POPULATION GENETIC STUDIES OF SPECIATION IN THE PLANT GENUS *STEPHANOMERIA* (ASTERACEA)

by

NATASHA ANN SHERMAN

(Under the Direction of JOHN M. BURKE)

ABSTRACT

The chapters of this dissertation are designed to investigate speciation in the plant genus *Stephanomeria*. Genetic markers were developed, and population genetic studies performed. In the first study, the goal was to investigate: the putative homoploid hybrid origin of *Stephanomeria diegensis*; whether this species arose once or on multiple occasions; and which subspecies of the parental taxa were involved in the original speciation event. The data presented support a hybrid origin of *S. diegensis*. In addition, the results showed the *S. diegensis* most likely arose once, and that *S. exigua* ssp. *exigua* or *S. exigua* ssp. *deanei* were involved in the formation. It was not possible to identify the subspecies of *S. virgata* involved. The second study investigates the allopolyploid origin of *S. elata*. The number of origins was investigated, as was the level of differentiation between two recognizable morphotypes, termed the Northern and Southern morphotype. This work identified three origins, with two origins found in the Southern morphotype and one in the Northern morphotype. The third study analyzed population genetic diversity in a disjunct population of *S. exigua* ssp. *coronaria* termed the Frenchglen population. This population is of particular interest because it is a peripheral isolate that gave rise to the

endangered *S. malheurensis*. The Frenchglen population was found to be genetically robust, and unique from populations from the balance of the range of *S. exigua* ssp. *coronaria*.

INDEX WORDS: speciation, hybridization, population genetic, homoploid, allopolyploid, peripatric, peripheral isolate

Population Genetic Studies of Speciation in the Plant Genus *Stephanomeria* (Asteraceae)

by

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DEDICATION

This work is dedicated to my family, for all their love, support, and understanding.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Under the Biological Species Concept, speciation is the development of reproductive isolating mechanisms between lineages that share a common ancestor (Mayr 1942). The three basic forms of speciation are allopatric, peripatric, and sympatric. The most common of these is allopatric speciation, in which a geographic barrier of some sort prevents intermating amongst individuals that were formerly in repoductive contact. When this happens, the different populations will accumulate differences that may ultimately lead to reproductive isolation. Peripatric speciation is similar, but involves a peripherally isolated population that diverges to the point of achieving reproductive isolation. In contrast, sympatric speciation involves evolutionary divergence with the neospecies remain in close geographic proximity to one another. Sympatric speciation is thus typically driven by strong selection, karyotypic evolution, changes in ploidy level, or a shift in mating system (Kempf et al. 2009; Owenby 1950; Fukamachi et al. 2009).

Because the path to complete reproductive isolation is lengthy, diverging populations may experience secondary reproductive contact and hybridize before complete isolation has been achieved. There are a number of possible evolutionary outcomes when incompletely isolated taxa come back into reproductive contact. For example, these taxa might merge into a single, highly variable population (e.g., Grant

1963), one taxon might drive the other to extinction via genetic assimilation (e.g., Wolf *et al*. 2001), or secondary contact might result in the reinforcement of prezygotic reproductive barriers (reviewed in Howard 1993; Servedio & Noor 2003). Alternatively, hybridization can have creative outcomes, such as adaptive trait introgression (reviewed in Rieseberg and Wendel 1993; see also Bailey *et al.* 2009; Currat *et al.* 2008; Gagnaire *et al.* 2009, Kawakimi *et al.* 2009; Kim *et al.* 2008; Lepis *et al.* 2009; Wood & Nakazato 2009; Arnold 2004), or even hybrid speciation (reviewed in Rieseberg 1997).

