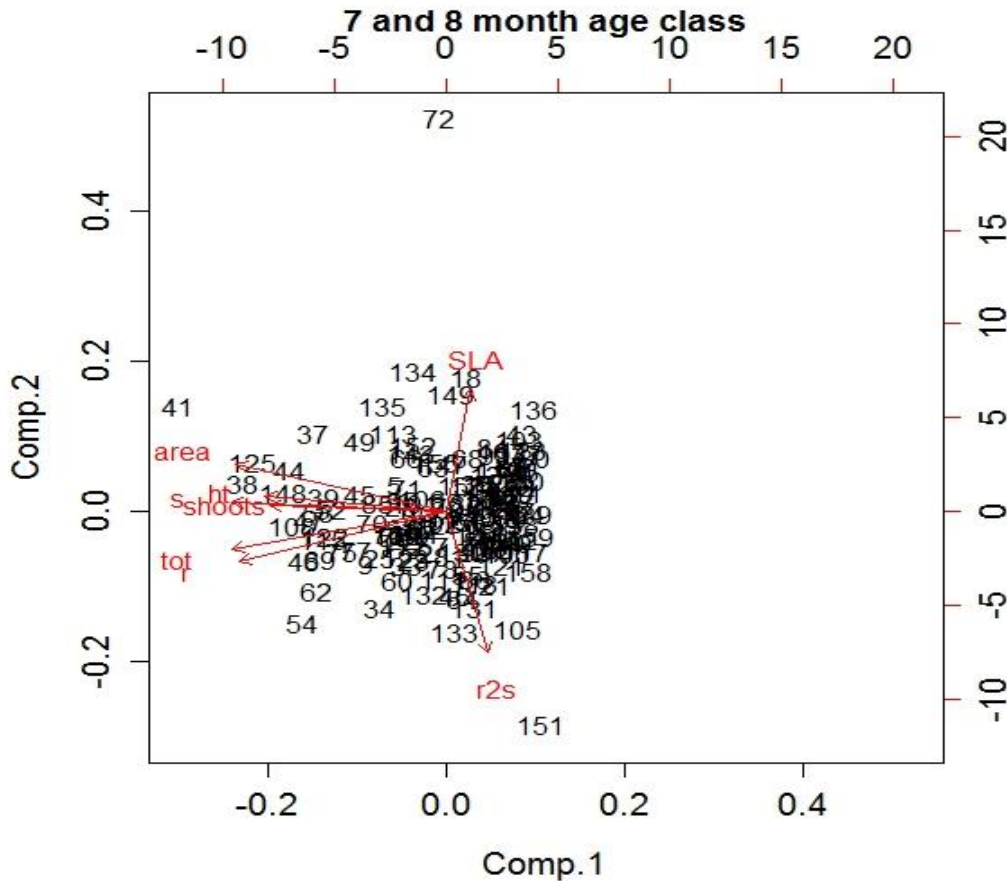


Figure 15. Biplot of loadings and components of the 7 & 8 month age class

Similar to the analysis of the 3 month old plants, the first principal component of the 8 month old plant analysis is heavily weighted on measurements of plant size (Table 12 and Fig. 15).

Component 1 also accounts for more than half of the variation in this dataset as well (Table 13).

Component 2 accounts for slightly less of the variation in this dataset (Table 13) but is still weighted similarly to the other analyses, weighted heavily by above-ground to below-ground biomass and the specific leaf area (Table 12 and Fig. 15).

An ANOVA was run on the first two components, the results are shown in table 14.

	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
population	11.69223	2.338447	5	55.29722	0.685036	0.636735
species	26.94871	13.47436	2	65.88077	3.947241	0.024039
pop:species	19.55715	3.259525	6	48.72648	0.95486	0.465415

Table 14. ANOVA results for component 1 of the 7 & 8 month age class

In this analysis, there were significant effects between species. A boxplot (Fig. 16) shows that unknown plants sampled showed a narrower range of component 1 scores, more so at the higher end of scores indicating that plants of unknown maternal origin were typically smaller in size than both *Sisyrinchium* hybrids and *S. sarmentosum* plants.

