POPULATION SURVEYS, REPRODUCTIVE ECOLOGY, AND POPULATION GENETICS OF *SYNTHYRIS BULLII*, A RARE SPECIES

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Project Objective(s) as Described in Application:

(1) Continue to survey populations of *Synthyris bullii* in Illinois

(2) Collect infructescences from Illinois populations to assess reproductive success and fitness

(3) Collect genetic data on current and historic populations of *S. bullii*

Completed Project Description:

Synthyris bullii (Plantaginaceae), commonly called kittentails, is a prairie-savanna species endemic to the Midwestern United States. The species is considered rare throughout its range, and is listed as a state threatened species in Illinois. The purpose of this study was to determine if reproductive factors were contributing to the decline of this species; specifically, we examined reproductive success (e.g., fruit/seed set) and fitness (i.e., germination). In addition, we developed a protocol for genetic work to determine if inbreeding and reduced gene flow could

also be compromising the reproductive health of populations. During the previous 4 years (2008-2011), 9 populations of *S. bullii* were visited in northwestern Illinois to collect data on population size, including the numbers of flowering and sterile plants. At each population, 20 infructescences were randomly collected to measure fruit/seed set and seed germination. Lastly, small samples of leaf tissue were collected from 20 randomly selected plants to develop genetics protocols. Of the populations surveyed, we found fluctuations in population size between years, with no consistent pattern in terms of populations increasing or decreasing in number. Fruit/seed set and germination did not significantly correlate with population size; however, the data shows a visual trend suggesting that fruit/seed set may still respond positively to increasing population size. For the 8 microsatellite markers we attempted to cross-amplify, we successfully developed protocols that optimized 5 markers for *S. bullii*. These results show that reproduction in this species may vary significantly in response to population size, which could be problematic given fluctuations in population size between years. Protocols for genetics work developed during this project can be used in future research to determine if there is also a relationship between population size and population genetics (e.g., genetic diversity, inbreeding) in *S. bullii*.

Summary of the Project Accomplishments: (See attachment)

Budget: (See attachment)

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Introduction

When habitat is destroyed and converted for anthropogenic uses, native populations suffer from such catastrophic losses that many conservationists cite this as one of the primary cause of species decline (Sax & Gaines 2003). Any surviving populations are then susceptible to the effects of a fragmented landscape, with high extinction risk associated with reduced population size and increased isolation (Wilcox & Murphy 1985, Saunders et al. 1991, Jacquemyn et al. 2010).

Studies in conservation biology have emphasized the importance of large population size (Shaffer 1981, Lande 1993), especially as a factor in reproduction and fitness (Oostermeijer et al. 2003, Reed 2005). In particular, Allee effects associated with small population size may compromise an individual's ability to reproduce and thus decrease population growth rates (Lande 1988, Groom 1998, Oostermeijer et al. 2003).

Recovery efforts for small populations may also be limited by reduced genetic diversity among surviving individuals due to a combination of genetic bottlenecks, increased inbreeding, reduced gene flow, and genetic drift (Saunders et al. 1991, Young et al. 1996, Oostermeijer et al. 2003). Reduction of genetic diversity is predicted to compromise the viability of populations by reducing fitness and increasing expression of deleterious alleles among populations (Young et al. 1996, Reed & Frankham 2003, Remiro et al. 2008). Large population size and increased gene flow are predicted to minimize the genetic consequences of habitat fragmentation (Oostermeijer et al. 2003, Reed & Frankham 2003, Jacquemyn et al. 2010).

Recognizing the importance of population size and reproductive success in determining the viability of rare populations, we developed the following objectives for studying the rare plant species *Synthyris bullii* in Illinois: (1) continue monitoring 9 populations in Illinois, and (2) assess reproductive (e.g., fruit/seed set) and fitness (i.e., germination) differences among the 9 study populations. The third objective, which was stated in the original project proposal to "collect genetic data on current and historic populations of *S. bullii*," was modified due to time and monetary constraints. The revised third objective was to develop a protocol for assessing genetic diversity in populations of *S. bullii*. Specifically, the goals for this part of the project were to: (1) optimize microsatellite primers originally developed for a closely-related species, *Collinsia verna*, and (2) determine if sufficient variation existed at the cross-amplified loci to use these primers for further study.

Methods

Study Species: Synthyris bullii (Plantaginaceae; synonym *Besseya bullii* [Scrophulariaceae]), commonly called kittentails, is a perennial prairie-savanna plant endemic to the Midwestern United States (Figure 1). This species is characterized by a large basal rosette and a spiked inflorescence (Gleason et al. 1991). The flowers of *S. bullii* are protogynous (i.e., stigmas are receptive before pollen sheds), and the inflorescence flowers acropetally (i.e., maturing from the base towards the top; McKone et al. 1995). *S. bullii* is a self-compatible species and is visited by bees (McKone et al. 1995, pers. observation). The chromosome number of *S. bullii* is 2n = 24 (Kruckeberg and Hedglin 1963). This species is found on sand or gravel soils in both prairie and savanna habitats (Gleason et al. 1991). *Synthyris bullii* has been declared locally threatened or endangered in all states where it occurs (IA, IL, IN MI, MN, OH

[extirpated] and WI). This species was previously considered for federal protection in the 1980s, with population declines attributed to eroding habitat conditions.