Hybrid speciation can occur via one of two main pathways: homoploid hybrid speciation and allopolyploidy. During homoploid hybrid speciation, the chromosome number of the hybrid and its parents are the same, and other forces (such as the segregation of sterility factors or genes underlying ecologically-important traits) must be invoked to explain the existence of reproductive isolation (reviewed in Rieseberg 1997; Gross and Rieseberg 2005). Alternatively, allopolyploid species contain a full chromosome complement from each parent, thereby resulting in reproductive isolation from its parental taxa due to a difference in ploidy level (reviewed in Ramsey & Schemske 1998; Otto & Whitton 2000; Soltis & Soltis 2009). While hybridization is widespread in plants (Mallet 2005), the conditions under which homoploid hybrid speciation can occur are stringent (McCarthy et al. 1995; Buerkle et al. 2000, 2003). This mode of speciation has thus generally been regarded as rare. Nonetheless, there are a number of well supported examples of homoploid hybrid speciation (Arnold 1997; Rieseberg 1997; Gross and Rieseberg 2005). In this dissertation, I describe several population genetic investigations of hybridization and speciation in the flowering plant genus *Stephanomeria*.

The genus *Stephanomeria*, one of ca. 1100 genera within the sunflower family (Compositae or Asteraceae; Jeffrey 1993), is composed of six annual and ten perennial species. These species are predominantly self-incompatible, and have a base chromosome number of $n = 8$ (Gottlieb 1969). These species have a relatively broad distribution in the western United States. For example, the range of *S. virgata* extends from central Mexico through southern Oregon, USA. Another annual species, *S. exigua*, has an even broader distribution, spanning central Mexico through southern Oregon, and reaching as far inland as Utah.

Stephanomeria virgata is comprised of two subspecies, *S. virgata* ssp. *pleurocarpa*, which occurs through out the range of this species, and *S. virgata* ssp. *virgata* which is more limited in distribution, only occurring in northern Mexico and southern California, USA. In contrast, *S. exigua* is composed of five subspecies. The most common of these is *S. exigua* ssp. *coronaria*, whose distribution spans northern Mexico through Southern Oregon, and from the Channel Islands east through Utah. Throughout it's range, *S. exigua* ssp. *coronaria* exhibits substantial phenotypic variation (Gottlieb 2006). Along the northern margin of this subspecies, there is a single disjunct population (known as the "Frenchglen" population) that is isolated from the balance of the species range by ca. 150 km. This population occurs in a desert near Burns, Oregon (Gottlieb 1973), and is sympatric with the only known population of the endangered endemic *S. malheurensis*, which is likewise an annual species.

Since the late 1960s, *S. diegensis* has been viewed a putative homoploid hybrid species, having most likely arisen as a result of interbreeding between *S. exigua* and *S. virgata*. While these putative parental species are separated by strong reproductive

barriers, F_1 hybrids between them exhibit ca. 10% pollen fertility (Gottlieb 1969, 1971) and they are known to hybridize in regions of overlap. The possibility of a hybrid origin of *S. diegensis* was first hypothesized because this species combines several morphological characters that distinguish *S. exigua* and *S. virgata*, though it also exhibits some transgressive (i.e., extreme) characters, such as the number of florets per inflorescence and length of lateral pinnae along the pappus bristles. The hypothesis of a hybrid origin was later supported by allozyme data (Gallez and Gottlieb 1982), though clear genetic evidence of hybridity has been lacking. In terms of geographic distribution, the range of *S. diegensis* falls entirely within the region of overlap between *S. exigua* and *S. virgata* in southern California, USA. Crossability with both of its putative parents is low, and pollen fertility in F_1 hybrids with its putative parents indicates that *S. diegensis* is the most strongly reproductively isolated of all annual *Stephanomeria* species (Gottlieb 1969). Interestingly, this pattern of increased reproductive isolation in hybrid species has been documented in other study systems (e.g., Rieseberg 2000; Lai et al. 2005).

Unfortunately, homoploid hybrid species are difficult to unambiguously identify, and only a handful of cases have been substantiated utilizing molecular approaches. In some cases, these hybrid species have very restricted ranges (e.g., Arnold 1993), whereas in others they have achieved a more widespread distribution, either through a single initial hybridization event followed by range expansion or via multiple origins (e.g., Welch & Rieseberg 2002; Schwarzbach & Rieseberg 2002; James & Abbott 2005). As noted above, Gottlieb's (1969, 1971) initial conclusions regarding the hybrid origin of *S. diegensis* were later supported by allozyme data (Gallez & Gottlieb 1982), though this work was far from conclusive because the majority of loci surveyed were uninformative.

