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RESEARCH ARTICLE

Mixing source populations increases genetic diversity of restored rare plant populations

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The genetic diversity of germplasm used in reintroduction and restoration efforts can influence how resulting populations establish, reproduce, and evolve over time, particularly in disturbed and changing conditions. Regional admixture provenancing, mixing seeds derived from multiple populations within the same region as the target site, has been suggested to produce genetically diverse germplasm. Yet little empirical evidence shows how genetic diversity in germplasm resulting from this approach compares to source populations, or how it varies in restored populations. Here, we use neutral molecular markers to follow genetic diversity through production and use of germplasm when mixing multiple source populations in nursery production beds. *Castilleja levisecta* is a rare species experiencing inbreeding depression in remaining populations, with a federal recovery plan requiring the re-establishment of populations in areas where it has been extirpated. Specifically, we track diversity from wild-collected source populations through different production approaches and reintroductions using two propagule types. We show that measures of genetic diversity, inbreeding, and relatedness change during the production and use of material produced with a regional admixture provenancing approach, with the step at which source populations are mixed and germplasm type used influencing whether all source populations are equally represented. While genetic diversity increased throughout the process, inbreeding and relatedness increased in nursery production beds but decreased in reintroductions, with the lowest inbreeding and relatedness in populations restored using seeds rather than plugs. The results highlight the importance of taking an integrated approach informed by research when planning and implementing reintroductions with mixed-source germplasm.

Key words: mixed-source germplasm, nursery production beds, regional admixture provenancing, reintroduction, source population

Implications for Practice

- When using a regional admixture provenancing approach to produce genetically diverse germplasm for restoration use, the decision to mix populations before or after nursery production can influence the likelihood that all source populations are represented in restored populations.
- Planting each source population in separate but adjacent nursery production rows, and mixing produced seeds immediately prior to restoration activities, led to restored populations that had desired representation of all source populations. The same was not true when source populations were mixed prior to planting in nursery beds.
- Propagule type used (seed versus plug) may influence the inbreeding and relatedness of plants in a restored population: the lowest inbreeding and relatedness was found in restorations using seeds rather than plugs.

Introduction

Restoring a rare species to a community from which it has been extirpated requires a detailed recovery plan, ideally developed by a multidisciplinary team of resource managers and scientists (Seddon et al. 2007; IUCN/SSC 2013). A key aspect of developing a recovery plan involves identifying and securing appropriate source material to use for a reintroduction (Godefroid et al. 2016). Global guidelines for implementing reintroductions, developed by the International Union for the Conservation of Nature's Species Survival Commission (IUCN/SSC 2013) recommend that source material contain adequate genetic diversity and that the source population is as geographically and ecologically close to the reintroduction site as possible. These guidelines also stipulate that potential sources may be inappropriate if plants are inbred, if they contain low genetic diversity, or if they are not adequately

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adapted to the reintroduction site, as these genetic characteristics may negatively impact the fitness of the reintroduced population (Ottewell et al. 2016). In these instances, the IUCN/SSC guidelines indicate a more radical sourcing strategy may be necessary involving mixing multiple founder populations to maximize diversity and increase likelihood that some individuals survive under novel conditions.

Research supports the use of genetically diverse plant material for restoration efforts because genetically diverse populations are generally more resilient to disease, stress, and climatic extremes (Burdon 2001; Reusch et al. 2005; Guarino et al. 2011), more likely to persist and spread (Hughes & Stachowicz 2004; Crawford & Whitney 2011), and support more ecosystem services (Crutsinger et al. 2006; Souza et al. 2017). Yet in practice many reintroduced plant populations have lower genetic diversity than remnant populations (Fant et al. 2008; Neale 2012). This has been attributed to low diversity in the source population (Williams & Davis 1996; Liu et al. 2008), the use of too few founders (Smulders et al. 2000), or unintentional diversity losses during the propagation of source material in a nursery setting (Dolan et al. 2008). In the few cases where reintroductions are more diverse than remnant populations, strategies involving addition of plant material over multiple years (Godefroid et al. 2011; Neale 2012) or use of multiple source populations (Williams & Orth 1998; Gustafson et al. 2002) were employed. Although mixing source populations remains controversial because of the potential for outbreeding depression resulting from genetic incompatibilities among mixed populations (Waller 2015), many guidelines are available to assess outbreeding depression risks (Frankham et al. 2011; Weeks et al. 2011), and recent meta-analyses highlight the benefits of mixing (Frankham 2015, 2016).

Mixing populations from multiple sources within a geographically or ecologically similar area is increasingly recommended to both restore common plant species (Havens et al. 2015) and reintroduce rare species (Weeks et al. 2011; Frankham et al. 2017), an approach recently termed *regional admixture provenancing* (Bucharova et al. 2019). These recommendations emphasize the value of genetic diversity to address the challenges of climate change, novel disturbances that have altered site conditions, recent population isolation, and lack of genetically appropriate material. Mixing source material may occur at the reintroduction site (e.g. seeds or seedlings from multiple populations sown or planted) or away from the site (e.g. growing source populations in a nursery setting and allowing them to cross, producing a large quantity of seeds that is more diverse and presumably less inbred than any single source population). Producing seeds in a nursery setting may be necessary when insufficient plant material is available from source populations, or when source populations have limited diversity or show signs of inbreeding depression (Broadhurst et al. 2017).