Population Surveys: During 2008 to 2011, 9 populations of *S. bullii* were surveyed annually in northern Illinois (Figure 2). At each population, the total number of individuals were counted and recorded. Individuals were categorized as either flowering or sterile; if flowering, the total number of inflorescences per plant was also noted. Additionally, local habitat quality was characterized for each population according to amount of woody encroachment, recent management activity, etc. (Figure 3).

Reproductive Success and Fitness: At each of the 9 study populations, a single infructescence was collected from 20 randomly selected individuals. Infructescences were stored in paper bags during transport, and fruit/seed set was assessed in the laboratory. Seeds collected from each population were also placed on petri dishes with moistened filter paper in a growth chamber, then monitored for a period of 30 days for evidence of germination.

Population Genetics - Appendix 1:

(1) *Sampling:* For this project, 4 populations from northern Illinois were selected (Table 1). In each population, a small sample of leaf tissue (<3 cm) was removed from 20 randomly selected plants. Leaf samples were placed in coin envelopes, allowed to completely dry, then stored at room temperature (25°C).

(2) *DNA Extraction:* Approximately 0.20 g of dried tissue was removed from each of the leaf samples. The dried tissue from each individual was pulverized into a fine powder using liquid nitrogen and a mortar and pestle. Samples were blended together and separated into 6 replicates. DNA was extracted using a QIAGEN DNEasy Plant Mini Kit. After extraction,

DNA concentration and purity was assessed by examining samples with a NanoDrop. Extracted DNA was stored at -20°C.

(3) *Cross-Amplification of Microsatellite Primers:* Microsatellite primers developed for *C. verna* were tested for the ability to cross-amplify in the study species, *S. bullii*. A total of 8 microsatellite primers were used in polymerase chain reaction (PCR) with the extracted *S. bullii* DNA. Specifically, the technique of gradient PCR was used to determine temperatures that were optimal for annealing and amplification. PCR products were visualized via gel electrophoresis using a 1% agarose-based gel and 100V for 30 minutes.

Results and Discussion

Population Surveys: Populations of *S. bullii* were surveyed in 4 years from 2008 to 2011 (Table 1). A one-way ANOVA showed that populations differed in total number of plants ($F=5.409$, $p<0.001$). The 4-year mean total number of plants for NachusaKit was significantly higher compared to the other populations with the exception of LoMoF105 and Fulton (Table 2). When analyzing the proportion of flowering plants in populations with a one-way ANOVA, we did not find differences among populations ($F=1.553$, $p=0.188$). In addition, neither total numbers of plants nor proportion of flowering plants showed significant differences among years ($F=1.027$, $p=0.394$; $F=1.960$, $p=0.141$, respectively; Table 2). However, a considerable drop in the number of flowering plants within several of the populations (e.g., PalisadesOzz in 2010, PalisadesOak in 2010; Table 1) suggests that some populations may be vulnerable to decline due to reproductive stochasticity.

Reproductive Success and Fitness: Depending on the population, the 4-year mean fruit and seed set ranged from 0 to 87% and 10 to 26%, respectively. One-way ANOVAs show significant differences for fruit set ($H=133.165$, $p<0.001$) and seed set ($F=11.250$, $p<0.001$)

among populations (Figure 4; Table 3). In addition, fruit and seed set differed significantly among years (H=54.790, p<0.001; F=7.820, p<0.001, respectively; Table 3). Fruit/seed sets were significantly lower in 2011 for all three Palisades populations compared to other populations. The observed variation in fruit/seed set could reflect difference in pollinator availability or resource allocations among populations and years. In addition, low seed set associated with *S. bullii* is a concern as it could be affecting recruitment within populations. Seed germination did not show significant differences among populations (H=13.097, p=0.109), but differences were found per year (H=48.428, p<0.001) (Table 3). The year 2008 had the lowest seed germination compared to 2009, 2010, and 2011 (Table 3). Detailed demographic studies should be conducted to determine the level of recruitment with *S. bullii* populations.

To examine the relationship between reproductive output, fitness and population size, mean fruit set, seed set, and germination were calculated for each population and compared to total number of flower individuals per population using a Pearson's correlation per year. Though the correlation was non-significant for all study years (Table 4), there is a visual trend that suggests fruit and seed set may increase with increasing population size (Figure 5 a-b), whereas there was no apparent correlation between germination and population size (Figure 5c).

Population Genetics: The results of the gradient PCR showed 6 of the microsatellite markers successfully cross-amplified, with 2 primers failing to anneal at any of the tested temperatures (Table 5). Of the primers that successfully amplified, 5 showed signs of polymorphism (i.e., multiple bands at a given locus) and can be considered suitable for future research (Figure 6).

Presentation/Publication of Results

Partial results from this study have been presented at scientific meetings. A total of 8 presentations have resulted from this grant (* = graduate student; ¥ = undergraduate):

2012. Environmental mediation of plant-pollinator interactions and decline of the rare prairie endemic *Besseya bullii* (Plantaginaceae), with K. Chi* (principal author). Botanical Society of America, St. Louis, Missouri.