Moreover, Gallez & Gottlieb (1982) relied on limited geographic sampling and only included three of the five *S. exigua* subspecies in their initial study. In the first chapter of this dissertation, I test the hypothesis of a homoploid hybrid origin of *S. diegensis*, investigate the possibility of multiple origins, and seek to identify the most likely parental subspecies.

Within the annual taxa, there is also a single, self-compatible tetraploid species known as *S. elata* (*n* =16; Gottlieb 1969). *Stephanomeria elata* exhibits regular chromosome pairing and disjunction, with no multivalents or other observable meiotic abnormalities (Gottlieb 1969). The apparently disomic inheritance of individuals within this species is thus most consistent with an allopolyploid origin. Based on overall morphological similarity as well as species ranges, Gottlieb (1969) suggesting that the most likely parents of *S. elata* are *S. exigua* and *S. virgata*. Interestingly, there are two identifiable morphotypes of *S. elata* (Northern and Southern), which could reflect either differentiation following polyploidization or multiple independent origins of *S. elata* (Gottlieb 1972). Historically, it was presumed that polyploids would be genetically depauperate due to the rare event of genome doubling after the hybridization events (Soltis & Soltis 2009). It has, however, since been shown that multiple events of polyplodization, followed by crosses amongst the descendants of these separate origins, might result in a genetically robust new species with high evolutionary potential (Soltis & Soltis 2009; Stebbins 1950; Levin 1983; Osborn et al. 2003). In the second chapter of this dissertation, I investigate the possibility of multiple origins of *S. elata*, and also characterize population-level differentiation within and among the Northern and Southern

morphotypes. I also report on the genome size of individuals from multiple populations of both morphotypes of *S. elata* as well as its presumptive parental species.

Finally, although *Stephanomeria* has a wide overall distribution, there is still interest in preserving peripherally-isolated populations of individual species, as these populations might prove to be genetically distinct. The protection of such populations thus has the potential to preserve novel variation that could facilitate future adaptation, especially in the face of a changing environment. The third chapter of this dissertation thus focuses on the population genetics of the Frenchglen population of *S. exigua* ssp. *coronaria* as well as the recently-derived, sympatrically-occuring *S. malheurensis*. This work is placed in the context of diversity across populations/subspecies throughout the range of *S. exigua*, thereby providing unique insights into the conservation value of the Frenchglen population as well as *S. malheurensis*.

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CHAPTER 2

POPULATION GENETIC ANALYSIS REVEALS A HOMOPLOID HYBRID ORIGIN OF *STEPHANOMERIA DIEGENSIS* (ASTERACEAE)[1]

[1] SHERMAN, N.A. and J.M. BURKE. Population Genetic Analysis Reveals a Homoploid Hybrid Origin of *Stephanomeria diegensis* (Asteraceae). *Molecular Ecology*.2009. 97 (4):403-408. Reprinted here with permission of Wiley Blackwell.

Abstract

Homoploid hybrid speciation has generally been viewed as a rare evolutionary phenomenon, with relatively few well-documented cases in nature. Here, we investigate the origin of *Stephanomeria diegensis*, a diploid flowering plant species that has been proposed to have arisen as a result of hybridization between *S. exigua* and *S. virgata*. Across the range of *S. diegensis*, all individuals share a common chloroplast haplotype with *S. virgata* while showing a greater affinity for *S. exigua* in terms of nuclear genetic diversity. A principal coordinates analysis (PCO) based on the nuclear data revealed that *S. diegensis* is most similar to each parent along different axes. Moreover, a Bayesian clustering analysis as well as a hybrid index-based analysis showed evidence of mixed ancestry, with approximately two thirds of the *S. diegensis* nuclear genome derived from *S. exigua*. These results provide strong support for a homoploid hybrid origin of *S. diegensis*. Finally, contrary to the finding that homoploid hybrid species are typically multiply-derived, our results were most consistent with a single origin of this species.

