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Battle of the Buttercups: Investigating Hybrid Zone Dynamics in a *Ranunculus*
Species Complex

by

Jessica Persinger

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

Thesis Committee:
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Portland State University
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Abstract

Plants pose a well-known challenge to the biological species concept because hybridization is a common occurrence. Range dynamics have a crucial role in determining whether species are given the opportunity to interbreed or not. Successful hybridization can be of particular concern when considering range-limited species and their persistence. In my thesis research, I examine the apparent hybrid zone between the southern Oregon endemic *Ranunculus austro-oreganus*, a candidate threatened species due to its limited range, and its widespread congener, *Ranunculus occidentalis*, whose range spans from California to Alaska. The discovered contact zone was recognized in populations which contained apparent intermediate ventral petal coloration phenotypes. First, I utilized morphological quantification to understand phenotypic variation. Phenotype quantification based on ventral petal coloration and leaf trichome density have confirmed the presence of intermediate populations. Second, I confirmed extensive introgression through nuclear genome surveys with Genotyping-by-Sequencing despite structure analyses indicating the presence of two genetic subgroups. Third, 50 outlier loci were analyzed across geographic clines where results suggest the width of the hybrid zone spans 20km. Sampled populations of *R. austro-oreganus* and *R. occidentalis* display evidence of

extensive, ongoing gene flow and this species complex provides a unique example of genetic exchange between variable phenotypes.

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Introduction

Understanding how to classify and define species is a fundamental question in evolutionary biology. Many concepts have been developed to delineate species, such as the biological species concept, where different species are defined as those that are unable to reproduce and make viable offspring (Mayr, 1942). Although the biological species concept is frequently used, a number of other species concepts have been deployed to better understand species classifications (e.g. phylogenetic, phenetic, ecological, genealogical). A number of challenges present when attempting to delineate species regardless of which concept is used, and species are notably more difficult to delineate when hybridization is ongoing. Hybridization has been defined as reproduction between genetically distinct populations (Barton and Hewitt, 1985), and when gene flow between species is ongoing these zones of contact can be understood as a genetic gradient between two species (Anderson, 1949). Major geological events, such as glaciations, create a unique opportunity for researchers to investigate lineages that have undergone divergence through glacial retreat. Geographically separated lineages have the potential to come back into proximity with one another and form hybrid zones (Mayr, 1963; Remington, 1968), allowing for observations in regard to species classifications.

Since the early 1900s, we have recognized the potential for hybridization to contribute to plant adaptation and diversification (Lotsy, 1916; Anderson, 1949; Anderson and Stebbins, 1954; Arnold, 1992; Rieseberg, 1997; Abbott *et al.*, 2013), and hybrid zones provide an opportunity to document adaptive introgression (Taylor *et al.*, 2015). Plants are ideal candidates for understanding congeneric species coexistence due to their limited capabilities for movement and dispersal and their ability to respond to microhabitat variation (Kay *et al.*, 2016). The limited movement of plant populations creates a troubling scenario when it is predicted that they may need migrate up to >1km per year to continue living in an environment that is suitable as climate is changing (Corlett and Westcott, 2013). Plants can either adapt, migrate or die in response to a rapidly changing environment (Aitken *et al.*, 2008), and hybrid zones provide an opportunity to document adaptive introgression (Taylor *et al.*, 2015), which may be a solution that lineages will utilize in order to overcome a changing climate. Although gene flow may infuse populations with beneficial genetic variation, preventing excessive gene flow into populations of range-limited species from their widespread congeners is necessary for their continued independent divergence and eventual development of strong isolating barriers (Arnold, 1997; Sobel and Streisfeld, 2015). The ultimate fate of range-limited species will depend largely on the strength of isolating barriers and the dynamics of hybrid

zones that may develop in regions of contact (Levin, 2003). Therefore, it is important to describe hybridization dynamics and patterns of gene flow in suspected hybrid zones to better understand species ecological and evolutionary trajectories.

Intermediate phenotypic forms have frequently been used to identify where species form a hybrid zone (e.g. Briggs 1962; Martin and Cruzan 1999; Gay *et al.*, 2008; Morales-Rozo *et al.*, 2017), and after a hybrid zone has been recognized, more detailed questions about gene flow and introgression between differentiated lineages can be investigated (Howard *et al.*, 1997; Kay *et al.*, 2018). Examining genomic differentiation and level of introgression between species in a hybrid zone provides an opportunity to disentangle the historical context of the species and better understand the longer-term outcomes of hybridization (Guichoux *et al.*, 2013; Harrison and Larson, 2014). Hybridization between species can occur through historical gene flow, contemporary gene flow, or a combination of both. Genetic markers, most commonly single-nucleotide polymorphisms (SNPs), are often used to understand genetic variation (Petit *et al.*, 2004) and are useful for understanding different time scales of gene flow. In order to utilize mitochondrial DNA (mtDNA) as a genetic marker in plants, a number of obstacles have had to be overcome (Palmer, 1992), but mtDNA has still been used in phylogenetic, phylogeographic, and population genetic studies

(Puorto *et al.*, 2001; Johnsen *et al.*, 2017). Chloroplast DNA (cpDNA) markers have been preferred amongst plant scientists as the genome is highly conserved across species and does not recombine (Palmer, 1987) which enables studies regarding historical range dynamics (Heuertz *et al.*, 2004). Nuclear DNA (nDNA) changes at a relatively fast rate due to genetic recombination, thus variants can provide detailed information about patterns of contemporary introgression and the dynamics of a hybrid zone (Barton and Hewitt, 1985). A recombining genome presents the opportunity to examine non-random associations of alleles on the same chromosome (linkage disequilibrium; LD) persisting through recombination events. LD estimates are higher when two distinct populations first hybridize and will consequently decay over time due to recombination effects (Reich *et al.*, 2001).