While approaches to increase diversity and alleviate inbreeding in plant materials for restoration and reintroduction are advancing on multiple continents, including Europe (Bucharova et al. 2019), North America (Dolan et al. 2008), and Australia (Broadhurst et al. 2017), little empirical data are available to evaluate the effects of nursery production of mixed-source plant materials on the diversity of the resulting crop used to implement reintroductions. Multiple steps in the production process have been identified where unintentional changes in diversity may occur (Basey et al. 2015; Espeland et al. 2017), but few have been investigated in real-world settings, making it challenging to understand the likelihood and relative strength of potential genetic bottlenecks in the production process. The few studies available illustrate the impacts of different methods of collecting and cleaning wild-collected seeds on the genetic diversity of produced material (Silen & Osterhaus 1979; Konnert & Ruetz 2003; Kettle et al. 2008), and highlight the high potential for evolution over multiple generations, particularly in short-lived selfing species relative to outcrossing perennial species (Nagel et al. 2019). None have followed genetic diversity from multiple wild populations through the production and use of mixed-source plant materials for reintroduction.

Another critical gap in the literature is how propagule type (i.e. seeds vs. plugs) used may influence genetic diversity of the restored population. While seed-based reintroductions allow for the inclusion of a high number of genetically diverse propagules, these populations can have higher extinction risk and slower population growth (Dalrymple et al. 2012). Conversely, the use of seedlings or larger plants allows greater control over the genetic diversity incorporated in the first generation of a reintroduction, but also involves more time and financial investment. Use of seedlings or larger plants also presents numerous steps in the production process that could unintentionally erode genetic diversity (Basey et al. 2015). We are not aware of studies that have directly addressed the genetic benefits or risks of using different types of propagules in a reintroduction, particularly when multiple sources are used, despite the need for these types of studies to help guide best practices.

In this study we test for changes in genetic diversity through production and use of mixed-source plant material, tracking diversity from wild collection of four source populations through two different production approaches and 11 reintroductions using two propagule types (seeds and plugs). We use a well-studied threatened species with documented self-incompatibility, strong inbreeding depression between related individuals, and heterosis in progeny from between-population crosses (Kaye & Lawrence 2003), justifying the use of a regional admixture provenancing approach for reintroduction efforts. We use neutral molecular markers to identify and track source populations, genetic diversity, and inbreeding through different production approaches and restoration propagule types. We ask: (1) Do measures of genetic diversity and inbreeding change during the production and use of mixed-source plant material? (2) Are source populations represented throughout production and use of mixed-source plant material? and (3) Does the production approach (mixing populations in the nursery vs. at the reintroduction site) or type of propagule used (seed vs. plug) impact genetic diversity, inbreeding, and source population representation in reintroduced populations? We expect that genetic diversity metrics will be higher and inbreeding will be lower in reintroduced populations relative to source populations.

Methods

Study Species and Populations

Castilleja levisecta Greenm. (Orobanchaceae) is a short-lived (usually 5–6 but up to 10–15 years) herbaceous perennial endemic to the prairies of western Oregon, Washington, and British Columbia (Caplow 2004). It is a generalist facultative-hemiparasite (Wentworth 2001) but does not require a host for reproduction when in cultivation (Kaye & Lawrence 2003). The species produces bright yellow flowers that require outcrossing for seed set, primarily by species of *Bombus* (Kaye & Lawrence 2003) and *Halictus* (S. Waters 2019, Center for Natural Lands Management, personal communication). Habitat loss due to agricultural conversion, real estate development, and expansion of conifer forest into prairies resulted in the loss of populations throughout the range and it was last reported from Oregon's Willamette Valley in 1938 (Gamon 1995). Only 11 wild populations remain: 10 in the islands of the Salish Sea in Washington and British Columbia and 1 on the mainland in Washington. The recovery plan for this federally listed threatened species includes ex situ seed production and reintroduction into the historic range both in Washington and the Willamette Valley in Oregon (USFWS 2000; Caplow 2004). A common garden study at 10 sites throughout the historic species range (nine in the Willamette Valley) using plants from six remnant wild populations showed that habitat similarity between source and garden sites predicted plant success, but genetic diversity and population size of the source population, and geographic distance, did not (Lawrence & Kaye 2011).

Production of Mixed-Source Plant Material

Seeds were collected from four remnant populations in Washington State identified as ideal source populations: Naas (N), Ebey's Landing (E), and Fort Casey (C) from Whidbey Island in north Puget Sound, and Rocky Prairie (R) on the mainland in south Puget Sound (Table 1; Fig. 1). The three island populations (N, E, and C) were selected based on their common garden performance, while Rocky Prairie (R) was included as the largest and geographically closest population to reintroduction sites, despite relatively lower performance and slightly different morphology in the common garden study (Lawrence & Kaye 2011).