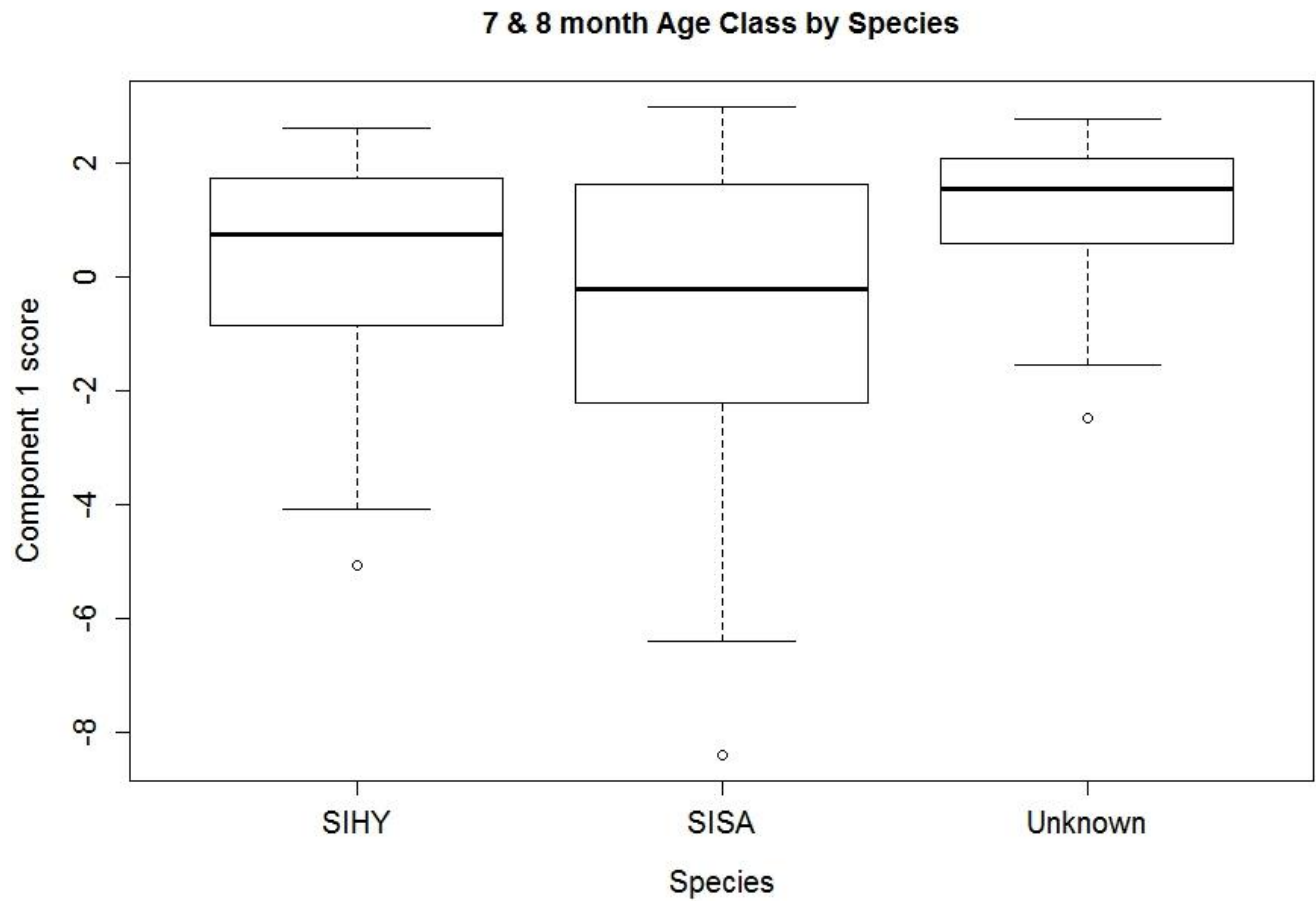


Figure 16. Component 1 scores divided by species for the 7 & 8 month age class

	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
population	3.194469	0.638894	5	145	0.528115	0.754708
species	1.900099	0.950049	2	145	0.785319	0.457904
pop:species	2.491882	0.415314	6	145	0.343302	0.912829

Table 15. ANOVA results for component 2 of the 7 & 8 month age class

Table 15 shows that there were no significant differences in component 2 scores for any effects in the 7 & 8 month age class plants.