2012. Effects of shading, seed longevity, and soil mixes on germination of *Besseya bullii* – a rare species in Illinois, with M. Curtis* (principal author). Illinois State Academy of Science, Galesburg, Illinois.

2012. Presence and intensity of pre-dispersal seed predation in response to habitat quality, with M. Leja¥ (principal author) and K. Chi*. Illinois State Academy of Science, Galesburg, Illinois.

2012. Effects of habitat quality on floral morphology and reproductive output in the rare plant *Besseya bullii* (Plantaginaceae), with S. Chavez¥ (principal author), M. Collins¥**,** and K. Chi*. Illinois State Academy of Science, Galesburg, Illinois.

2012. Effects of microclimate on the reproductive biology of *Besseya bullii* (Plantaginaceae), a rare plant, with D. Robinson ¥ (principal author) and K. Chi*. Illinois State Academy of Science, Galesburg, Illinois.

2011. Shading effects on the reproductive ecology of *Besseya bullii*, a rare species, with K. Chi* (principal author), M. Collins¥, and D. Abou-El-Seoud¥. XVIII International Botanical Congress**,** Melbourne, Australia. (poster)

2011. Environmental and demographic effects on reproduction in the rare species *Besseya bullii* (Plantaginaceae), with K. Chi* (principal author). Botanical Society of America, St. Louis, Missouri.

2011. Shading effects on the reproductive ecology of *Besseya bullii*, a rare species, with K. Chi* (principal author), M. Collins¥, and D. Abou-El-Seoud¥. Illinois State Academy of Science, Charleston, Illinois. (poster)

In addition, these results are included in manuscripts that are currently in preparation for publication in peer-reviewed scientific journals. Lastly, in accordance with permit agreements for research, data collected during this study were also submitted as part of annual reports to the U.S. Fish and Wildlife Service for the two “LoMo” populations and The Nature Conservancy for the two “Nachusa” populations.

Future Work

Protocols developed in this project will be used in future studies to examine population genetics in *S. bullii*. With the 5 microsatellite markers that were optimized in this study, we will be able to measure genetic diversity (e.g., heterozygosity, allelic richness) and inbreeding in remnant populations. In addition, research questions and preliminary methods have been developed to examine connectivity among areas where *S. bullii* occur to determine how landscape factors could be impacting the reproductive success of this species. Results from future population genetics studies should provide further insight into potential causes of decline in *S. bullii*. In addition, flow cytometry will be used to further explore population genetics, including intraspecific genome size variation associated with degree of isolation and habitat.

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Table 1. Survey data from 9 Illinois populations during a 4-year period. Number of flowering plants ("Flower") as well as total number of individuals is shown for each population. * Indicates populations used for population genetics research.

	2008		2009		2010		2011	
Site Name	**Flower**	**Total**	**Flower**	**Total**	**Flower**	**Total**	**Flower**	**Total**
LoMoF105 *			511	1893	158	777	127	620
LoMoRail *	304	762	398	1407	220	601	186	514
NachusaKit *	500	1000	271	1485	708	2654	70	858
NachusaTell *	85	389	79	96	66	335	69	135
HarlemHills	93	209	79	431	78	196	54	143
Fulton	106	472	47	972	23	1489	35	534
PalisadesMush	239	635	175	350	42	161	55	140
PalisadesOzz	20	40	8	68	0	42	13	42
PalisadesOak	253	451	92	357	18	191	89	176

Table 2. Four year mean (and standard error of the mean [SEM]) values for population metrics per population and year.

1) Total number of plants

a) Population

Population	Mean	SEM
LoMoF105	1096.67	400.738
LoMoRail	821	201.975
NachusaKit	1499.25	407.646
NachusaTell	238.75	72.446
HarlemHills	244.75	63.703
Fulton	866.75	235.376
PalisadesMush	321.5	114.672
PalisadesOzz	48	6.683
PalisadesOak	293.75	66.552

b) Year

Year	Mean	SEM
2008	494.75	107.607
2009	784.333	224.414
2010	716.222	284.405
2011	351.333	95.016

2) Proportion of flowering plants

a) Population

Population	Mean	SEM
LoMoF105	0.226	0.022
LoMoRail	0.352	0.0246
NachusaKit	0.258	0.0892
NachusaTel	0.437	0.147
HarlemHills	0.351	0.0576
Fulton	0.0885	0.0465
PalisadesMush	0.383	0.0489
PalisadesOzz	0.232	0.11

PalisadesOak	0.355	0.109

b) Year

Year	**Mean**	**SEM**
2008	0.403	0.0447
2009	0.296	0.0782
2010	0.2	0.0472
2011	90.312	0.0547

Table 3. Four year mean values (and standard error of the mean [SEM]) for reproductive/fitness metrics per population and year.