Introduction

Under the Biological Species Concept, speciation is defined as the development of reproductive isolating mechanisms between lineages that share a common ancestor (Mayr 1942). Because the path to complete cross-incompatibility is often lengthy, lineages that have begun to diverge may experience secondary reproductive contact. There are a number of possible evolutionary outcomes when incompletely isolated taxa come back

into reproductive contact. For example, the taxa might merge into a single, highly variable population (e.g., Grant 1963), or one taxon might drive the other to extinction via genetic assimilation (e.g., Wolf *et al*. 2001). If, on the other hand, the incipient reproductive barriers are sufficiently strong and hybrid progeny perform poorly, selection against individuals that mate with the 'wrong' type might result in the reinforcement of prezygotic reproductive barriers (reviewed in Howard 1993; Servedio & Noor 2003). Alternatively, hybridization can have creative outcomes, including the introgression of alleles from one taxon into another (reviewed in Rieseberg and Wendel 1993; see also Bailey *et al.* 2009, Currat *et al.* 2008, Gagnaire *et al.* 2009, Kawakimi *et al.* 2009, Kim *et al.* 2008, Lepis *et al.* 2009, Wood & Nakazato 2009), the origin of novel adaptations (reviewed in Arnold 2004), and even hybrid speciation (reviewed in Rieseberg 1997).

The most common form of hybrid speciation is allopolyploidy, in which a cross between two species gives rise to a hybrid lineage that carries the full complement of chromosomes from both parental species. In such cases, the hybrid lineage will be reproductively isolated from its parents due to the increase in chromosome number (reviewed in Ramsey & Schemske 1998; Otto & Whitton 2000). Alternatively, hybrid speciation can occur without a change in chromosome number – a phenomenon known as homoploid hybrid speciation (reviewed in Rieseberg 1997; see also Rieseberg *et al.* 2003, Gross & Rieseberg 2005). The most widely accepted model of homoploid hybrid speciation is the recombinational model of Stebbins (1957) and Grant (1958), wherein reproductive isolation results from the production of novel combinations of sterility factors following hybridization between chromosomally or genetically divergent parental species. The resulting hybrids are interfertile with one another but are at least partially

isolated from both of their parents, and thus free to evolve independently. Alternatively, hybridization between two ecologically distinct taxa can result in the production of a unique homozygous recombinant type isolated by external, rather than internal, barriers (Grant 1981).

While hybridization is widespread in plants (Mallet 2005), the conditions under which homoploid hybrid speciation can occur are stringent (McCarthy et al. 1995, Buerkle et al. 2000, 2003). This mode of speciation has thus generally been regarded as rare. Nonetheless, the taxonomic literature includes a number of proposed instances of homoploid hybrid speciation (Arnold 1997, Rieseberg 1997). Unfortunately, homoploid hybrid species are difficult to unambiguously identify, and only a handful of cases have been substantiated using molecular approaches. In some instances, these hybrid species have very restricted ranges (e.g., Arnold 1993), whereas in others they have achieved a more widespread distribution, either through a single initial hybridization event followed by range expansion, or via multiple origins (e.g., Welch & Rieseberg 2002, Schwarzbach & Rieseberg 2002, James & Abbott 2005). Here we investigate the origin of *Stephanomeria diegensis*, a putative homoploid hybrid species with a range that spans much of coastal southern California.

The genus *Stephanomeria*, one of ca. 1100 genera within the sunflower family (Compositae or Asteraceae; Jeffrey 1993) is composed of six annual and ten perennial species and has a base chromosome number of n = 8. In his initial systematic treatment of the genus, Gottlieb (1971) identified the annual *S. diegensis* as a putative hybrid species, having most likely arisen as a result of interbreeding between *S. exigua* and *S. virgata*. These latter species are polytypic annuals that are composed of five and two subspecies,

respectively. Both species have broad ranges in the western United States, and F_1 hybrids between them exhibit ca. 10% pollen fertility (Gottlieb 1969).