Lineages can be characterized through the measurements of genomic diversity, and these data can inform the dynamics within a region of contact. Outlier loci, such as loci under selection, can be used to understand signatures of past introgression events throughout contact zones (e.g. Guichoux *et al.*, 2013) and to understand the level of divergence that lineages may be experiencing. Outlier SNP data can then be used to measure allelic frequencies in populations, where large differences in allelic frequencies would suggest that selection is acting upon the genome. Changes in allele frequencies amongst populations is

commonly viewed through cline analyses utilizing population genomic data (Qi *et al.*, 2014; Stankowski *et al.*, 2017; Esquerré *et al.*, 2019; Scott *et al.*, 2019), but can be carried out in a number of ways (Gompert *et al.*, 2017). Geographic cline analyses are especially useful for integrating a spatial understanding of hybrid zones, allowing clines associated with both morphological and genomic data to be analyzed (Arntzen *et al.*, 2016; Riemsdijk *et al.*, 2019). Steep, narrow, and concordant clines would suggest a hybrid zone is being maintained by both strong selection and dispersal; conversely, gradual, wide, and displaced clines would indicate selection against hybrid individuals is low (Barton and Gale, 1993). Geographic cline data can then infer a directionality of a hybrid zone with the addition of LD analyses where asymmetrically higher levels of LD would suggest the hybrid zone would move in the direction of higher estimates (Wang *et al.*, 2011).

Study System

In this study, I examine morphological and genomic variability between two perennial buttercups whose distributions and morphology suggests ongoing hybridization. The widespread species, *Ranunculus occidentalis* Nutt. (Western buttercup; Ranunculaceae), occurs across an extensive geographic region, stretching from southern California to Alaska, whereas *Ranunculus austro-*

oreganus L.D. Benson (Southern Oregon buttercup) occupies a small range in Jackson County, Oregon and is considered a candidate threatened species (Fig. 1; Kagan *et al.*, 2016). *R. austro-oreganus* was separated out from the *R. occidentalis* complex and described as a new species in 1954 (Benson) on the basis of its unique flower color, bulbous leaf bases, and a distinct geographic distribution. Both species are members of section *Ranunculus* within the genus (Benson, 1954); hybridization and incomplete barriers to crossing are common within sections of *Ranunculus* (Benson, 1948; Hörandl *et al.*, 2005; Baltisberger and Hörandl, 2016). Analyses of chloroplast genomes confirm that *R. austro-oreganus* and *R. occidentalis* are closely related (Li *et al.*, 2019) and display evidence of historical gene flow (Appendix A). Additionally, greenhouse crosses between the two species have shown successful seed production and germination, indicating minimal barriers to reproductive success (unpublished data).

These two species appear to be interbreeding in a region where their ranges come into contact, in a narrow hybrid zone near the edge of a proposed suture zone in southern Oregon, USA (Swenson and Howard, 2004). These species have been found to occur in sympatry within similar ecogeographic regions. While the flowers of both species appear yellow from above, they are easily distinguished in the field based on the dark orange ventral petal color of *R.*

austro-oreganus in contrast to the yellow ventral petals of *R. occidentalis* (Fig. 2A). Yellow petal coloration is associated with the presence of carotenoids and flavonoids (Grotewold, 2006; Ono *et al.*, 2006), and the presence of an orange color indicates the additional presence of anthocyanins (Eugster and Märki-Fischer, 1991; Berman *et al.*, 2016). The putative hybrid zone was identified based on the presence of large numbers of plants displaying a range of ventral petal colors; this zone occupies a narrow region covering Upper Table Rock near Medford, Oregon (Fig. 1). Populations of the two species in the hybrid zone and allopatric populations in the region were sampled to confirm patterns of petal coloration and leaf trichome density measurements across the species' ranges in the Rogue River Valley. This information is supplemented with assays of nuclear genome variation to understand the putative hybrid zone dynamics.

Research conducted in the region suggests that past glaciation has greatly influenced the distribution of genetic variation of flora and fauna (Soltis *et al.*, 1997). This species complex may be a product of divergent lineages from the last glacial maximum coming into secondary contact. It is possible that gene flow into populations of *R. austro-oreganus* from *R. occidentalis* would aid in adaptation depending on the cost of dispersal and the strength of genetic drift (Polechová, 2018) because of the limited range *R. austro-oreganus* occupies. It is also possible that these species are more recently diverged where the range-

limited species has originated by budding (peripatric) speciation from *R. occidentalis* since the last ice age. It is worth noting that, in addition to the unknown historical context between these lineages, it is also unknown whether the hybrid zone between these populations is stationary or moving. Understanding the dynamics within this hybrid zone, or lack thereof, have implications for conservation biology when working with species that have limited ranges.

By quantifying phenotypic data and estimation of genomic variability in the putative hybrid zone, I will address the following questions: 1) what happens phenotypically when these two taxa come into contact, 2) what is the extent of the suspected hybrid zone, and 3) is there evidence of hybrid zone expansion?

Methods

Morphological Differentiation

Observations of plants populations in the field and in the greenhouse suggested that *Ranunculus austro-oreganus* and *Ranunculus occidentalis* display differences in their ventral petal color and in the density of leaf trichomes. These two morphological traits were quantified across populations to characterize the geographic distribution of these traits across populations in the region of the presumed hybrid zone. Each population analyzed was assigned a species classification based on the apparent ventral petal coloration observed in the field. Three petals from three separate flowers and two leaves (one basal and one cauline) were collected in the field from each plant in two separate flowering seasons. Petals and leaves were collected in the field from 28 populations (6 *R. austro-oreganus*, 11 *R. occidentalis*, and 11 putative hybrids) in 2017, and petals were collected from an additional two populations of *R. austro-oreganus* and two hybrid populations in 2019. Leaves and petals were placed in coin envelopes in the field and dried with silica beads.