There were a total of only 11 plants in the 9 and 10 month age class (Table 1d). An ANOVA seemed inappropriate given the low sample size. The plants were used instead to give a better idea of the growth patterns of the populations in the study.

Discussion: Limitations

Sample size was the study's greatest limitation. The 5 month age class was the largest sample with 269 plants (Table 1b). Most maternal IDs had three representatives with the exception of the *S. idahoense* plants, which had up to 6 representatives. Considering that most plants used were selected because of the higher seed count and high germination rates, the datasets don't represent a large proportion of plants found at each population. Tables 1a through 1d show the number of plants represented in each population/species combination. Distribution between population and species was not balanced. While these limitations did not affect how the analysis was conducted, it does limit the applicability of the results and the post-hoc analysis.

Uneven distribution and missing population x species combinations also affected the analysis. Using a mixed effects ANOVA allowed the analysis to account for more of the variation in the dataset, factoring in variability found between plants of the same maternal ID. However, the mixed effects ANOVA did not allow me to analyze the main effects in R to determine where significant differences were. For example, only *S. idahoense* was represented in Peterson Prairie so comparison of species was not possible since *S. idahoense* was absent from all other sites. The use of the mixed effects model further complicated how the factors interacted and also seemed to contribute to the difficulty of a post-hoc analysis. Since the missing group combinations were not due to lack of sampling or poor design, there are only a few ways this problem could be remedied. Representation of species within a population will not change, the study would either have to eliminate the random effect or limit the inference population to simply, the plants sampled rather than to the entire population of *Sisyrinchium*. Considering that the study was meant to serve as an indication for populations in the field, the second workaround seems like a less viable option.

Older age classes were more unevenly distributed between population and species. In these datasets, maternal representation was not guaranteed two plants per parent. Relatively low totals for plants in just the 7 month age class complicated analysis of the dataset. Analysis of this dataset alone would have led to low degrees of freedom and the use of a different statistical test. Combining the 7 month age class with the 8 month age class allowed me to use the 7 month age class data while increasing the representation of maternal lines across sites and increasing the total sample size of the dataset (Table 1c). Plants from both age classes seemed very similar in

size when observed in the greenhouse and displayed the same amount of variation as the 8 month age class. Although the improvement in representation for population/species was small, representation for population and species as separate factors improved to at least 2 plants per factor. While the distributions were not ideal, ANOVA tests are known to be robust to unequal sample sizes so an analysis on the combined group was conducted.

Fewer plants germinated earlier in the study, leading to lower numbers in the 9 and 10 month classes (Table 1d) and why an analysis was not performed on those datasets. Combining the two datasets did not help at all as there were 11 plants total for the combined datasets. The only solution to this problem would be to let plants grow for longer and germinate more plants to sample, drawing out the experiment further than feasible.

The image analysis, as performed, was another limitation in this study. Using a camera and an image processing program greatly increased the ease and consistency of measurements but doing so also had shortcomings. Difficulties occurred when attempting to analyze poor photos. Camera settings were automatic, allowing for less control of lighting and depth of field, though



Figures 17 & 18. Inputs to ImageJ showing the approximations and inaccuracies associated with this method

both were corrected in post processing. Composition of the photo was the main problem; measurements not taken in the center of the photo were difficult to distinguish against the dark lighting around the edges and due to the curvature of the lens, objects near the edges are more prone to distortion. For leaves in darker areas of the photos (Figures 17 and 18), a fair amount of estimation was required to attempt to quantify the area of the leaf. Since the program measures

dark areas (dark leaves : white paper), there was a tradeoff between how much of the leaf was shaded and how much of the edge was shaded to make up for the lack of leaf shading.