The seed collected was used for nursery production of mixed-source seed lots. Seeds were sown in spring 2008 at Webster Forest Nursery near Olympia, Washington, U.S.A. (hereafter Washington Nursery or WASH), and spring 2010 at the Corvallis Plant Material Center near Corvallis, Oregon (hereafter Oregon). Each nursery used a different approach to mix seed sources. At Washington Nursery, seed production beds were planted using plugs grown from seed derived from all four wild populations, and it is not possible to track the source population of individual plants. Additional plants were planted each year depending on what was available, and as a result the final proportion of seed used from

Table 1. Summary of all *Castilleja levisecta* populations studied including population ID, state, number of individuals included in analyses (*N*), source population (if applicable), and genetic diversity indices, including number of alleles (Na), expected number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), the inbreeding coefficient (*F*), and relatedness (r_{LR}). Populations are listed by population type (wild, nursery, Oregon plug reintroduction, Oregon seed reintroduction, and Washington seed reintroduction).

Population	*ID*	*State*	*N*	*Source*	*Na*	*Ne*	*Ho*	*He*	F	r_{LR}
Wild (population size)										
Naas (841)	N	WA	30	N/A	3.86	2.16	0.51	0.51	−0.03	0.16
Rocky Prairie (7,240)	R	WA	30	N/A	3.29	2.02	0.39	0.43	0.17	0.24
Ebey's Landing (4,612)	E	WA	35	N/A	3.29	2.18	0.41	0.41	0.00	0.14
Fort Casey (1,196)	C	WA	30	N/A	3.86	2.79	0.50	0.54	0.11	0.10
Nursery										
Naas (Oregon)	NO	OR	25	N	3.57	2.28	0.45	0.52	0.11	0.14
Rocky Prairie (Oregon)	RO	OR	24	R	3.86	2.31	0.40	0.50	0.22	0.21
Ebey's Landing (Oregon)	EO	OR	25	E	4.14	2.20	0.41	0.47	0.17	0.11
Fort Casey (Oregon)	CO	OR	25	C	3.71	2.57	0.51	0.54	0.13	0.12
Washington	WASH	WA	30	N, R, E, C	4.86	3.29	0.53	0.62	0.15	0.08
Oregon plug reintroductions (year established)										
Finley Plug (2011)	OP1	OR	29	NS, RS, ES, CS	5.14	2.76	0.50	0.53	0.06	0.03
Baskett Slough (2012)	OP2	OR	30	NS, RS, ES, CS	4.71	2.83	0.64	0.57	−0.12	0.04
Cardwell Hill (2011)	OP3	OR	30	NS, RS, ES, CS	4.71	2.67	0.62	0.56	−0.08	0.05
Lupine Meadows (2011)	OP4	OR	30	NS, RS, ES, CS	4.71	2.46	0.53	0.51	−0.05	0.10
Oregon seed reintroductions (year established)										
Finley Seed (2011)	OS1	OR	30	NS, RS, ES, CS	4.71	2.81	0.52	0.52	0.00	0.03
Beazell (2010)	OS2	OR	34	NS, RS, ES, CS	5.29	3.23	0.64	0.60	−0.09	0.01
Baskett Slough (2011)	OS3	OR	30	NS, RS, ES, CS	5.00	2.73	0.62	0.56	−0.10	0.04
Lupine Meadows (2011)	OS4	OR	30	NS, RS, ES, CS	4.86	2.68	0.59	0.57	−0.04	0.02
Washington seed reintroductions (year established)										
Tenalquot (2010)	WS1	WA	30	Web	5.14	2.97	0.54	0.61	0.12	0.06
Wolf Haven (2011)	WS2	WA	30	Web	5.29	3.14	0.60	0.61	0.02	0.09
Glacial Heritage (2010)	WS3	WA	30	Web	5.86	3.52	0.55	0.61	0.09	0.04