Morphological characters were quantified using a Leica MZ16 stereomicroscope and ImageJ software (v.1.52c). Ventral petal color was quantified across 28 populations (a total of 532 individuals) by capturing images at 40x magnification. ImageJ was used to convert each petal image to 8-bit gray

scale using weighted RGB conversions to maintain luminosity ($R \times 0.30$, $G \times 0.59$, $B \times 0.11$), where the yellow color is then perceived as lightness (high gray value) and the red/brown character is darkness (low gray value). A 500px^2 selection was made over the center of the image, with the goal of representing a section of the petal without any of the background in the selection. The ImageJ measurement tool was used to capture the mean gray-scale value of the selection, where every pixel had a value from 0 to 255, black to white. Variation in leaf hair coverage was quantified in the same manner as petals, for one basal and one cauline leaf from the 28 populations, resulting in 1,184 leaf samples. Each leaf image was converted to 8-bit gray scale and the mean gray-scale value was measured over a standardized area of 0.785 mm^2 placed in the center of the leaf. Leaf and petal data were analyzed using linear models with SAS Proc GLM (2007) to test for differences between the three species classifications: the two *Ranunculus* species and the putative hybrid populations. Differences analyzed were conducted with sampling location nested within species/hybrid designations. The variance in ventral petal color (gray value) within populations was examined to assess the geographic distribution of hybrid populations. For leaf gray value analysis, leaf position was nested within species and location. Residuals from these models displayed approximately normal distributions.

DNA Isolation and Sequencing

Leaf tissue was collected from 20 individuals in each of 48 populations from *R. austro-oreganus*, *R. occidentalis*, and their putative hybrids through the 2015-2018 field seasons. All plant material was stored in coin envelopes and were dried and kept in silica gel until DNA extraction. Total genomic DNA was extracted following the Qiagen DNeasy 96 Plant Kit protocol (Qiagen, Germantown, MD). Isolated DNA was quantified using a Qubit 4 fluorometer (Invitrogen, ThermoFisher Scientific, Waltham, MA), and purified DNA samples were stored at -20°C. Individuals with a DNA concentration greater than 20 ng/ul were prioritized and selected for Genotyping-by-Sequencing (GBS), a cost-effective strategy for genome-wide variant discovery between restriction enzyme cut sites in nDNA (Elshire *et al.*, 2011). To confirm DNA quality 100ng of DNA for 10% of samples were digested with EcoRI (GAATTC) and incubated at 37°C for 2 hours. Digested and undigested samples were then run in separate lanes on a 1% agarose gel for 60 minutes and imaged to confirm digest success. DNA isolations from 125 *R. austro-oreganus*, 174 *R. occidentalis*, and 79 putative hybrid individuals (16, 22, and 10 populations respectively, ranging from 7-8 individuals per population) were chosen to be sequenced. Four 96-well plates, each containing a blank for control, were submitted on dry ice to the Biotechnology Center at University of Wisconsin-Madison for enzyme

optimization, library construction, and sequencing (HiSeq 1x100, 96 samples per lane). Enzyme optimization indicated that ApeKI (GCWGC) would be most effective and was subsequently used to prepare all GBS libraries.

GBS data were filtered for a minimum quality and analyzed for genotyping using a mock reference to identify variants. Sequencing data was processed with GBS-SNP-CROP v.4.0 (Melo *et al.*, 2016), a bioinformatic pipeline utilized to implement quality control, generate a mock reference, and genomic variants were identified. All computing processing was carried out using the Coeus High Performance Computing (HPC) cluster at Portland State University. The four plates of GBS data were quality filtered, trimmed, and demultiplexed separately. Reads were trimmed and quality filtered using Trimmomatic (Bolger *et al.*, 2014) and trimmed according to the following guidelines: their average quality score dropped below 30 in a 4bp sliding window, leading and trailing bases were trimmed if their quality score dropped below 30, and any reads following trimming with a length under 32bp were discarded. Seven individuals without sufficient data (<200KB file size) were removed at this stage, four blank wells and three individuals.

The mock reference was created by finding a consensus between two individuals collected from populations classified as *R. occidentalis* that were suspected to be outside of the putative hybrid zone based on petal coloration

(populations 'OldStageRd1' and 'OldStageRd2'); two individuals were used to reduce erroneous reads in the mock reference. The VSEARCH clustering algorithm (Rognes *et al.*, 2016) was used for reads that were above the 93% nucleotide identity cutoff and the minimum acceptable length for a mock reference cluster was 32bp. All 377 individuals were then aligned to the mock reference through the use of BWA-mem (Li and Durbin, 2009). Variant SNPs were recognized through the use of SAMtools (Li *et al.*, 2009) where a minimum acceptable phred score base call quality and alignment quality of 30 was required. A variant discovery matrix was then created by identifying SNPs between the mock reference and all individuals used. The variant discovery matrix was then filtered with the following parameters, as recommended for diploid data in the GBS-SNP-CROP pipeline (Melo *et al.*, 2016): a minimum of 75% of individuals needed to have the variant present with an average read depth ranging from 3-200 and a minimum of three individuals supporting the existence of an alternate allele. Non-primary allele reads were removed if the secondary allele percentage was below 80% across all individuals. The read depth required to call an individual a homozygote when the secondary allele depth was equal to zero was a minimum of 5 reads. If there was a secondary allele present the minimum read depth was 20 in order for an individual to call the individual homozygous, with a minimum ratio of primary to alternate reads of 0.3.

SNPs with a proportion of heterozygous individuals greater than 0.5 or a minor allele frequency below 0.05 were then removed with TASSEL (Bradbury *et al.*, 2007) as these were likely a result from misalignment or a collapse of homologous regions in the mock reference, consequently not representing true variants. Once filtered, a subset of 2196 SNPs remained and were used in subsequent analyses.

Genetic Clustering

I analyzed genetic clustering and structure in a variety of ways to understand the separation of allelic frequencies among populations. Population heterozygosity and SNP frequency measurements were estimated with Adegenet v.2.1.0 in R (Jombart, 2008). A PCA matrix of individuals' genotype frequencies was constructed using Hierfstat (Fig. 4; Goudet, 2005). Genetic clusters were estimated using a Bayesian framework with the program Structure (Pritchard *et al.*, 2000). The number of possible subpopulations (K) was estimated by examining K values ranging from 1-23, each having ten replicates containing a burn-in of 25,000 and MCMC of 25,000. Options to "use sampling locations to assist clustering (locprior model)" and "use given populations at the initial condition for population origins (start at pop info)" were flagged to improve the model. The optimal K was found using the Evanno method (Evanno *et al.*, 2005)

and CLUMPAK (Kopelman *et al.*, 2015) assisted in the visualization of the Structure output.