The possibility of a hybrid origin of *S. diegensis* was first invoked because this species combines several morphological characters that distinguish *S. exigua* and *S. virgata*, though it also exhibits some transgressive (i.e., extreme) characters (e.g., the number of florets per inflorescence and length of lateral pinnae along the pappus bristles). In terms of geographic distribution, the range of *S. diegensis* falls entirely within the region of overlap between *S. exigua* and *S. virgata*. Finally, crossability with both of its putative parents is low. In fact, pollen fertility in F_1 hybrids between *S. diegensis* and its putative parents indicate that it is the most strongly reproductively isolated of all annual *Stephanomeria* species (Gottlieb1969). Interestingly, this pattern of increased reproductive isolation in hybrid species has been documented in other study systems (e.g., Rieseberg 2000, Lai et al. 2005).

Gottlieb's (1969, 1971) initial conclusions regarding the origin of *S. diegensis* were later supported by allozyme data (Gallez & Gottlieb 1982). More specifically, the *S. diegensis* gene pool was found to include alleles from both *S. exigua* and *S. virgata*, and there was a paucity of unique *S. diegensis* alleles. Unfortunately, the majority of loci surveyed in that work were uninformative because the putative parental species shared the same majority allele. Thus, while the results were consistent with a hybrid origin, they were far from conclusive. Beyond this, Gallez & Gottlieb (1982) relied on relatively limited geographic sampling and only included three of the five *S. exigua* subspecies in their study. As noted above, *S. exigua* and *S. virgata* co-occur and hybridize throughout the distribution of *S. diegensis*, yet the possible role of multiple origins in producing the

widespread range of their putative hybrid daughter species has never been investigated. Moreover, the identity of the parents of *S. diegensis* (in terms of particular subspecies of *S. exigua* and *S. virgata*) remains unknown. Here we use chloroplast and nuclear DNA markers to test the hypothesis of a hybrid origin of *S. diegensis*, investigate the possibility of multiple origins, and identify the most likely parental subspecies.

MATERIALS AND METHODS

Plant Materials and DNA Extractions

Buds and/or achenes (single-seeded fruits) were collected from twenty-five populations of *S. exigua*, twelve populations of *S. virgata*, and nine populations of *S. diegensis* spanning the known range of this species. The collection sites ranged from southern San Diego County, California north through Mariposa County, California (Figure 1; Supplemental Table 1). Because *S. exigua* comprises five subspecies, and *S. virgata* comprises two subspecies, care was taken to include samples from all taxa throughout the range. Buds collected in the field were preserved in a saturated sodium chloride, 30% CTAB solution, shipped back to the lab, and stored at -20° C until extractions were completed (Rogstad 1992). Voucher specimens were collected for one or two individuals per population and were deposited at the University of Georgia herbarium. Total genomic DNA was isolated from 219 *S. exigua*, 79 *S. virgata*, and 79 *S. diegensis* individuals. DNA was isolated from either the preserved buds or from buds of plants grown in the greenhouse grown from field-collected achenes using a modified CTAB-based protocol (Doyle & Doyle 1990).