1) Fruit Set

a) Population

Population	Mean	SEM
LoMoF105	0.867	0.0183
LoMoRail	0.838	0.0215
NachusaKit	0.831	0.0169
NachusaTel	0.832	0.0212
Fulton	0.771	0.0191
HarlemHills	0.828	0.0157
PalisadesMush	0.74	0.0244
PalisadesOzz	0.467	0.0514
PalisadesOak	0.64	0.0225

b) Year

Year	Mean	SEM
2008	0.805	0.0132
2009	0.793	0.0138
2010	0.831	0.0178
2011	0.695	0.0174

2) Seed Set

a) Population

Population	Mean	SEM
LoMoF105	0.212	0.0133
LoMoRail	0.255	0.0155
NachusaKit	0.241	0.0144
NachusaTel	0.243	0.013
HarlemHills	0.215	0.0185
Fulton	0.145	0.0107
PalisadesMush	0.174	0.0111

PalisadesOzz	0.0987	0.0166
PalisadesOak	0.150	0.0138

b) Year

Year	**Mean**	**SEM**
2008	0.237	0.031
2009	0.223	0.00882
2010	0.216	0.00964
2011	0.17	0.00826

3) Germination

a) Population

Population	**Mean**	**SEM**
LoMoF105	0.89	0.0492
LoMoRail	0.916	0.0307
NachusaKit	0.942	0.0264
NachusaTel	0.934	0.0186
HarlemHills	0.935	0.0322
Fulton	0.866	0.0454
PalisadesMush	0.883	0.0249
PalisadesOzz	0.845	0.0325
PalisadesOak	0.863	0.0394

b) Year

Year	**Mean**	**SEM**
2008	0.779	0.0234
2009	0.953	0.0196
2010	0.953	0.0117
2011	0.93	0.0164

Table 4. Pearson's correlation r and p-values for the correlation between population size and fruit set, seed set, and germination during 4 years.

Year	Fruit Set		Seed Set		Germination	
	r	p	r	p	r	p
2008	0.454	0.306	0.847	0.153	0.286	0.492
2009	0.442	0.201	0.375	0.285	-0.528	0.144
2010	0.401	0.284	0.573	0.107	0.283	0.461
2011	0.367	0.332	0.234	0.545	0.290	0.449

Table 5. Microsatellite primers cross-amplified with *S. bullii*. Optimal annealing temperatures are shown for each primer, as well as the suitability of primers for future research.

Primer Name	Polymorphic?	Optimal Annealing Temperature(s)	Appropriate for Future Research?
Cove A107	Yes	54°C-56°C	Yes
Cove A119	No	None	No
Cove A125	Yes	54°C-55°C	Yes
Cove A134	No	54°C-59°C	No
Cove B2	Yes	52°C-56°C	Yes
Cove B105	Yes	53°C-58°C	Yes
Cove B116	Yes	54°C	Yes
Cove C8	No	None	No

Figure 1. A *Synthyris bullii* plant producing two inflorescences.

Figure 2. Map of the 9 Illinois populations of *S. bullii* used for the study.