Outlier Loci

Loci potentially affected by selection pressures (i.e. divergent selection, genetic hitchhiking) can aid in the estimation of hybrid zone clines by indicating genetic structure (Nosil *et al.*, 2009; Gompert and Buerkle, 2011). The outlier loci that may be under selection can be especially useful for assessing differences in relative gene flow when gene flow is high (Guichoux *et al.*, 2013), as suspected in the two *Ranunculus* species. Outlier loci are typically identified through tests of genetic differentiation (e.g. F_{st}) that is higher than expected under a neutral distribution (Beaumont and Balding, 2004). F_{st} estimates using BayeScan v2.1 (Foll and Gaggiotti, 2008) failed to identify outlier loci in this system. Alternatively, outlier loci were identified through the use of morphological differentiation by analyzing four populations, two *R. austro-oreganus* and two *R. occidentalis*, based on their lowest variance populations and ventral petal color measurements. These four populations are suspected to have the least amount of introgression given their phenotypic measurements. Outlier loci in this study were identified and defined as SNPs with moderate allelic frequency differences (>0.3) between the two groupings populations, suggestive of ongoing

interspecific genetic differentiation. Additional SNPs were identified as being outlier loci with the requirement of putative species classifications having moderate allelic frequency differences (>0.3) between all *R. austro-oreganus* and *R. occidentalis* populations. These criteria identified a total of 61 outlier SNPs out of the original 2196 detected.

Geographic Cline Analysis

Geographic clines can be used to understand the dynamics in a hybrid zone for different traits and genetic parameters. Steep clines across a study region would suggest that the characters examined are under selection pressure, whereas gradual clines would indicate weak selection. Cline estimation was carried out using a maximum likelihood approach for both of the morphological traits and each of the 61 outlier loci. These data were analyzed across a single latitudinal geographic distance where a population location's latitude was putatively found to be the "center" of the hybrid zone (population UTR9).

Population distance to the center of the hybrid zone was found by utilizing each longitude value and measuring to the center on a projected latitude line using ArcMap v.10.7.1 (Table B1; ESRI, Redlands, CA). Cline models were estimated with the R package HZAR (Derryberry *et al.*, 2014); grayscale measurements for each populations' petal color and trichome density along with allelic frequency at

each locus were assessed. A null model, which tests the hypothesis that there is no cline in the sampled region, along with 15 alternative cline models were analyzed for each locus. Each model was run on a separate seed and were run with a MCMC length set to 100,000 with a burn-in of 10,000. All cline models were checked for convergence and the best-fit model (lowest AIC score) was selected to extract summary statistics and the maximum-likelihood clines for each variant. Cline center and width were then estimated by averaging the statistics from the significant outlier loci cline models.

Linkage Disequilibrium

The nuclear genome undergoes recombination during meiosis which allows for linkage disequilibrium estimates to identify populations that have been subjected to recent introgression. The 50 loci that were found to have significant geographic cline models were selected to be analyzed, as these were loci most differentiated across the study region and would likely show traces of LD in hybrid populations. The 50 loci were gathered from the raw VCF file output from GBS-SNP-CROP and were converted to an Arlequin input file (PGDSpider v.1.1.5; Lischer & Excoffier, 2012) where the data were organized at the population level. Linkage disequilibrium analyses were then conducted in Arlequin v.3.5 (Excoffier and Lischer, 2010). The loci were treated as genotypic

data with the gametic phase known. 10,000 steps in Markov Chain were run along with 100,000 dememorization steps between pairwise loci. Total counts of pairwise loci in each population that were found to be in significant linkage disequilibrium were summarized to be compared across geographic space.

Results

Morphological Variation

Strong differences were found between the two *Ranunculus* species and the putative hybrid populations for two phenotypic traits: ventral petal color, as anticipated by field observations, and leaf trichome density, a variable trait first discovered and described by this research. Ventral petals of *R. austro-oreganus* were much darker compared to *R. occidentalis*, and hybrids were intermediate ($F_{2/25} = 61.36$, $P < 0.0001$; Fig. 2), as expected due to the use of the trait to define species and hybrids. Leaves of *R. austro-oreganus* were much brighter (denoting more leaf trichomes) than *R. occidentalis*, and the hybrids were intermediate between the two species ($F_{2/25} = 5.03$, $P = 0.0146$; Fig. 2). There were significant differences among sampling locations for petal gray values ($F_{25/501} = 14.79$, $P < 0.001$) and leaf hair gray values ($F_{25/28} = 21.52$, $P < 0.001$). The difference for gray value between basal and cauline leaves was not significant ($F_{28/1005} = 0.78$, $P = 0.793$ for leaf position nested within species*location tested over the error).

The variance in ventral petal color corresponds with the location of the putative hybrid zone (compare Figs. 1 and 3), but there is also evidence of highly variable measurements in populations of *R. austro-oreganus* at the southeastern edge of its range (Agate Lake; Fig. 3). Overall, there was a strong

correspondence between the mean ventral petal color and the variance in color (based on gray scale) which was anticipated by the field observations that led to each population's species classification. Populations having intermediate coloration display the highest variance in color (Fig. 4; quadratic model adjusted $R^2 = 0.66$, $F_{2/27} = 29.2$, $P < 0.0001$).

Nuclear DNA

After filtering, the GBS-SNP-CROP pipeline identified 2196 variants among 377 individuals in comparison to the consensus mock reference. The largest peak in the Delta K value from the Structure analysis indicates K=2 as the optimum number of genetic clusters; a second peak in the Delta K value is present at K=11 (Fig. B1). The subpopulation structure of K=2 does not show distinct differences across the geographic space (Fig. 5), although a trend of increasing admixture is apparent in eastern populations. Western populations, largely composed of *R. occidentalis*, display strong population structure. Similarly to the structure analysis, the PCA suggests that there is a separation into two groupings (Fig. 6), although there is separation occurring within populations. PC 1 explains 2.51% of the genetic variation and PC 2 explains 2.24%. There was a steep decline in eigenvalues for the remainder of the principal components.