Chloroplast DNA Analysis

Seven regions were amplified from the chloroplast genome using the polymerase chain reaction (PCR) and then sequenced for three individuals of each species (GenBank accession numbers: GQ429013-GQ429054). The regions amplified were: *atp*B-*rbc*L, *psb*B-*psb*F, *trn*K-*rpl*32, *rpl*36-*inf*A-*rps*8, *pet*N1-*psb*M2 section of *trn*C-*trn*D, *trn*L(UAA)5'exon-*trn*F(GAA), *trn*L-*rpl*32, (Chiang *et al.* 1998, Hamilton 1999, Johnson and Soltis 1995, Kress *et al*. 2005, Lee and Wen 2004, Taberlet et al. 1991, Timme *et al.* 2007). Reaction volumes were 20 µl. Each reaction contained 20 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl, 100 µM each deoxynucleotide triphosphate, 0.1 µM forward primer, 0.1 µM reverse primer, and one unit of *Taq* DNA polymerase. Cycling conditions followed a touchdown protocol to reduce non-specific binding (Don *et al.* 1991), as follows: initial denaturation at 95°C for 3 min; followed by 10 cycles of 30 s at 94°C, 30 s at 65°C (annealing temperature was reduced by 1° per cycle), and 45 s at 72°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C; and a final extension time of 20 min at 72°C. To prepare for DNA sequencing, 10 µl of each PCR product was incubated at 37°C for 45 m with 0.8 units of Shrimp Alkaline Phosphatase and 4 units of Exo-nuclease I (USB, Cleveland, OH). Enzymes were then denatured by heating to 80°C for 15 minutes. Purified PCR products (0.5 – 2 µl depending on approximate concentration) were then sequenced with the primers used for the initial amplification. DyeNamic (Amersham, Piscataway, NJ) chemistry was used for the sequencing following the manufacturers' protocols with minor modifications.

Unincorporated dyes were removed from the sequencing reactions with Sephadex (Amersham, Piscataway, NJ) cleanup and sequences were resolved on a Basestation (MJ

Research, San Francisco, CA) automated DNA sequencer. Sequences were aligned using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI), and putative species-specific sequence differences were identified. All sampled individuals were analyzed via PCR-RFLP of the *trn*L(UAA)5'exon-*trn*F(GAA) region, which exhibited a putative species-specific *Rsa*I restriction difference between the *S. exigua* and *S. virgata* samples (see below). PCR conditions were as described above, and restriction digestions were conducted as suggested by the manufacturer (Promega Corp. Madison, WI). Restriction fragments were then separated by gel electrophoresis and visualized by staining with ethidium bromide.

Simple Sequence Repeat Markers and Genotyping

Simple sequence repeat (SSR) primers were designed from 288 SSR-bearing *Stephanomeria* expressed-sequence tags (N.A. Sherman & J.M. Burke, unpublished data). These primer pairs were then tested for amplification and polymorphism on DNA samples from eight individuals across the three species (three each from *S. exigua* and *S. virgata*, and two from *S. diegensis*). This resulted in the identification of seventeen SSR markers that amplified reliably across taxa, exhibited polymorphism, and were easily scorable (Table 1).

All seventeen loci were amplified using a modification of the three-primer PCR protocol outlined by Schuelke (2000; see Wills et al. 2005). PCR reaction volumes were 14 μl. Each reaction contained 10 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl, 100 μM each deoxynucleotide triphosphate, 0.02 μM forward primer (with an M13 -29 sequence tail [5'-CACGACGTTGTAAAACGACA-3']), 0.1 μM reverse primer, 0.1 μM fluorescently-labeled M13 -29 primer, and one unit of *Taq*

DNA polymerase. The fluorescent labels used were HEX and TET, and cycling conditions were as above. Amplicons were diluted 1:50 or 1:150 (depending on product intensity in the original screen) and visualized on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA) with MapMarker 1000 ROX size standards (BioVentures, Murfreesboro, TN) included in each lane to allow for accurate fragment size determination. Alleles were called using the software package GeneMarker v. 1.70 (SoftGenetics, State College, PA).

Data Analyses

Utilizing the SSR data, descriptive population genetic statistics were calculated for each taxon using GenAlEx v. 6.1 (Peakall & Smouse 2006). These values included percentage of polymorphic loci, mean number of alleles per locus, and gene diversity (calculated as Nei's [1978] unbiased expected heterozygosity; H_e). Relationships amongst taxa were then graphically assessed via principal coordinate analysis (PCO; again using GenAlEx) using the covariance matrix with data standardization of genetic distance. Neighbor-joining trees were constructed in PHYLIP 3.67 (Felsenstein 2007) using a distance matrix from MSAnalyzer (Dieringer & Schlotterer 2003) based on Nei et al.'s (1983) genetic distance (calculated from the allele frequency data). Nodal support was calculated with 1000 bootstrap replicates using the CONSENSE program in PHYLIP 3.67 (Felsenstein 2007).