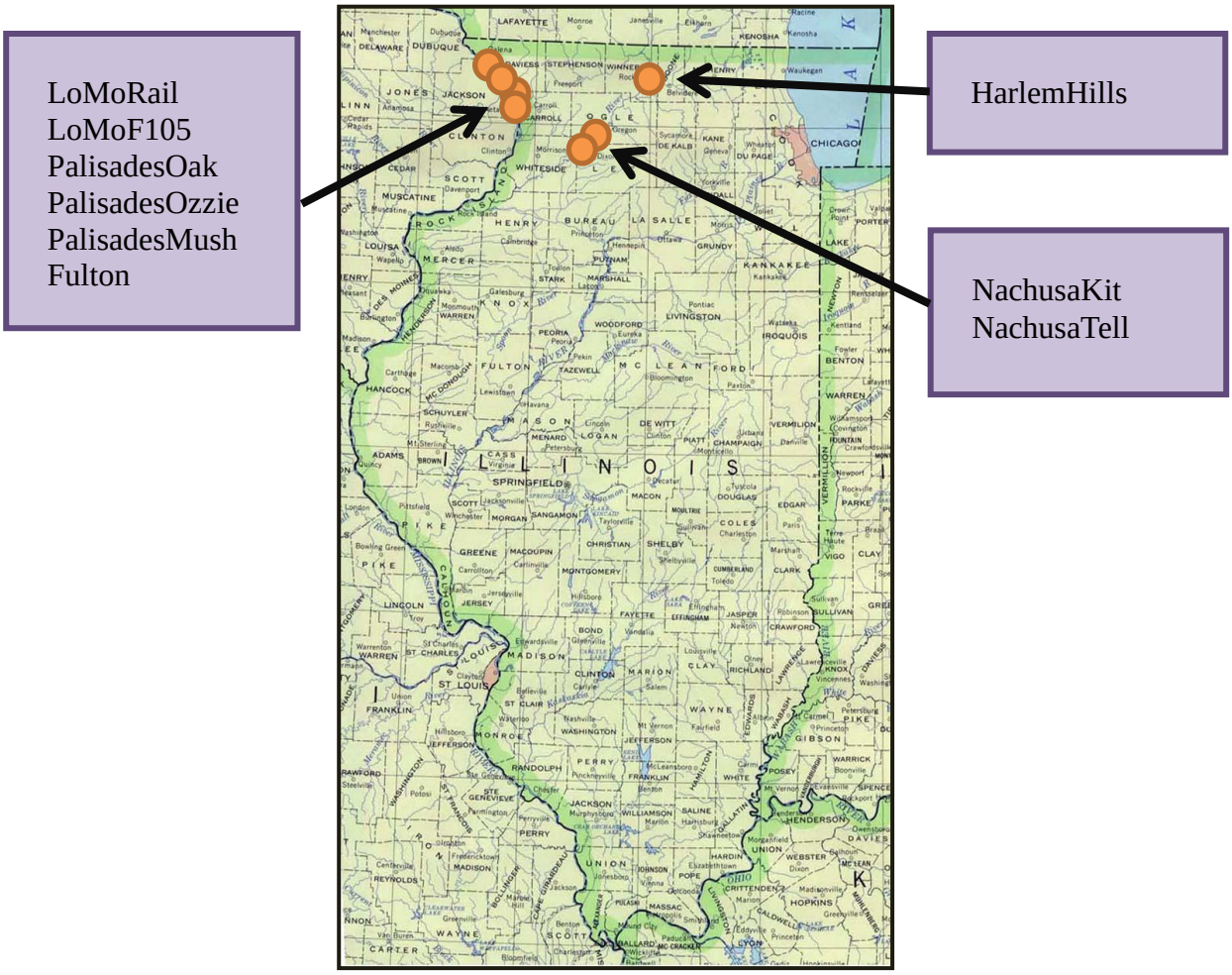


Figure 3. Examples of different types of habitats where populations of *S. bullii* were found: (a) dry-mesic prairie, (b) oak savannah, and (c) woodland.

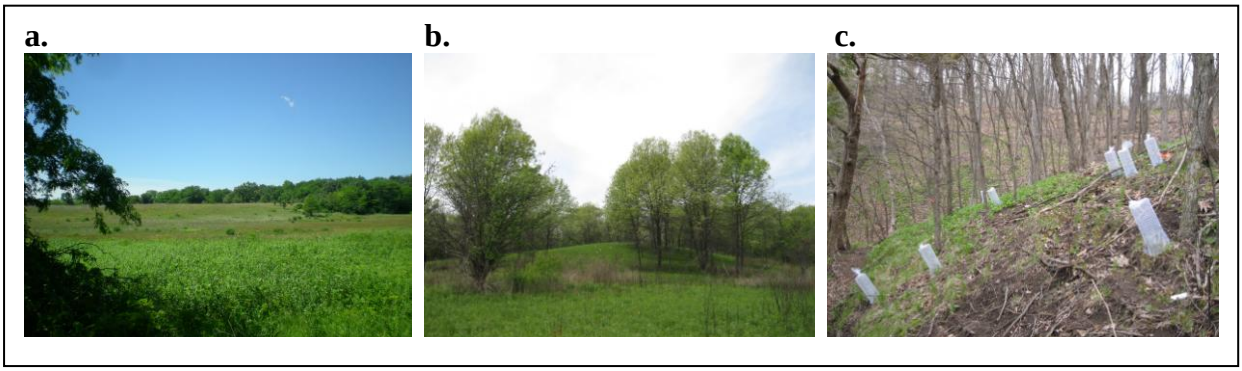


Figure 4. (a) Fruit set, and (b) seed set for 9 Illinois populations of *S. bullii* during 4 years.