Discussion: Interpretation

Principal Component Analysis

Loadings from the principal component analysis of all datasets indicated that below-ground biomass, above-ground biomass, total biomass and leaf area were all strongly related across all age classes (Tables 2, 7. and 12). All are direct measures of plant size and all measurements were unmodified from data collection except for total biomass, which can be thought of as linearly correlated with above-ground and below-ground biomass. Component 1 was consistently weighted heavily by these 4 measurements. Height was also strongly related to plant size although in the 5 month age class, it contributed to both component 1 and component 2. Interestingly, the proportion of variance explained by component 1 was near 60% for all principal components, despite the varying results of the ANOVA tests (Tables 4, 9 and 14).

The second principal component was consistently weighted by above-ground to below-ground biomass and plant above-ground surface area to above-ground mass (what was referred to as specific leaf area, Tables 2, 7 and 12). Component 2 accounted for between 14% and 17% of the total variance (Tables 3, 8 and 13), a somewhat narrow range, indicating consistency between age class measurements. Consistency of loadings and proportion of variances across datasets seem to be a good result, although not explicitly stated in any statistical literature found.

ANOVA results

Based on previous studies discussed in the introduction, differences were not expected to appear in morphologic characteristics between species designations or populations. Significant results of the ANOVA were a bit of a surprise though low sample sizes and the use of the mixed effects model seemed to dismiss most of these results.

Three Month Age Class

A two way mixed effects ANOVA analysis of the first principal component of the 3 month age class plants found no significant differences for any of the factors (Table 4), conforming nicely to expectations. From experience, many plants were difficult to distinguish based on size at such

an early stage. Although there are no direct citations for this, logically, plant size can also be thought of primarily as a response to the environment, indicating that the environment is relatively constant for all plants and that no unique characteristics exist in individual plants at this stage.

The second component on the other hand, showed significant differences between species and population x species at $\alpha = 0.05$ and moderately significant differences between populations at $\alpha = 0.10$ (Table 5). The unbalanced design of the study, as discussed above, complicated further analysis in R and only allowed a complete Tukey contrast on the interactions. The presence of an interaction seems to have had strong effects on the main effects and may be another reason post-hoc analysis is limited to the interactions. While the ANOVA indicated significant differences in both of the main effects, due to the problems discussed previously, the test may have given bad results, despite the level of significance being relatively high. Bar plots of both main effects seem to support this conclusion (Figures 8 and 9); no populations exhibit largely higher or lower component 2 scores for both main effects of population and species. *S. idahoense* plants did exhibit a narrower range of scores (Figure 9), probably due to the fact that *S. idahoense* plants were represented by one population and had relatively fewer samples.

For the interaction effects, most scores were higher than $p = 0.5$. Only three contrasts scored p-values lower than 0.2 while none scored lower than $p = 0.05$ (Table 6). Clackamas Meadow hybrids were involved in the three interactions with p-values less than 0.2. Although not significant, a bar plot of the interaction effects and component 2 seems to support the results of the Tukey approximation. *Sisyrinchium* hybrids from Clackamas Meadow appeared to have scored significantly lower than all other population x species combinations (Figure 10). Low sample size associated with the site and species combination probably increased the variance of the combination. High variability within other population x species combinations also probably contributed to the insignificant contrasts.

Five Month Age Class

Results of the ANOVA on component 1 of the five month age class indicated that there were no significant differences for the factors at $\alpha = 0.05$ (Table 9). The effect of species was marginally significant. A bar plot of the data shows that *S. idahoense* plants scored somewhat lower than other species (Figure 12), although not quite outside of the range of variability of the other

species, probably resulting in only marginal significance. Results of the ANOVA on component 2 indicated significant differences between species and population x species effects. A bar plot of component 2 scores shows that *S. idahoense* plants scored higher (Figure 13), meaning that *S. idahoense* plants had longer leaves and lower biomass ratios.