The hypothesis of a hybrid origin of *S. diegensis* was next investigated using the admixture model of the Bayesian clustering program STRUCTURE ver. 2.2 (Pritchard et al. 2002, Falush et al. 2003) following the approach of James & Abbott (2005). Given that the neighbor-joining results for *S. exigua* and *S. virgata* showed these species to be

distinct (Figure 2 and see below), the STRUCTURE analysis was performed with $K = 2$ clusters and individuals of the two parental species were treated as "learning samples" with the USEPOPINFO feature turned on. For the purpose of this analysis, *S. diegensis* was treated as an unknown, and STRUCTURE was used to infer the ancestry of all individuals in the dataset. This analysis was performed assuming independent allele frequencies across populations with an initial burn-in period of 20,000 replicates and 50,000 MCMC iterations. This analysis was repeated and the results were found to be consistent across five runs.

The genomic composition of *S. diegensis* individuals was further investigated using a maximum-likelihood hybrid index score estimated using the program HINDEX (Buerkle 2005) following the methods of Gross et al. (2007). Briefly, each *S. diegensis* individual was assigned a hybrid index score, ranging from 0 (*S. virgata*-derived) to 1 (*S. exigua*-derived) based on its genotype and the allele frequencies of its putative parents. Because a hybrid species will be a stabilized mosaic of the two parental genotypes, one might expect a subset of the loci to be derived from one parent with the balance being derived from the other parent, rather than observing segregating variation at each locus. Hybrid index scores were calculated on a per-locus basis such that a putative parental origin could be assigned to each locus. Per-locus hybrid index scores were then averaged for each population and loci were considered to be *S. exigua*-derived if the value was greater than 0.60 or *S. virgata*-derived if the value was less than 0.40. These locus assignments were only made if they were consistent across six or more of the nine *S. diegensis* populations. To double-check the single locus results, hybrid indices were re-calculated for all *S. diegensis* individuals using either: (1) the putative *exigua*-derived loci,

or (2) the putative *virgata*-derived loci. This allowed us to test whether or not the suites of loci assigned to each parent provided consistent results across all *S. diegensis* populations.

Finally, in order to investigate the particular parental subspecies that may have been involved in the origin of *S. diegensis*, neighbor-joining trees were constructed separately for *S. diegensis* along with each of its putative parents. These analyses utilized the subset of loci that were assigned to the parent of interest using HINDEX. In other words, *S. exigua* and *S. diegensis* were analyzed using the *S. exigua*-derived markers, whereas *S. virgata* and *S. diegensis* were analyzed using the *S. virgata*-derived markers. These trees were constructed as described above.

RESULTS

Chloroplast DNA Variation

Sequence analysis of the seven cpDNA regions revealed very low levels of polymorphism. Across a combined 5 kb of sequence, there were 2 polymorphic sites as well as 5 indels spanning 1-31 bp each. Overall, only two sites showed an apparent species-specific difference between *S. exigua* and *S. virgata*, and only one of these (in *trn*L(UAA)5'exon-*trn*F(GAA)) corresponded to a readily available restriction enzyme recognition site. Expanded genotyping of 374 individuals across multiple populations of all subspecies of both *S. exigua* and *S. virgata* confirmed the species-specific nature of this polymorphism, with 100% of *S. exigua* individuals harboring an additional cut site in this region, and 0% of *S. virgata* individuals harboring this cut site (Table 2). Genotyping

of *S. diegensis* revealed that all 79 individuals across the nine sampled populations carried the *S. virgata* haplotype.