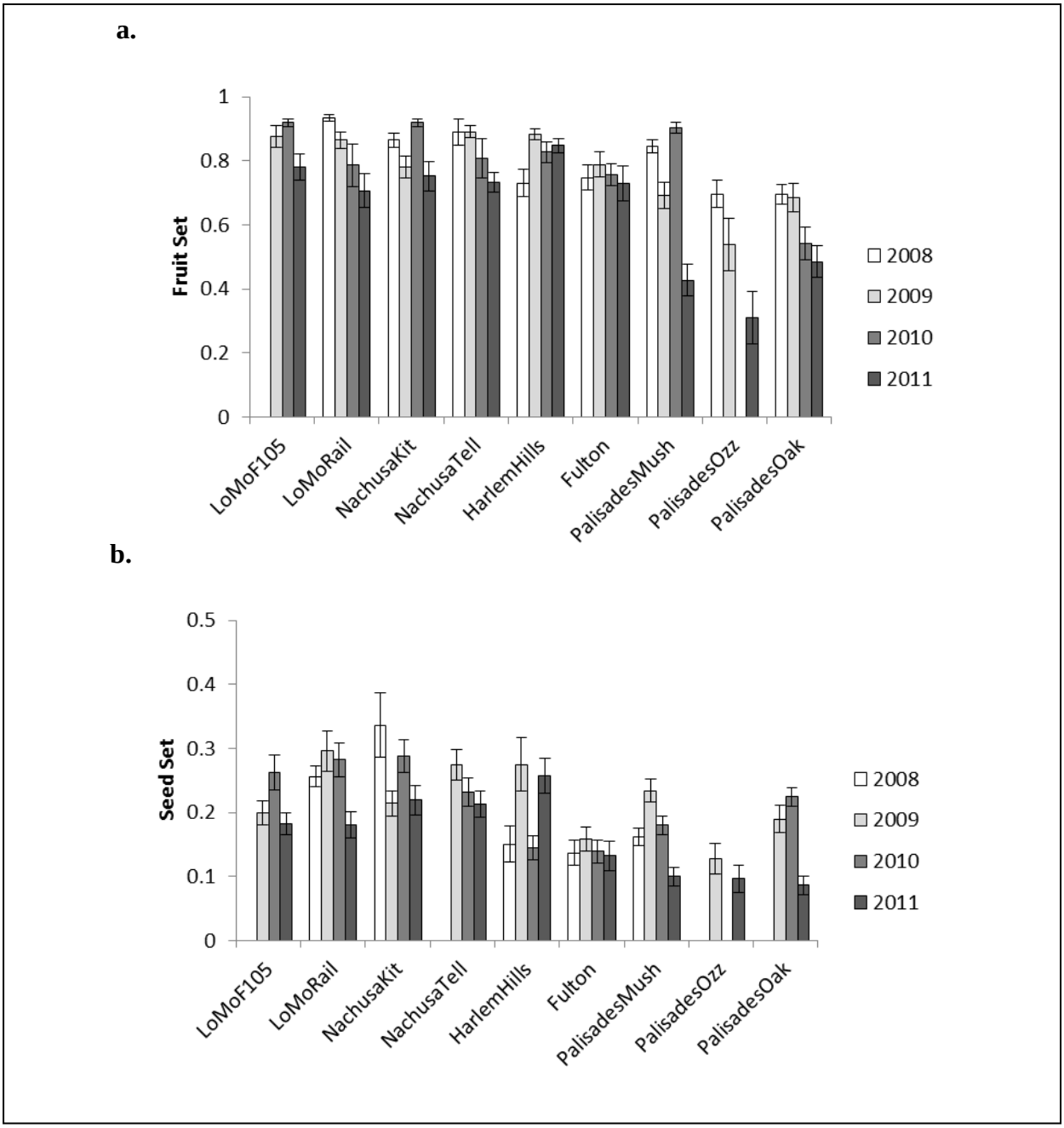


Figure 5. (a) Fruit set, (b) seed set, and (c) germination as a function of population size for 9 populations for 4 years.