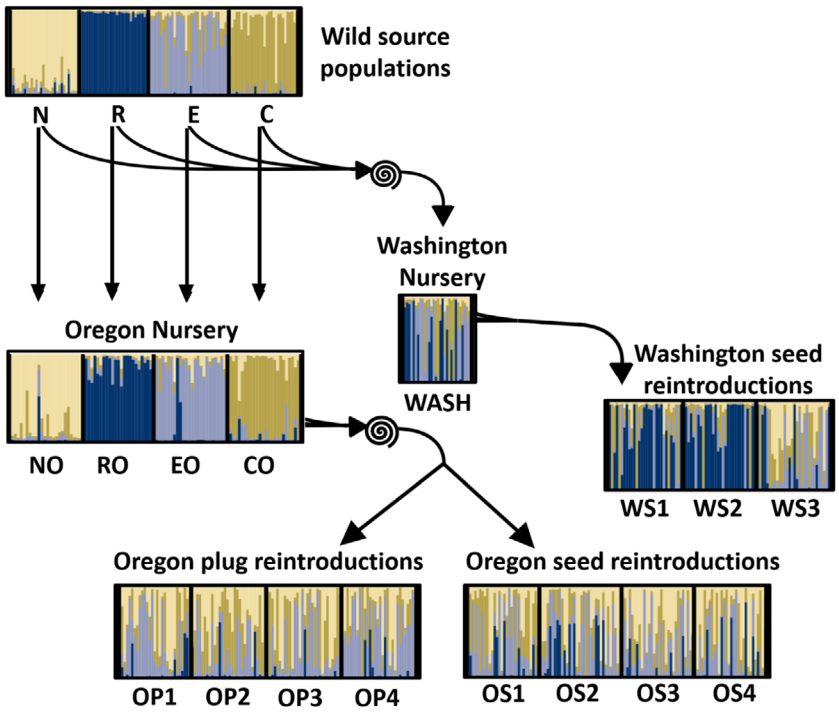

Figure 1. Use of regional admixture provenancing approach to produce seeds of *Castilleja levisecta* for reintroduction. Four populations were wild-collected, grown in two nurseries (one in Oregon, maintained in adjacent but separate beds, and one in Washington, not maintained separately). Seed produced from these nurseries were used in 11 reintroductions. Seed lots were allowed to mix in nursery settings, and resulting seeds were used for reintroductions by seed or grown into plugs for reintroductions. See Table 1 for site information.

each source population is unknown. Beds were started with plugs produced from Rocky Prairie in 2007, with more Rocky Prairie and smaller quantities of Naas and Fort Casey plants added in 2008, plugs from Ebey's Landing were added in 2009, and additional plugs from Naas, Ebey's Landing, and Rocky Prairie were added in 2010. Plants were not maintained in rows by source population, but open space in the production bed rows were filled each year with whatever plugs were available. It is not possible to estimate the final proportion of each source population installed into the production beds, but by the time of harvest in 2010 and 2011 (harvested once for all reintroductions carried out that year), the source population that likely had the greatest number of planted plugs was Rocky Prairie, followed by Ebey's Landing, then Naas, with likely very few plants sourced from Fort Casey. By contrast, in Oregon the seeds from each source were kept separate, grown into source-identified plugs, with individual sources planted in adjacent rows at the same time. In Oregon, the percentage of each population planted was: Naas = 29%, Ebey's Landing = 32%, Fort Casey = 27%, and Rocky Prairie = 12%.

Material from Washington and Oregon nurseries was installed into multiple reintroduction sites in both states from fall 2010 to 2012 (Table 1). Three reintroductions in Washington were started from direct sowing of mixed-source seed from the Washington Nursery (Washington seed reintroductions), while four reintroductions were started in Oregon from seed produced at the Oregon Nursery (Oregon seed reintroductions). In Oregon, an additional four reintroductions were started by out-planting Oregon-grown plugs (Oregon plug reintroductions). For each reintroduction year, a single mixed-population seed lot harvested from the Oregon seed production beds was used in plug- and seed-based reintroductions. At the time of sampling, we assumed all nursery material was one generation removed from wild populations (e.g. no reproduction within the nursery), although it is possible that a few individuals within the beds were derived from self-sown seed). We also assumed that all reintroduction material was one generation removed from the nursery.

DNA Extraction and Microsatellite Amplification

Thirty leaf samples were randomly collected from 21 populations (Table 1) in June 2013, including: four original source populations (N, R, E, C); five seed production beds (one in Washington [WASH], four from source-identified rows in Oregon [NO, RO, EO, CO]), three reintroductions from Washington seed (WS1-3); four reintroductions from Oregon seed (OS1-4); and four reintroductions started from plugs grown at Oregon (OP1-4). Collected leaves were dried with silica gel and sent to Chicago Botanic Garden, where DNA was extracted in 2014 using a modified 2× cetyltrimethylammonium bromide protocol (Doyle & Doyle 1987).

Forty-two sets of microsatellite primers developed for a related species, *Castilleja sessiliflora* (Fant et al. 2013), were tested. Seven reliably amplified *C. levisecta* were polymorphic.

DNA was amplified using polymerase chain reaction (PCR) with fluorescently tagged forward primers (Sigma-Aldrich, St. Louis, MO, U.S.A.). PCR reactions were made in a 10-μL volume containing the following: 2 μL genomic DNA, 5 μL MyTaq Master Mix (Bioline, Taunton, MA, U.S.A.), 0.125 μL bovine serum albumin (10 mg/mL), 0.1 μL forward primer, 0.1 μL reverse primer, and 2.675 μL DNA-grade water. The PCR conditions were 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, 52°C for 40 seconds, 72°C for 1 minute; and a final extension of 72°C for 4 minutes. Microsatellite products were analyzed and manually scored using a CEQ 8000 Genetic Analysis System version 9.0 (Beckman Coulter, Brea, CA, U.S.A.).

Statistical Analysis

Genetic indices were calculated in GenAlEx (Peakall & Smouse 2006), including three measures of genetic diversity (number of alleles (Na), effective alleles (Ne), and expected heterozygosity (He)), one measure of inbreeding (F), and Lynch and Ritland's (1999) Relatedness (r_{LR}). Among population groups, analyses of variance were used to compare differences in genetic indices using R statistical package (R Development Core Team 2019). For these analyses, populations were assigned to one of five groups: 1) source, 2) nursery, 3) Oregon plug reintroductions, 4) Oregon seed reintroductions, and 5) Washington seed reintroductions. Population differentiation (Fst) was calculated for the four source populations using GenAlEx. Patterns of differentiation among all populations were inferred with Bayesian clustering analysis (number of genetic clusters, K) using Structure version 2.2 (Pritchard et al. 2000). Ten independent runs per K were analyzed using a burn-in period of 10,000 iterations for $K = 1–20$. The value of K that best described the data was found using the rate of change in the log-likelihood probability of data between corresponding K values (ΔK) (Evanno et al. 2005) as executed in Structure Harvester (Earl & vonHoldt 2012).