The four populations used to identify outlier loci revealed 56 loci of interest for cline analyses (Table B2). An additional seven loci, two of which overlapped with the allopatric SNP subset, were additionally identified to be outlier loci of interest due to moderate allelic frequency differences found between all *R. austro-oreganus* and *R. occidentalis* populations (Table B2). A total of 61 variants were classified as outlier loci and used for cline analyses.

Geographic clines estimated for morphological traits and nuclear loci displayed varying results. Petal color and trichome density showed highly differentiated clines, where petal color shows a steep cline near the proposed center of the hybrid zone and trichome density shows a gradual change over geographic space (Fig 7). Of the 61 outlier loci examined, 50 loci were found to have significant cline models (Fig. B2) with variable cline center and width estimates (Table B2). The average cline center was estimated to be 2710 meters East from the proposed center (Fig. 8) and the average cline width was estimated to be 19696 meters (+/- 9848 meters from the center; Fig. 9; Table B2). The estimated hybrid zone spans the entirety of the morphological sampling extent (Fig. 10). Total counts of significant pairwise loci found to be in linkage disequilibrium showed a general trend of increasing across the putative hybrid zone geographic space (Table B1; Fig. 11). to be compared across geographic space.

Discussion

In this study, a combination of morphological characters and DNA analyses were used to characterize variation across a contact zone between two buttercups, *R. austro-oreganus* and *R. occidentalis*. Image analyses of petal coloration confirmed that there are intermediate phenotypes in suspected hybrid populations, a common occurrence in hybrid zones (Kálmán *et al.*, 2004). Leaf trichome density analyses were additionally discovered to support the intermediate population measurements and classifications. Populations were identified based on ventral petal coloration during field collections and the image analysis confirms those groupings. However, populations classified based on the prevalent phenotypes display evidence of extensive introgression of nuclear loci based upon GBS analyses. Additionally, intermediate trichome density measurements support the differentiation in species classifications as we did not categorize species based on this trait; trichome density of the suspected hybrid populations is significantly different from both *R. occidentalis* and *R. austro-oreganus*. The geographic clines resulting from the morphological analyses indicate considerable evidence that both traits vary across this hybrid zone. The cline derived from ventral petal color data displays a very steep cline shape suggesting that selection is strongly acting upon this trait. Floral traits under selection have been found to be associated with ecotypic variation (Stankowski

et al., 2017) and this possibility must be considered for this system. On the other hand, trichome density has a much more gradual cline shape suggesting weaker selection, although leaf traits are also known to be involved in ecotypic variability (Kay *et al.*, 2018).

The morphological characters observed in the study are both known for their adaptive importance. It was hypothesized that the darker ventral petal coloration may be derived from anthocyanins, which are known for ultraviolet radiation protection (Mori *et al.*, 2005). Additionally, petal variation may be driving pollinator preferences (Meléndez-Ackerman, 1997; Pansarin and Amaral, 2008; Anderson *et al.*, 2009) which could influence selection pressures. The increase in trichome density in *R. austro-oreganus* also has the potential to contribute against ultraviolet radiation (Karabourniotis *et al.*, 1992; Skaltsa *et al.*, 1994). An increase in trichome density can additionally increase the leaf boundary layer, thus decreasing leaf temperature and diffusion of both water vapor and carbon dioxide (Ehleringer and Mooney, 1978). A reduction in transpiration rate can lead to higher leaf temperatures because the leaves are less able to cool themselves (Ripley *et al.*, 1999), although abundant trichomes may aid in drought stressed plants by absorbing water and decreasing adverse drought related effects (Grammatikopoulos and Manetas, 1994). *R. austro-oreganus* may be considered more fit for a changing climate because it contains both adaptive traits (darker

petal coloration and higher trichome density) which may be due to a historical range where these traits were favored (i.e. higher elevation, higher UV radiation exposure, less rainfall, etc.).

We identified a contact zone between two *Ranunculus* species, located in Medford, Oregon. The contact zone is composed of two genetic clusters where there is a substantial level of shared variation among the 2196 nuclear SNPs identified. These results fail to provide a clear separation of genetic clusters between these two species, signifying extensive admixture. This study system exemplifies far more admixture and less population structure than other hybridization studies conducted in a similar spatial extent (e.g. Zamudio and Wieczorek, 2007; Sung *et al.*, 2018). There is a second peak in Delta K values suggesting that substructure exists within the two genetic clusters and may be present in response to ecotypic differences (Andrew and Rieseberg, 2013). The second peak in Delta K values should be strongly considered given the known tendency for structure analyses to suggest $K=2$ (Janes *et al.*, 2017). The principal components analysis displayed similar results to the structure analysis, where the first two principal components described the most genetic variations at approximately 4.75%. Western populations, largely composed of *R. occidentalis*, appear to contain less genetic variation amongst individuals sequenced.

Cline models derived from 50 nuclear SNPs display varying steepness, where steeper clines suggest selection acting upon those genomic regions. Estimation of the cline center confirms field observations where the center occurs east of Upper Table Rock and the width of the hybrid zone spans the entirety of the longitudinal sampling area used for the morphological analyses. The longitudinal sampling scheme implemented in this study may be misleading to the estimates of the hybrid zone. It is possible that the geographic distance parameter could be fine-tuned to better represent species distributions to better disentangle population structure that may exist. Changes to the distance parameter could change both the center and width estimates, but the inference of ongoing gene flow would not change. Structure analyses, hybrid zone extent estimates, and linkage disequilibrium counts all suggest that *R. austro-oreganus* populations are experiencing extensive gene flow with *R. occidentalis*. This could have long-term implications for the range-limited species, as its range may become furtherly limited. Although the fate of the range-limited species is dim, it's possible that the adaptive traits that *R. austro-oreganus* exhibit will provide hybrid vigor to populations in this contact zone (Anderson and Stebbins, 1954).