The Tukey approximation for contrasts showed that *S. idahoense* plants were involved in all significant interactions (at $p < 0.05$) and most of the marginally significant interactions ($p < 0.15$, Table 11). *S. idahoense* mainly contrasted from *S. sarmentosum* (all populations except those from South Prairie). According to these results, growth measurements may be used to distinguish between maternal *S. idahoense* and *S. sarmentosum* at this stage of development. As with the limitations to the previous data, the applicability of the results is reduced due to the fact that *S. idahoense* was represented by one population (Table 1b). Component 2 only accounts for 16% of the variation in the dataset (Table 8), further reducing the applicability of the results. *S. idahoense* plants also contrasted strongly from *Sisyrinchium* hybrids from Falls Creek Horse Camp and marginally contrasted from Clackamas Meadow *Sisyrinchium* hybrids (Table 11) indicating that hybrids from Clackamas Meadow and Falls Creek Horse Camp are more similar to *S. sarmentosum* than *S. idahoense*. No other significant contrasts were observed in the post-hoc analysis. No significant differences were found within populations with the exception of Peterson Prairie; only Peterson Prairie showed significant contrasts between *Sisyrinchium* hybrids and *S. idahoense* to *S. sarmentosum*. *S. idahoense* plants scored higher with component 2 which was inversely weighted with above-ground to below-ground biomass (Figure 13). As component 2 increased, above-ground to below-ground biomass decreased, meaning above-ground portions of *S. idahoense* plants had more biomass. Differing measurements between *S. idahoense* and *S. sarmentosum* indicates differing growth strategies between the two plants. *S. sarmentosum* seem to allocate more energy to the below-ground section of the plant as early as 5 months into the growing season. *S. idahoense* invest more energy in shoot and leaf production at this stage, possibly as a result of the short growing season in its native environment.

Seven and Eight Month Age Class

Analysis of the seven and eight month age class plants indicated significant differences between species. Unfortunately, no *S. idahoense* from Peterson Prairie germinated early enough in the study and was not a part of this dataset. Looking at a bar plot of component 1 scores (Fig. 16),

plants of unknown parentage scored higher, translating to larger leaves and a lower biomass ratio, but did not stand out from the other two species in the dataset. Interaction effects due to the mixed effects model likely had something to do with the significant result, even though there were no significant interactions in the dataset.

All three analyses indicated different effects affecting morphological traits across different pairwise comparisons, despite the similarities in the principal component analysis loadings. The variability of morphological traits in *S. idahoense*, *S. sarmentosum* and their hybrids is evident as shown by the three analyses in this study. This study may have simply missed the period of rhizome formation. More morphological differences may occur between species at later stages of growth, although given the inconclusive results, further analysis is not recommended. Time spent measuring leaf area and biomass was on the scale of hours for a fraction of the population. Acquiring more conclusive results would require a larger sample of more maternal ID's so that a greater proportion of maternal ID's from each population are represented.

In addition, it seems impractical to conduct similar measurements in the field. In addition to the time requirement, measurements also required a very controlled environment that would not be easy to achieve in the field.

Conclusion

This study was not able to identify significant differences in morphologic characteristics between population and species. Significant differences did occur between plants in the 5 month age class but not between the species and population combinations the study was intended to distinguish between, except in one population where *S. idahoense* was significantly different from *S. sarmentosum*. Specifically, the study failed to find significant differences between *Sisyrinchium* hybrids and co-occurring species of *S. idahoense* and *S. sarmentosum*. While the result is not what I had expected, it was not surprising. As stated at the beginning of the discussion of the ANOVAs, it was expected that there would be no significant differences between species designations. The expectation proved true in this study and echoed the results of Wilson's study [2000]. Distinct alleles identified in Raven's study [2006] as well as distinct separation in phylogenetic trees [Chaveau et. al. 2011, Karst and Wilson 2012] may serve to illustrate the range of genetic variability observed in *S. idahoense*. Further studies on floral characteristics may be able to identify differences between the two species and their hybrids.

Another question to consider is where the suspected hybrids obtained their mixed genetics from if *S. idahoense* was found to co-occur at only one site (Peterson Prairie). Little information exists on how the flowers are pollinated. In his study, Henderson [1976] noted that “Cross pollination in natural populations is accomplished by solitary bees of the family Megachilidae.” A study on the foraging ranges of solitary bees found that foraging distances were less than 1km for all species studied [Gathmann and Tschardt, 2002]. With distances between populations used in this study at least 14km, it is unlikely that cross pollination is occurring by solitary bees. A study by Greenleaf et. al. [2007] concluded that bee foraging range decreased as body size decreased, further supporting the idea that cross pollination is unlikely to occur between sites given the small size of the flowers and the resulting limitation on pollinator size. While it is very likely that undocumented *S. idahoense* plants occur near and between sites, the presence of hybrids seems to indicate the high diversity of genetic, floral and morphologic characteristics exhibited by *S. idahoense*.