Results

Genetic Variation and Inbreeding

A total of 57 alleles were identified across seven loci in 617 individuals. All loci were polymorphic in each population except one (D103) at Ebey's Landing. The average number of alleles per locus ranged between 2.9 and 5.9 (mean = 4.4), while effective alleles per locus ranged from 2.0 to 3.5 (mean = 2.7). Expected heterozygosity ranged from 0.41 to 0.62. Eight private alleles were found within six populations across five loci. There was also variation in degree of relatedness and inbreeding within samples. The inbreeding coefficient ranged from −0.12 to 0.22, while relatedness between samples ranged from unrelated (0.01) to equivalent to half sibs (0.24).

Diversity in Remnant Populations

All wild-source populations showed similar levels of genetic diversity (i.e. number of alleles, average = 3.57) and expected heterozygosity (average = 0.47; Table 1; Fig. 1). Average inbreeding (F) was 0.06, with Rocky Prairie and Fort Casey having moderately high inbreeding (F = 0.17 and 0.11, respectively). Population size was not significantly correlated with genetic diversity (number alleles, number effective alleles, effective heterozygosity) or inbreeding coefficient.

Differentiation in Remnant Populations

Pairwise genetic distances showed moderate differentiation among source populations (Fst = 0.09–0.36), even among the three sites on Whidbey Island (Fst = 0.09–0.18), despite how geographically close these populations are to each other (1.8–4.0 km apart). The most genetically distinct population was Rocky Prairie (Fst = 0.27–0.36), which is 120 km from the other three populations. Structure Harvester identified four genetic groupings (K) as the best explanation of data; at $K = 4$, the four source populations were easily distinguishable from each other (Fig. 1). The lowest assigned membership for a source population to a single cluster was Ebey's Landing at 74%, with Naas, Rocky Prairie, and Fort Casey assigned clusters at 87%, 96%, and 82%, respectively.

Tracking Diversity Metrics and Inbreeding Through Production and Use

There was a significant increase in genetic diversity metrics from source through reintroduced populations, including average number of alleles ($F_{4,15} = 15.91$, $p = <0.001$), average effective alleles ($F_{4,15} = 4.06$, $p = 0.02$), and expected heterozygosity ($F_{4,15} = 4.43$, $p = 0.01$) (Table 1; Fig. 2). As a result, restored populations had significantly higher average genetic diversity than individual source populations. There was no significant difference in diversity metrics between populations restored from different nurseries (Oregon or Washington) or using different types of material (seed or plug).

There was also a significant difference between source and reintroduced populations for measures of inbreeding ($F_{4,15} = 8.45$, $p = 0.001$) and relatedness ($F_{4,15} = 4.95$, $p = 0.01$) (Table 1; Fig. 2). Inbreeding was highest in source populations, all nursery populations, and reintroduced populations in Washington, and lowest in Oregon seed reintroductions. By contrast, relatedness between individuals was highest ($r_{LR} > 0.1$) for source populations and nursery plants in Oregon, and lowest for Oregon seed reintroductions. Moving from source population to nursery beds, mean inbreeding coefficient increased across all populations (average $F = 0.06$ and 0.16, respectively), although this shift was not significant. However, relatedness remained high for all populations except Washington Nursery, where source populations were mixed. Moving from nursery beds to reintroduced populations showed a significant drop in both inbreeding coefficient and relatedness for all samples except the inbreeding coefficient for Washington seed reintroductions. There was no significant difference in inbreeding or relatedness between restored populations regardless of source nurseries (Oregon or Washington) or propagule type (seed or plug). Populations reintroduced using seeds from Washington tended to have higher inbreeding than those reintroduced using seeds or plugs from Oregon (average $F = -0.06$ vs. 0.08).