Conclusions

In summary, evidence of a hybrid zone has been found between the widespread buttercup species, *R. occidentalis*, and the range-limited *R. austro-oreganus*. The data reported in this study are virtually the only information available on the biology of *R. austro-oreganus*, which is being considered for threatened species listing (Kagan *et al.*, 2016). The phenotypic patterns provide support for the presence of intermediate populations, where selection appears to be acting upon ventral petal coloration. Furthermore, geographic clines display evidence of a hybrid zone spanning 20km across Jackson County, Oregon, where selection appears to be acting upon phenotypic traits along with a number of genomic loci as indicated by steep clines. Linkage disequilibrium analyses indicate that the hybrid zone is moving east towards the range-limited species. Genetic analyses suggest extensive contemporary introgression throughout the contact zone. Depending on the historical relationship between these species, the movement of the hybrid zone could suggest: 1. that the range-limited species will continue to experience genetic swamping by its widespread congener, or 2. the range-limited species is expanding its range into that of its widespread congener. Further analyses are required to fully understand the dynamics of this contact zone.

Results of future work could be improved by incorporating genomic information and abiotic factors in an ecological niche modeling approach to evaluate intra-specific response and local adaptation to environmental factors (e.g. Swenson, 2006; Barnard-Kubow *et al.*, 2015; Fitzpatrick and Keller, 2015). Abiotic factors such as soil composition, annual rainfall, elevation, and local temperatures may be influencing the ranges that these populations are well adapted to and may be maintaining phenotypic differentiation. Given that these species apparently may possess distinct, separate adaptations to the same environment, they may also have varying responses to climate change, anthropogenic stressors, and hybridization event outcomes. Future work expanding on greenhouse crosses between the species and experimental common garden studies have great potential to contribute to our understanding of the abiotic variables promoting growth in each species. Additionally, future studies could focus on investigating the potential association between genomic and phenotypic data to better understand traits potentially important in reproductive isolation and/or adaptive introgression (e.g. Sung *et al.*, 2018). The *Ranunculus* species complex presented here should be considered a strong candidate system for better understanding contact zones that could have important implications for directing conservation efforts, especially in the case of range-limited species.

Figures

Figure 1. Sampling locations for the *Ranunculus occidentalis* (yellow), *R. austro-oreganus* (orange), and their putative hybrids (green) in Jackson County, Oregon. The inset shows the geographic range of *R. occidentalis* in green and of *R. austro-oreganus* (small blue circle). Upper Table Rock (UTR) is labeled for reference.

Figure 2. Morphological differentiation measured in the average gray values for petal color (A) and trichome density (B) in *Ranunculus austro-oreganus*, *R. occidentalis*, and their putative hybrids. Putative hybrids were found to be significantly intermediate between *R. austro-oreganus* and *R. occidentalis* populations for both ventral petal coloration and trichome density.

Figure 3. The geographic distribution of populations of *Ranunculus occidentalis* (yellow markers), *R. austro-oreganus* (red markers), and their hybrids (orange markers) in Jackson County, Oregon showing the mean (marker color based on gray value) and the variance (marker size) in ventral petal color.

Figure 4. The relationship between the mean and the variance in ventral petal color for populations of *Ranunculus occidentalis* (yellow markers), *R. austrooreganus* (orange markers), and hybrids (green markers) in Jackson County, Oregon.

Figure 5. The K=2 minor cluster plot where sampled populations are organized across, west to east, the longitudinal geographic space of the putative hybrid zone.

Figure 6. PCA generated with the R package adegenet and using the 2196 loci from GBS-SNP-CROP. Each dot represents a sequenced within a population and population identification is denoted by the color of the dot and ellipse surrounding the total population. PC 1 (x-axis) and PC 2 (y-axis) are displayed, as suggested by the corresponding eigenvalues.

Figure 7. Morphological geographic clines across the putative hybrid zone, longitudinally. These clines are based on morphological differences quantified in grayscale where (A) displays ventral petal color and (B) displays trichome density.

Figure 8. Histogram of estimated center of hybrid zone where the blue line indicates the average based upon significant cline models.

Figure 9. Histogram of estimated width of hybrid zone where the blue vertical line indicates the average width value divided by two ($\pm$ the center). The x axes in the upper panels have been truncated for better visualization (Table B2).

Figure 10. Populations whose nuclear DNA were analyzed. Hybrid zone center estimate (pink line) and width (green box) based on cline analyses is displayed.

Figure 11. Number of loci pairs that were found to be in linkage disequilibrium in each population (black dots) across the putative hybrid zone.

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Appendix A. Haplotype Identification

Samples from 14 *R. austro-oreganus*, 14 *R. occidentalis*, and 11 putative hybrid populations were collected throughout Jackson County, Oregon. Libraries, primarily with 20 individuals per population, were created using KAPA HyperPlus Library Preparation Kit. Samples were suspended in 10mM Tris-HCl and kept at -20°C until capture preparation, where a MyBaits capture array (Arbor Biosciences) to target specific regions that are conserved amongst ~20 full chloroplast genomes as described in Kohn *et al.* (2017). The samples were transported on ice to Oregon Health and Science University (Portland, Oregon) where Illumina sequencing was used to obtain cpDNA for 46 Single Sample Libraries (SSLs) and 51 Pooled Population Libraries (PLs; Table A1).

Reference chloroplast genomes were constructed and annotated for both *R. austro-oreganus* (NCBI accession no. KX639503) and *R. occidentalis* (NCBI accession no. KX557270). Shotgun sequenced reads for both species were used to construct complete chloroplast genomes and adapters were trimmed using cutadapt v.1.1 (Martin, 2011) and trimmed for quality using Trimmomatic v.0.39 (Bolger *et al.*, 2014). *Ranunculus macranthas* (NCBI accession no. NC_008796) was used for reference guided assembly, where bwa mem (Li and Durbin, 2009) was used to align the raw shotgun sequenced reads to the reference. The final *R. occidentalis* genome was used as the reference for the *R. austro-oreganus*

genome assembly. Once the raw reads were mapped the reference, the reads were then filtered using SAMtools (Li *et al.*, 2009). The sorted bam file was then converted to a fastq file using Picard Toolkit (2019). SPAdes (Bankevich *et al.*, 2012) was then used to scaffold raw reads to for manual assembly. Error correction on the final circularized genome consisted of indexing the trimmed and filtered reads in order to re-align to the draft genome by utilizing bwa mem once again. Various SAMtools tools were then used to view the raw reads in comparison to the consensus read, and any discrepancies were fixed. DOGMA (Wyman *et al.*, 2004) was then used to annotate the two finalized genomes and Sequin v.16.0 (National Center for Biotechnology Information, 2019) allowed for GenBank submission ready products.