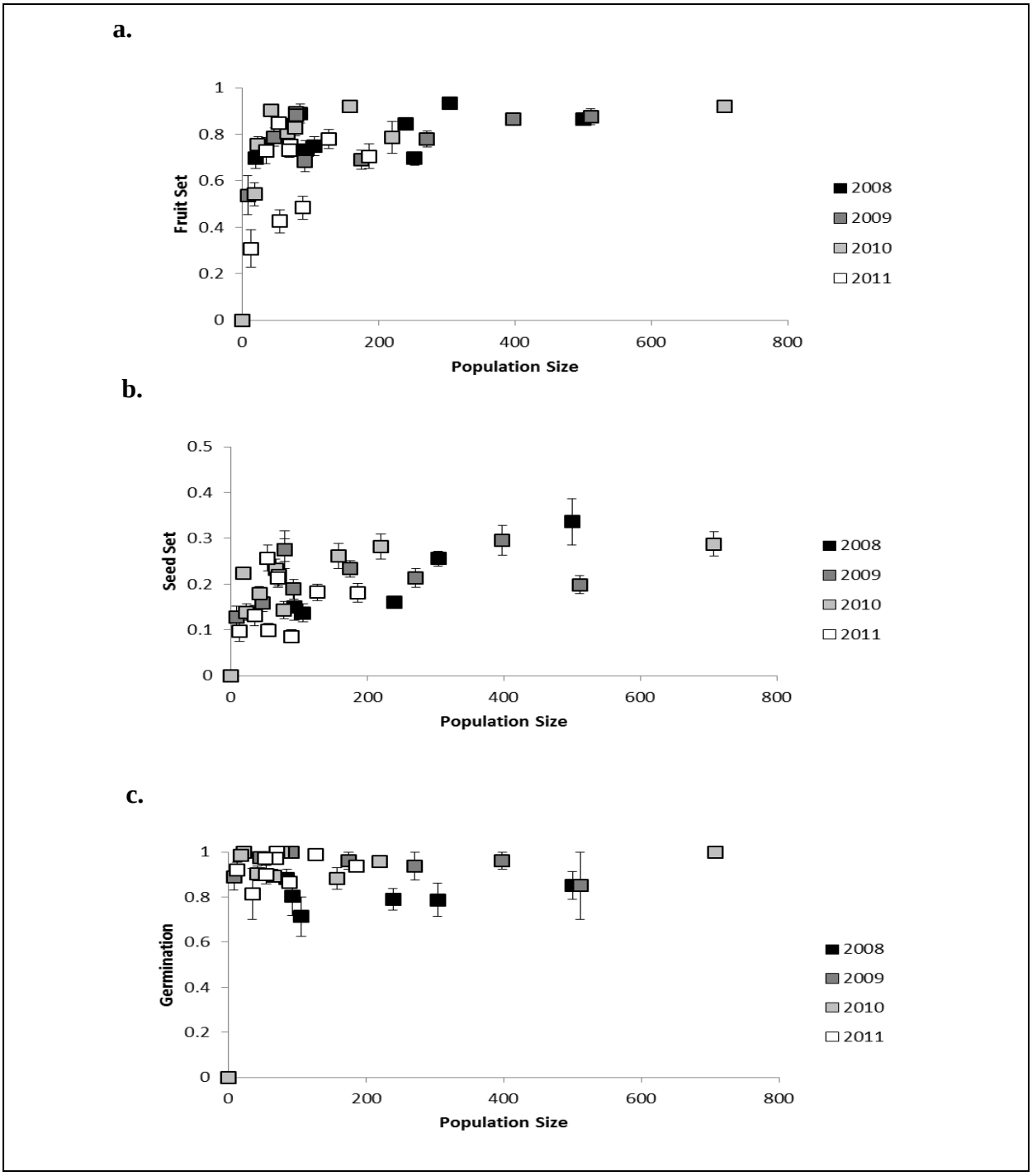
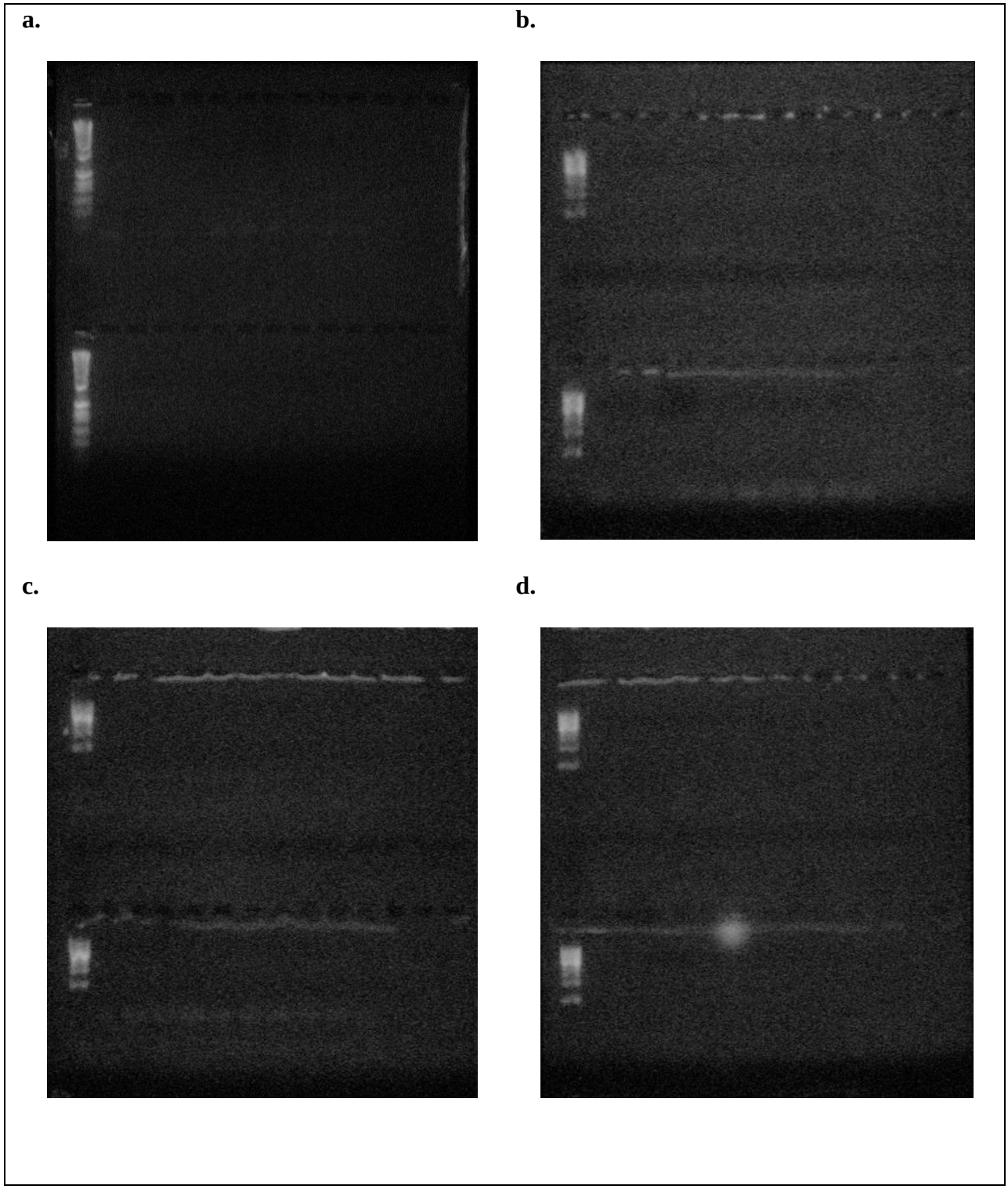


Figure 6. Results of gradient PCR products for: (a) A107 and A119, (b) A125 and A134, (c) B2 and B105, and (d) B116 and C8.



Appendix 1: DNA Extraction and Cross-Amplification of Microsatellite Primers

DNA EXTRACTION

(1) Extract DNA using the "mini" preparation in the Qiagen DNEasy Plant Mini Kit (pg. 24 of the instruction booklet).

(2) Follow all steps as printed in the manual except for the process to crush the plant tissue (steps 1-6). To pulverize the plant tissue, place a mortar in an ice bath. Weigh out 20 mg of dry leaf tissue, then add to mortar. Pour a small amount of liquid nitrogen over the leaf tissue and grind with a pestle until you have a fine powder (do not allow tissue to warm and turn into a liquid state). You may have to add liquid nitrogen and grind several times before achieving the right consistency.