Further research after the writing of the discussion revealed that rhizome formation did not occur in *S. sarmentosum*. Henderson [1972], in his original thesis mentioned

“‘Stems rooting at the nodes’ penned by Suksdorf on the type specimen has caused some confusion. Neither Greene nor Bicknell could find reason for this statement. My examination of 2 natural populations of *S. sarmentosum* reveals no nodal rooting habits” [p. 160].

The confusion may have stemmed from another section in Henderson’s thesis [1972] where he writes (Of the roots of the section *Bermudianae*) “Most of the Northwest taxa have coarse, fibrous roots varying in color from straw to dark brown, but some plants of Southwest Oregon have a rhizome with thick yellow roots” [p. 16]. This new information does not invalidate the purpose of this study but rather, strongly supports the finding that no significant differences exist in morphologic characteristics of *S. sarmentosum*, *S. idahoense* and their hybrids. Minute differences may exist between morphological characteristics at specific stages of growth between *S. idahoense* and *S. sarmentosum* but are not enough to distinguish the species and their hybrids.

References

- Bicknell, E.P. 1899. Studies in *Sisyrinchium* IV: *S. angustifolium* and Related Species of the West and Northwest. Bulletin of the Terrey Botanical Club 26 (8): 445-457
- Bower, A. and S.N. Aitken. 2008. Ecological Genetics and Seed Transfer Guidelines for *Pinus Albicaulis* (Pinaceae). American Journal of Botany 95(1): 66-76
- Chauveau, O., L. Eggers, C. Raquin, A. Silverio, S. Brown, A. Couloux, C. Cruaud, E. Kaltchuk-Santos, R. Yockteng, T.T. Souza-Chies and S. Nadot. 2011. Evolution of Oil-Producing Trochomes in *Sisyrinchium* (Iridaceae): Insights from the First Comprehensive Phylogenetic Analysis of the Genus. Annals of Botany: 1-26.
- Cordell, S., G Goldstein, D. Mueller-Dombois, D. Webb, P.M. Vitousek. 1997. Physiological and Morphological Variation in *Metrosideros polymorpha*, a Dominant Hawaiian Tree Species, Along an Altitudinal Gradient: The Role of Phenotypic Plasticity. Oecologia 113: 188-196
- DeWoody, J and V. Hipkins. 2015. Molecular Genetic Variation Does Not Correspond to Species Identification in the *Sisyrinchium sarmentosum* – *S. idahoense* complex in Oregon and Washington. USDA Forest Service, National Forest Genetics Laboratory Project #258.
- Gallagher, J.L., G.F. Somers, D.M. Grant and D.M. Seliskar. 1988. Persistent Differences in Two Forms of *Spartina alterniflora*: A Common Garden Experiment. Ecology 69(4): 1005-1008
- Gathmann, A. and T. Tschardt. 2002. Foraging Ranges of Solitary Bees. Journal of Animal Ecology 71: 757-764
- Greenleaf, S. S., N. M. Williams, R. Winfree and C. Kremen. 2007. Bee Foraging Ranges and Their Relationship to Body Size. Oecologia.
- Henderson, D. M. 1972. A biosystematic study of the genus *Sisyrinchium* (Iridaceae) in the Pacific Northwest. Doctoral Thesis, University of Washington.
- Henderson, D.M. 1979. A Biosystematic Study of Pacific Northwestern Blue-eyed Grasses (*Sisyrinchium*, Iridaceae). Brittonia 28: 149-176
- Hitchcock, C.L., J.W. Thompson and A. Cronquist. 1973. Vascular Plants of the Pacific Northwest: Volume 1: Vascular Cryptogams, Gymnosperms and Monocotyledons. University of Washington Press.
- Karst, L. and C.A. Wilson. 2012. Phylogeny of the New World Genus *Sisyrinchium* (Iridaceae) Based in Analyses of Plastid and Nuclear DNA Sequence Data. Systematic Botany 37(1): 87-95
- Kim, S.H., H. Kinmonth-Schultz and J. Cantillo. 2014. Protocol: Leaf Area using Photographs and ImageJ in Plant Ecophysiology; Laboratory Manual. P.29-34