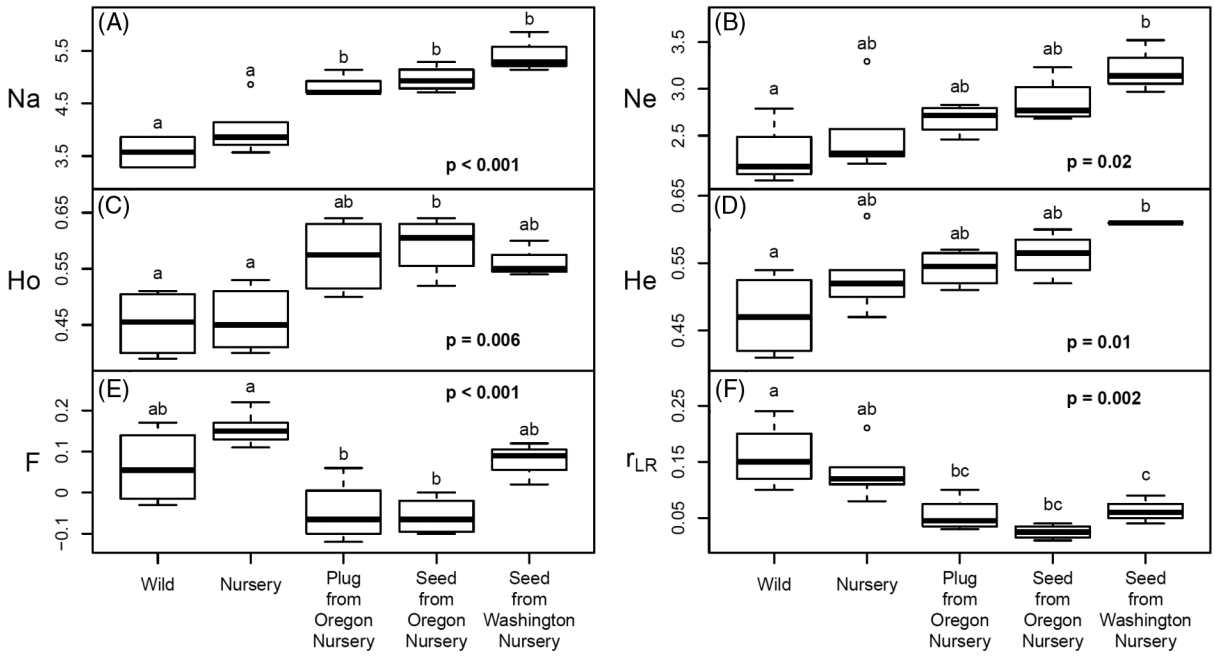

Figure 2. Results of analyses of variance for five population types of *Castilleja levisecta*. Mean values for (A) number of alleles (Na), (B) number of effective alleles (Ne), (C) observed heterozygosity (Ho), (D) expected heterozygosity (He), (E) inbreeding coefficient (*F*), and (F) relatedness (r_{LR}). A post hoc Tukey test showed significant differences between groups at $p < 0.05$.

Table 2. Likelihood of genetic cluster representation within each population of *Castilleja levisecta* studied when $K = 4$. The four genetic clusters correlate to the four wild populations labeled at each column heading. Clusters are bolded for each population when their representation is 25% or greater.

		Genetic cluster			
Population	*ID*	*N*	*R*	*E*	*C*
Wild					
Naas	N	**87%**	4%	4%	6%
Rocky Prairie	R	1%	**96%**	1%	1%
Ebey's Landing	E	8%	1%	**74%**	16%
Fort Casey	C	13%	2%	3%	**82%**
Nursery					
Washington	WASH	5%	**34%**	**37%**	24%
Naas (Oregon)	NO	**84%**	3%	4%	9%
Rocky Prairie (Oregon)	RO	4%	**84%**	9%	4%
Ebey's Landing (Oregon)	EO	9%	7%	**78%**	7%
Fort Casey (Oregon)	CO	9%	4%	6%	**81%**
Washington seed reintroductions (year established)					
Tenalquot (2010)	WS1	5%	**57%**	16%	22%
Wolf Haven (2011)	WS2	3%	**71%**	14%	12%
Glacial Heritage (2010)	WS3	**28%**	20%	**30%**	23%
Oregon plug reintroductions (year established)					
Finley Plug (2011)	OP1	**31%**	6%	**38%**	**25%**
Baskett Slough (2012)	OP2	**35%**	6%	**26%**	**34%**
Cardwell Hill (2011)	OP3	**33%**	3%	**30%**	**34%**
Lupine Meadows (2011)	OP4	**26%**	6%	**48%**	20%
Oregon seed reintroductions (year established)					
Finley Seed (2011)	OS1	24%	11%	**31%**	**34%**
Beazell (2010)	OS2	**32%**	17%	**30%**	22%
Baskett Slough (2011)	OS3	**41%**	8%	**32%**	19%
Lupine Meadows (2011)	OS4	**37%**	14%	**26%**	24%

At Oregon, structure results at $K = 4$ show that the majority of plants in each of the four beds matched to the same genetic cluster as their source populations (Fig. 1). By contrast, many individual plants in the reintroductions sourced from Oregon (plugs and seeds) were split between two genetic clusters, suggesting outcrossing among populations in the nursery beds. Regardless, when the average percent assignment to clusters was calculated across plants, they matched the objectives of the nursery and reintroduction, with equal representation of Naas, Ebey's Landing and Fort Casey (26–35%), and small number of Rocky Prairie (3–6%; Table 2). Reintroductions started with seed showed similar results as plugs, although seed-based reintroductions had slightly larger variation in population representation than plugs, both for the three Whidbey Island populations (range of representation from 19 to 41%) and Rocky Prairie (range of representation from 8 to 17%).