Sequence reads were aligned to the *R. occidentalis* reference genome following the methods found in Kohn *et al.* (2017). Genomic variants were identified within the chloroplast genome. The SNPs were filtered for quality and support. A total of 31 haplotypes were discovered across the putative hybrid zone (Fig. A1) with extensive haplotype sharing between the species (Fig. A2).

Figure A1. Haplotype frequencies belonging to each of the 51 populations across the putative hybrid zone. Pie charts are outlined in the corresponding species classification (*Ranunculus occidentalis*, yellow; *R. austro-oreganus*, orange; Putative Hybrids, green).

Figure A2. Haplotype network based on genomic cpDNA SNPs collected across 51 populations (1,009 pooled individuals) in Jackson County, Oregon. The 31 unique haplotypes are each represented by a pie chart, which displays the total number of individuals identified as the haplotype and the fraction of individuals classified as each species (*Ranunculus occidentalis*, yellow; *R. austro-oreganus*, orange; Putative Hybrids, green). Haplotypes are separated by nodes with hash marks depicting the number of SNPs separating the nearest neighboring haplotype(s).

Table A1. Pooled populations (PLs) included in chloroplast sequencing where ~20 individuals were pooled per population. The number of single sample libraries (SSLs) processed from each population is listed in the last column.

Location Name	Latitude	Longitude	Species	No. Individuals in Pool	No. SSLs
AgateLake1	42.42131	-122.77464	Ranaus	20	1
AgateLake2	42.41217	-122.77715	Ranaus	20	2
Brownsboro1	42.46189	-122.74269	Ranaus	20	1
Brownsboro2	42.46016	-122.73991	Ranaus	20	1
ButteFallsBLM	42.49589	-122.69261	Ranocc	20	1
ChulaRock	42.47684	-122.83836	Ranaus	20	1
Dawgs	42.46175	-122.94286	Hybrid	20	2
Denman	42.46143	-122.88158	Ranaus	20	1
EaglePoint	42.47236	-122.79391	Ranaus	20	1
EmigrantLake	42.14664	-122.62058	Ranaus	20	1
Emu	42.45259	-123.00207	Ranocc	20	2
GlassLane	42.50557	-122.89159	Ranocc	20	1
GoldHill	42.4357	-123.05159	Ranocc	20	1
GrizzlyPeak	42.2375	-122.57323	Ranocc	20	0
Imperatrice1	42.23313	-122.70831	Ranaus	20	1
Imperatrice2	42.23402	-122.69521	Ranaus	20	1
JacksonvilleForkTrail	42.31339	-122.98202	Ranocc	20	0
JulietAve	42.48932	-122.87351	Hybrid	19	1
LaurelGlenCreek	40.55361	-122.45389	Ranocc	20	1
LowerRogueMountain	42.41634	-123.1547	Ranocc	20	0
LowerRogueSwitchback	42.41631	-123.15229	Ranocc	20	0
LTR1	42.46822	-122.94569	Ranocc	20	1
LTR2	42.45583	-122.952	Ranocc	0	1
LTR3	42.46069	-122.94842	Ranocc	20	1
LTR4	42.45164	-122.95132	Ranocc	20	1
NoRiverAccess	42.44974	-123.00276	Ranocc	20	1
OldStageRd1	42.39008	-122.98995	Ranocc	20	1
OldStageRd2	42.42134	-123.04127	Ranocc	20	1
PerryRd	42.51147	-122.9353	Ranocc	20	1
PhoenixWaterTower	42.2793	-122.80553	Ranocc	20	0
RogueSide	42.4518	-123.12539	Ranocc	20	0
RoxyAnn1	42.34795	-122.78654	Ranaus	11	2
RoxyAnn2	42.35124	-122.79394	Ranaus	20	1
SamsValleyPrivate	42.49821	-122.97446	Ranocc	19	0
SkyKing	42.04928	-122.59246	Ranaus	20	1
SurreyRoadNorth	42.2866	-122.88306	Ranocc	20	0
SurreyRoadSouth	42.28329	-122.88406	Ranocc	19	0
Touvelle	42.44286	-122.88145	Ranaus	20	1
UpperRiverRoad1	42.45661	-123.02602	Ranocc	20	1
UpperRiverRoad2	42.45409	-123.00842	Ranocc	20	1
UTR1	42.46838	-122.88453	Hybrid	20	0
UTR2	42.4707	-122.89983	Hybrid	11	1
UTR4	42.46797	-122.89055	Hybrid	20	1
UTR6	42.46758	-122.88822	Hybrid	20	1
UTR7	42.46665	-122.88222	Hybrid	20	1
UTR8	42.47507	-122.91609	Hybrid	20	1
UTR9	42.47011	-122.91144	Hybrid	20	1
UTR-Radar	42.47706	-122.91624	Hybrid	20	1
UTR-West	42.46593	-122.91493	Hybrid	20	1
Whetstone	42.41838	-122.90896	Ranaus	20	1
Whetstone2	42.41227	-122.91065	Ranaus	20	1
WhiskeyShastatownTrinity	40.64819	-122.57411	Ranocc	20	1

Appendix B. Nuclear DNA

Figure B1. Genetic clustering approximated with Structure. A-D displays output statistics. Peaks in Delta K value (A) indicate optimum values of K.

Figure B2. The 50 significant cline models from HZAR. Allelic frequency (0-1) is measured on the y-axis and estimated for each population across geographic distance (meters; x-axis).