- Knoke, D. and D. Giblin (2014). *Sisyrinchium idahoense*, Idaho blue-eyed grass. Burke Museum of Natural History and Culture. <<http://biology.burke.washington.edu/herbarium/>> Accessed [8-6-14]
- Lambers, H., F.S. Chapin III and T.L. Pons. 2008. Growth and Allocation in Plant Physiological Ecology. Springer Science and Business Media LLC.
- Raven, A. 2007. An Exploration of Possible Hybridization Between Pale Blue-eyed Grass and Idaho Blue-eyed Grass in Washington and Oregon. A report submitted to the USFS Gifford Pinchot National Forest, Challenge Cost Share Agreement 01-CS-11060301-023
- Ruchty, A. and A. Raven. 2009. Conservation Assessment for *Sisyrinchium sarmentosum* (Suks. Ex. Greene). USDA Forest Service Region 6 and USDoI Bureau of Land Management.
- Selling, R. and E. Guerrant. CPC National Collection Plant Profile: *Sisyrinchium sarmentosum*. Center for Plant Conservation. <<http://www.centerforplantconservation.org/collection/>> Accessed [8-6-14]
- US Department of Agriculture, Natural Resource Conservation Service (2014). Plant Profile: *Sisyrinchium sarmentosum* Suksd. Ex Greene mountain blue-eyed grass. Plants Database. <<http://plants.usda.gov/core/profile?symbol=sisa4>> Accessed [8-6-14]
- US Department of Agriculture, Natural Resource Conservation Service (2014). *Sisyrinchium idahoense* E.P. Bicknell, Idaho blue-eyed Grass. Plants Database. <<http://plants.usda.gov/core/profile?symbol=SIID>> Accessed [8-6-14]
- US Fish and Wildlife Service (2014). Species Profile: Mountain blue-eyed grass (*Sisyrinchium sarmentosum*). Environmental Conservation Online System. Accessed [8-6-14]
- Vitasse, Y., S Delzon, C. C. Bresson, R. Michalet, A. Kremer. 2009. Altitudinal Differentiation in Growth and Phenology Among Populations of Temperate-Zone Tree Species Growing in a Common Garden. Canadian Journal of Forest Resources 39: 1259-1269
- Warren, C.R., M. Tausz and M.A. Adams. 2005. Does Rainfall Explain Variation in Leaf Morphology and Physiology Among Populations of Red Ironbark (*Eucalyptus sideroxylon* subs. *Tricarpa*) Grown in a Common Garden? Tree Physiology 25:1369-1378
- Washington Department of Natural Resources (2014). *Sisyrinchium sarmentosum* Field Guide. Accessed [8-6-14].
- Wilson, B.L., D.L. Doede and V.D. Hipkins. 2000. Isozyme Variation in *Sisyrinchium sarmentosum* (Iridaceae). Northwest Science 74(4): 346-354

Software

- Bates, D., M. Maechler, B. Bolker and S. Walker. 2014. lme4: Linear Mixed-effects Models Using Eigen and S4. R package version 1.1-7, <<http://CRAN.R-project.org/package=lme4>>.

H. Wickham. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer New York.

Kuznetsova, A., P. B. Brockhoff and R. Haubo Bojesen Christensen. 2014. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-20. <<http://CRAN.R-project.org/package=lmerTest>>

Lenth, R. V. and M. Herv . 2015. lsmeans: Least-Squares Means. R package version 2.17. <<http://CRAN.R-project.org/package=lsmeans>>

R Core Team. 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <<http://www.R-project.org/>>

Rasband, W. ImageJ 1.48v. National Institutes of Health.