At Washington Nursery, the single mixed-source bed was comprised of plants grown from all four source populations, with structure results at $K = 4$ suggesting the production bed includes 5% Naas, 24% Fort Casey, 27% Ebey's Landing, and 34% Rocky Prairie source material (Fig. 1). Some individuals showed likely mixed parentage (e.g. they belonged to two genetic clusters), suggesting they were derived from self-sown seed resulting from a cross between source populations in the bed (Fig. 1). However, it is possible that these second-generation plants established between 2010/2011, when seed was collected for reintroduction sites included in our study, and 2013, the year leaves were collected from production beds for this study. Of the three Washington seed reintroductions, WS1 and WS2 had higher representation of Rocky Prairie (57–71%) when compared to the original seedbed, and a decrease in Ebey's Landing and Fort Casey (Table 2). The representation of Naas remained consistently low from nursery to reintroduction at these two sites. At WS3, the representation of Fort Casey was similar to WS1 and WS2, but the representation of Rocky Prairie was much lower, while both Naas and Ebey's Landing had large increases in representation (from 5 to 28% for Naas, and 16–30% for Ebey's Landing).

Discussion

Planting multiple seed sources together in nursery production beds (i.e. an admixture provenancing approach) is expected to produce seeds with increased genetic diversity and reduced inbreeding relative to original source populations. Our study provides the first empirical evidence that this provenancing approach can deliver on these expectations. By comparing two production nurseries, which combined populations at different stages in the production process and produced both seeds and plugs for restoration efforts, we demonstrate how this approach generally yielded expected genetic benefits across 11 different reintroduction sites. However, we found that mixing source populations at the reintroduction site, rather than in nursery production beds, was the most effective way to ensure all source populations are represented, while also ensuring high diversity and low relatedness and inbreeding in source material. Additionally, we identified differences in the representation of source material in reintroduced populations which may be related to propagule type. Specifically, plugs appear to deliver a more consistent representation of source populations in reintroductions, at least in the first generation, while use of seeds produces higher variability in source population representation. Future experimental work is needed to determine whether these patterns hold up in other species and systems.

Genetic Diversity and Inbreeding Through Production and Use

All measures of genetic diversity increased in reintroduced populations relative to wild populations (although this was not always significant). This is not unexpected, as *Castilleja levisecta* is an obligate outcrossing species, so as long as there are sufficient pollinator services available (Cane 2008), populations are likely to inter-breed in the nursery, increasing net genetic diversity of seed produced. One caveat is that microsatellite markers only measure neutral genetic variation (Whitlock 2014), so our results do not necessarily mean that similar changes occurred in adaptive variation. In fact, adaptive genetic variation may have been lost during the production process because selective pressures in a nursery setting are rarely similar to those in the wild (Espeland et al. 2017), although previous studies have shown that even plants cultivated over multiple generations can show regional variation (Bucharova et al. 2017). Yet, because only one generation passed from wild collection to our nursery production bed, the likelihood that adaptive diversity was lost in our study system is lower than for annual selfing species produced over multiple generations (Nagel et al. 2019).

We also found elevated inbreeding within source populations, which remained high or even increased in seed production beds (e.g. each wild source population had lower inbreeding than the same population grown in the Oregon Nursery). As a consequence, seed production beds had the highest average inbreeding coefficient of any stage in production. While increases in inbreeding are common when plants are maintained in cultivation for multiple generations (Stanford et al. 1960; Cowling 2013), our production beds mostly represent adults of first-generation material. Field-based studies have found higher inbreeding levels in seedlings relative to adults growing in natural populations, associated with greater representation of half and full sibs (Schaal & Levin 1976; Tonsor et al. 1993; Spigler et al. 2010), with inbreeding levels expected to decline when sampling the surviving adult population. This shift could be explained as selection against inbred individuals at the seedling stage. However, under relaxed selection within nursery conditions like ours, seedling survival is expected to be higher than under natural conditions, hence more inbred individuals may reach flowering than in the wild (Kettle et al. 2008). To address this, Konnert and Ruetz (2003) suggest collecting established wild seedlings to establish nursery production beds, because they have already been subjected to natural selection, maximizing diversity captured while minimizing the likelihood of including inbred individuals. This may be challenging in practice, and if a goal is to ensure maximum diversity of source populations, relaxed selection may be important to ensure all lines are maintained.

Both inbreeding and relatedness showed greatest declines when comparing reintroductions to seed production beds, likely due to outcrossing in the nursery. These shifts occurred across all propagule types, including plugs generated under the relaxed selection of nursery conditions, suggesting cross-pollination between populations. These results are supported by Structure results, showing that many individuals in the reintroduction were comprised of more than one genetic cluster, which was not the case for plants in the wild or nursery beds. However, cross-pollination also produces numerous F1 offspring with potentially higher fitness-associated heterosis, especially given many of the source populations show elevated inbreeding (Pickup et al. 2013). Hence, in addition to high levels of outcrossing, these changes in inbreeding and relatedness levels may be a result of elevated fitness of outcrossed individuals, selection against homozygous offspring (Spigler et al. 2010), or a combination of these factors.