Table B1. Sampling locations and number of individuals sequenced for GBS analyses, ordered from East (negative values) to West (positive values) relative to the proposed center of the contact zone (UTR9; PopID 33). Number of significant loci pairs detected in each population in the linkage disequilibrium analysis are reported.

PopID	Location Name	Latitude	Longitude	Species	Distance (m)	No. Individuals Sampled	No. Sig. Loci Pairs
41	LowerRogueMountain	42.41634	-123.15470	Ranocc	-19952.34	7	2
42	LowerRogueSwitchback	42.41631	-123.15229	Ranocc	-19754.67	8	2
43	RogueSide	42.45180	-123.12539	Ranocc	-17548.32	8	40
17	GoldHill	42.43570	-123.05159	Ranocc	-11495.20	8	6
11	OldStageRd2	42.42134	-123.04127	Ranocc	-10648.74	8	21
37	UpperRiverRd1	42.45661	-123.02602	Ranocc	-9397.93	8	28
38	UpperRiverRoad2	42.45409	-123.00842	Ranocc	-7954.36	8	12
36	NoRiverAccess	42.44974	-123.00276	Ranocc	-7490.13	8	62
7	OldStageRd1	42.39008	-122.98995	Ranocc	-6439.44	8	30
40	JacksonvilleForkTrail	42.31339	-122.98202	Ranocc	-5789.02	8	6
45	BrittCanyonTrail	42.31303	-122.97759	Ranocc	-5425.67	8	8
32	SamsValleyPrivate	42.49821	-122.97446	Ranocc	-5168.94	8	25
28	LTR4	42.45164	-122.95132	Ranocc	-3270.98	8	26
39	LTR3	42.46069	-122.94842	Ranocc	-3033.12	8	25
27	LTR1	42.46822	-122.94569	Ranocc	-2809.21	8	22
13	Dawgs	42.46143	-122.88158	Hybrid	-2577.09	8	46
25	PerryRoad	42.51147	-122.93530	Ranocc	-1957.01	8	33
19	UTR-Radar	42.47706	-122.91624	Hybrid	-393.70	8	8
34	UTR8	42.47507	-122.91609	Hybrid	-381.40	8	18
22	UTR-West	42.46593	-122.91493	Hybrid	-286.25	8	10
33	UTR9	42.47011	-122.91144	Hybrid	0.00	8	31
10	Whetstone2	42.41227	-122.91065	Ranaus	64.80	8	34
12	Whetstone1	42.41838	-122.90896	Ranaus	203.41	7	20
9	UTR2	42.47070	-122.89983	Hybrid	952.26	8	13
14	GlassLane	42.50557	-122.89159	Ranocc	1628.11	8	9
3	UTR4	42.46797	-122.89055	Hybrid	1713.41	8	27
30	UTR6	42.46758	-122.88822	Hybrid	1904.52	8	15
46	SurreyRoadSouth	42.28329	-122.88406	Ranocc	2245.73	7	34
44	SurreyRoadNorth	42.28660	-122.88306	Ranocc	2327.75	8	26
31	UTR7	42.46665	-122.88222	Hybrid	2396.64	8	26
2	Denman1	42.46175	-122.94286	Ranaus	2449.14	8	12
21	TouvilleStatePark	42.44286	-122.88145	Ranaus	2459.80	8	41
29	JulietAve	42.48932	-122.87351	Hybrid	3111.04	8	48
8	ChulaRockRd	42.47684	-122.83836	Ranaus	5994.07	8	27
47	PheonixWaterTower	42.27930	-122.80553	Ranocc	8686.81	8	18
24	RoxyAnn2	42.35124	-122.79394	Ranaus	9637.43	8	19
15	EaglePointHill	42.47236	-122.79391	Ranaus	9639.89	8	14
1	RoxyAnn1	42.34795	-122.78654	Ranaus	10244.38	8	25
35	AgateLake2	42.41217	-122.77715	Ranaus	11014.56	8	16
20	AgateLake1	42.42131	-122.77464	Ranaus	11220.43	8	40
4	Brownsboro1	42.46189	-122.74269	Ranaus	13840.99	8	63
5	Brownsboro2	42.46016	-122.73991	Ranaus	14069.00	8	48
18	Imperatrice1	42.23313	-122.70831	Ranaus	16660.86	8	42
23	Imperatrice2	42.23402	-122.69521	Ranaus	17735.33	7	32
48	ButteFalls	42.49589	-122.69261	Ranocc	17948.58	8	34
6	EmigrantLake	42.14664	-122.62058	Ranaus	23856.52	8	19
16	SkyKingCole	42.04928	-122.59246	Ranaus	26162.94	8	6
26	GrizzlyPeak	42.23750	-122.57323	Ranocc	27740.20	8	26

Table B2. Outlier loci listed in ascending order by locus ID that were identified by moderate allelic frequency differences (>0.3) and their corresponding cline center relative to the proposed center and cline width estimates from HZAR.

Locus ID	Cline Center (m)	Cline Width (m)
63509	-8531	3758
81577	-3284	84
81578	-3272	140
100508	-3305	165
100509	-3262	82
141124	-14686	41672
186072	-10428	36486
186081	1661	11566
206747	-3623	2001
226590	768	19576
237266	14951	74044
279873	1702	6356
283807	9263	174
283832	9493	163
319835	29876	100197
338172	2464	3527
367960	-5218	10224
508514	2268	11602
541591	-6679	4123
695100	28338	77793
801503	-20143	83682
827561	1235	7478
853875	-1134	4062
877641	3698	5780
1050556	-3286	14206
1055099	16	109
1063741	-9396	4666
1077197	-503	12381
1126411	554	1805
1202109	27387	96144
1208774	-4840	10340
1274729	54796	139669
1297605	6706	9059
1309002	-3851	11371
1323135	-646	256
2015129	3451	7502
2660025	-895	137
2660108	-379	110
3635428	-527	212
3706228	-350	93
3708575	4573	15733
3790767	-6361	9246
4657214	20153	121159
5209179	-816	773
6660961	11827	1488
8289457	1210	5062
10861261	3647	9778
13294110	9194	151
16494400	-542	244
24394488	2215	8388