(3) Continue with step 7 after grinding plant tissue.

CHECKING DNA QUALITY (USING ND-1000 NANODROP)

(1) The quality of the extracted DNA will be verified using a Nanodrop. On the computer, open the program "ND-1000."

(2) Select the type of sample: "Nucleic Acid."

(3) The program will request that the sample pedestals are clean and for you to add a water sample. Gently lift up the metal arm, add 2 ul of RNA-free water to the bottom sample pedestal, lift it back down gently, and then click the "OK" button on the computer.

(4) After it has processed this (the machine will make several clicking sounds), lift the metal arm, then very gently blot the sample pedestals (both upper and bottom sensors) with a Kimwipe. Add 2 ul of your buffer solution (whatever solution your DNA sample is being

stored in) to the bottom sample pedestal and click the "Blank" button on the screen. After the nanodrop is finished reading the sample, lift the arm and blot the sensors again with a Kimwipe.

(5) The ND-1000 is now ready to measure your sample. Load 2 ul of your DNA sample onto the bottom pedestal and click "Read." Remember to blot between samples with a Kimwipe.

(6) After you have finished measuring each of the DNA samples, compare the output for each sample. You are trying to find a sample that maximizes the concentration (ng/ul); however, you also want a "260/280" reading between the values of 1.8 and 1.9 to prevent contamination from proteins/carbohydrates.

PCR MIX

(1) The following recipe will make enough PCR mix for <u>1 sample</u> (with a total reaction volume of 25 uL).

Template DNA	1 uL	*(This is the DNA you extracted in earlier steps.)*
Primer-F (10 uM)	1.25 uL	*(The "forward" strand of the primer.)*
Primer-R (10 uM)	1.25 uL	*(The "reverse" strand of the primer.)*
dNTP (2 mmol)	2.5 uL	
MgCl2 (50mM)	2.5 uL	
5x Buffer	5.0 uL	
Water	11.4 uL	
Taq polymerase	0.1 uL	

(2) Multiply the amounts above by the number of samples you have (for example, if you have 10 samples, multiply all of the amounts by 10). This is the total amount of each reagent required for PCR.

(3) Dilute all reagents to the concentration listed above. Use the equation $M_1V_1=M_2V_2$ to calculate your dilutions. For example, if you are starting out with a 100uM solution, and you need 100 uL of a 10 uM solution, then:

$100\ uM * V_1 = 10\ uM * 100\ uL$

$V_1 = 10\ uL$

Combine 10 uL of solution with 90 uL of water to get 100 uL of a 10uM solution.

(4) Take all reagents and place in an ice bath, EXCEPT for the Taq polymerase. (Taq is extremely temperature-sensitive and should be kept in the freezer until ready to add to PCR mix.)

(5) First make a master mix of all ingredients EXCEPT for the template DNA and Taq polymerase. After these ingredients are combined in one centrifuge tub, aliquot portions (a total volume of 23.9 uL) into each well of your PCR plate. Add the template DNA and Taq to each well.

(6) When ready, use the following program to run your PCR:

1. Incubate at 94.0°C for 3 minutes.
2. Incubate at 94.0°C for 1 minute.
3. Incubate at X°C for 1 minute.

   (Where X = annealing temp. for the specific primer being used. See below.)
4. Incubate at 72.0°C for 1 minute.

5. Cycle to Step 2 for 35 more times.
6. Incubate at 72.0°C for 10 minutes.
7. Incubate at 10.0°C for forever.

Primer Name	**Optimal Annealing Temperature(s)**
Cove A107	54°C-56°C
Cove A125	54°C-55°C
Cove B2	52°C-56°C
Cove B105	53°C-58°C
Cove B116	54°C

Budget: The Revised Budget 1 was needed because funds were secured to cover salary and fringe benefits for Katherine Chi from another source and the reallocation of funds to conduct Objective 3: "Collect genetic data on current and historic populations of *S. bullii*," which was considered the main priority of the project. While we made considerable progress in terms of extracting DNA from leaf tissue collected in the field, we were not able to optimize primers for our study species. Because we were stalled on resolving optimization issues, we have used the funding to purchase the supplies to conduct flow cytometry in 2013, flow cytometry training and to cover travel cost to study sites (Revised Budget 2). Flow cytometry will allow us to determine the genome size for the species.

	Original Budget	**Revised Budget 1**	**Revised Budget 2**
Salaries and Wages	$1621	$0	$0
Fringe Benefits	$197	$0	$0
Materials and Supplies	$0	$900	$900
Travel	$0	$0	$854
Contractual Services	$0	$918	$64
Equipment	$0	$0	$0
Total Direct Costs	$1818	$1818	$1818
Indirect Costs	$182	$182	$182
Total Project Costs	$2000	$2000	$2000

D7176

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IN
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Chi, K.; B. Molano Population surveys, reproductive ecology, and population genetics of Besseya bullii, a rare species - $2,000 (11-018W)
Taylor, C.; J. Tiemann Diet of the Longnose Dace, (Rhinichthys cataractae), in Illinois - $1,185 (11-020W)
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