Impact of Propagule Used and Production Approach

We found little evidence that propagule type (seed or plug) impacted genetic diversity or inbreeding in our reintroduced populations. The only potential difference, based on sites using Oregon germplasm, is that the probability of maintaining desired representation of source populations is greater when planting plugs, while selection at the seed germination and establishment phase may be more likely to select for certain populations. It is unclear whether these slight differences in source populations in seeds versus plugs translated to any fitness impact for the reintroduced population. Thus the decision about which type of germplasm to use may depend on the amount of seed available and the long-term cost of using seeds versus plugs (Kaye & Cramer 2003; Guerrant & Kaye 2007). Transplants are often more reliable at supporting demographically successful reintroductions relative to seeds (Godefroid et al. 2011; Bowles et al. 2015), although this is not always the case (Albrecht & Maschinski 2012; van Katwijk et al. 2016). In a retrospective analysis of over 40 instances using outplanted plugs at four sites in Washington, only one had succeeded in establishing a new subpopulation that persisted and grew to exceed the number of plugs originally outplanted (Dunwiddie, unpublished data). In Oregon, several populations have demonstrated recruitment of new individuals from seed but again, only one grew to exceed the number of plants originally planted (Kaye, unpublished data). This is in contrast to the use of seeds, where many new populations, some exceeding 100,000 flowering individuals, have succeeded in both Washington and Oregon (Dunwiddie & Martin 2016; Kaye 2019).

Source Populations Represented Through Production and Use

At the Oregon Nursery, where source populations were intentionally planted and maintained in separate but adjacent nursery bed rows, we found no evidence of changes over 3 years between planting and sampling. However, at the Washington Nursery, results may indicate selection against the Naas population, which represented only 5% of the material collected despite all four source populations being included in the original planting. While we do not know the specific proportion of material from Naas included in the nursery, records indicate it was planted in greater numbers than Fort Casey, but our results indicate five times greater representation of the Fort Casey genetic cluster relative to the Naas cluster in the nursery bed at the time of our sampling. We conclude that Naas plugs outplanted in the Washington Nursery may have had disproportionately low survival.

All restored populations had some representation from all four of the original wild source populations. However, genetic composition was most consistent when the production beds were maintained in source-identified rows (i.e. Oregon Nursery). The least variation in source population representation occurred in reintroductions planted with plugs. The higher variability in representation of source populations in seed-derived restorations might suggest that selection is acting at the seed germination and establishment phases. This was particularly evident in the restored populations using seeds from Washington Nursery, which had the greatest variation in source population representation even among reintroductions conducted in the same year. This provides further evidence to suggest that selection acted at the reintroduction sites to favor the genetics of some source populations and not others. Additionally, if selection is indeed acting on seeds and shifting the representation of source populations at each site, it may help to explain why populations reintroduced using seeds were much more likely to be demographically viable over the long term. Further research is needed to understand whether selection is indeed driving variation in source population representation, and whether this influences the long-term viability of a population.

Additional Research Needed

One ongoing challenge associated with regional admixture provenancing (Bucharova et al. 2019) is determining best source populations to include in the mix, as well as where resulting germplasm should be planted. Future research to assess whether increasing or decreasing the number and identity of populations mixed would help to clarify the most effective approach for this and similar species, particularly in the context of climate change (Havens et al. 2015). There is likely a point at which mixing source populations from too broad an area will result in a deterioration of performance compared to the locally adapted type (Leimu & Fischer 2008; Hereford 2009), although climate change is rapidly shifting the conditions to which plants are adapted (Breed et al. 2013). It is also possible that mixing populations with chromosomal differences or that have been genetically isolated for 500+ years will lead to outbreeding depression, which may manifest in the first or second generation (Frankham et al. 2011). It is promising that mixing multiple source populations can improve fitness, diversity, and conservation outcomes of reintroduced populations of rare species with no measurable outbreeding effects (Rick et al. 2019). Mixing populations over areas of similar adaptation, such as within an ecoregion (Ward et al. 2008; Miller et al. 2011; Gustafson et al. 2018) or sub-portion of an ecoregion (Baughman et al.

2019), or a provisional seed zone (Bower et al. 2014), can increase the diversity of the plant materials available for restoration while minimizing risks of maladaptation.

One goal of *C. levisecta*'s recovery and reintroduction plans is to reintroduce genetically diverse populations to sites where the species has been extirpated (USFWS 2000; Caplow 2004). Our results support bringing multiple source populations together in a production setting to allow cross pollination, increasing genetic diversity and decreasing inbreeding and relatedness in propagules used for reintroduction. By incorporating genetic diversity from multiple populations within a nursery, it is possible to produce and use genetically diverse germplasm following a regional admixture provenancing approach. When seed production was maintained in source identified rows, there was greater control of the relative contribution of each seed source to the final reintroduction. Mixing is a bet-hedging strategy. In the short term, more genetic diversity is available for selection to act upon, potentially resulting in improved initial establishment, as well as improved long-term population viability and resilience to change (Reusch et al. 2005; Crawford & Whitney 2011; Wang et al. 2012; Hufbauer et al. 2013; Szűcs et al. 2014; Hufbauer et al. 2015; Szűcs et al. 2017). Furthermore, because previous research on this species illustrated that crosses between populations yielded plants with greater fitness (heterosis) than within-population crosses (Kaye & Lawrence 2003), this approach likely provided reintroduced populations with an additional fitness advantage.

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