Nuclear Variation

With the exception of *S. exigua* ssp. *macrocarpa*, all taxa exhibited polymorphism across all loci (Table 2). The mean number of alleles per locus in *S. exigua* ranged from a low of 3.1 across loci (range 1-6) in *S.exigua* ssp. *macrocarpa* to a high of 13.1 (range 7-24) in *S. exigua* ssp. *coronaria*. In *S. virgata*, there was an average of 9.1 (range 3-19) and 9.6 (range 4-15) alleles per locus in ssp. *virgata* and ssp. *pleurocarpa*, respectively. *Stephanomeria diegensis* exhibited an average of 11.5 (range 5-24) alleles per locus. Due to differences in sampling depth, however, variation in allele number must be interpreted with caution. In terms of overall levels of genetic diversity, H_e in *S. exigua* ranged from a low of 0.42 ± 0.07 (mean ± SE) in *S. exigua* ssp. *macrocarpa* to a high of 0.71 ± 0.03 in *S. exigua* ssp. *carotifera*. In *S. virgata*, the corresponding values were 0.68 ± 0.05 and 0.72 ± 0.05 in ssp. *virgata* and ssp. *pleurocarpa*, respectively. *Stephanomeria diegensis* had an average gene diversity of 0.64 ± 0.05.

The principal coordinate analysis (PCO) revealed that the three species (*S. exigua*, *S. virgata*, and *S. diegensis*) form relatively distinct groups (Figure 2). The subspecies within each of the putative parental species, however, exhibit extensive overlap. Overall, *S. diegensis* was more similar to *S. exigua* along the first coordinate (which explains 30.8% of the variation) and more similar to *S. virgata* along the second coordinate (which explains 24.3% of the variation).

The neighbor-joining tree based on data from all seventeen loci revealed that *S. exigua* and *S. virgata* are genetically distinct from one another (with 53.5 % bootstrap

support; Figure 3). When *S. diegensis* was added to this analysis, it formed a well-supported (99.9 % bootstrap support), monophyletic group most closely related to the *S. exigua* ssp. *exigua* populations (its position is indicated by the star in Figure 3). The addition of *S. diegensis* did not otherwise change the topology of the tree.

Consistent with the hypothesis of a hybrid origin, *S. diegensis* showed a signature of shared ancestry in the STRUCTURE analysis with the average *S. diegensis* individual exhibiting ca. 65% membership in the *S. exigua* group and ca. 35% membership in the *S. virgata* group (Figure 4). These results were generally consistent across populations. However, there were individuals within both *S. exigua* and *S. virgata* that did not cluster true to their presumptive species type. Note that, while 'population' information was provided to STRUCTURE in the form of species identifications for individuals of *S. exigua* and *S. virgata*, this information was only used to train the algorithm. As such, these *a priori* designations can be (and in some cases were) overridden by the genetic data. Because the morphology and cpDNA profiles of these 'mis-assigned' parental individuals were all consistent with their species designation, the unexpected placement of these individuals most likely reflects either introgressive hybridization or the retention of ancestral polymorphism.

Mean hybrid index scores for *S. diegensis* populations (estimated with all seventeen loci) ranged from 0.67 to 1.0, with an average of 0.87 ± 0.03 (mean ± SE). Eleven loci were identified as putatively *S. exigua*-derived, four loci were putatively *S. virgata*-derived, and two loci could not be assigned to either parent. In one case, this was due to a paucity of shared alleles whereas, in the other case, the hybrid index value fell between 0.40 and 0.60. The pooled *S. exigua*-derived loci gave a per-population average

hybrid index score for *S. diegensis* of 0.81–1 with an overall average value of 0.96 ± 0.02 across the species, and the pooled *S. virgata*-derived loci gave an average hybrid index score of 0.02–0.29 with an overall average value of 0.13 ± 0.03 across the species.

In the neighbor-joining trees constructed with the eleven *S. exigua*-derived loci, *S. diegensis* formed a monophyletic group with 97.8% bootstrap support (Figure 5a). This group was most closely associated with populations of *S. exigua* ssp. *exigua* and *S. exigua* ssp. *deanei*, though the overall topology of the tree was not well supported. For the neighbor-joining trees constructed with the four *S. virgata*-derived loci, *S. diegensis* once again formed a monophyletic group with 80.8% bootstrap support (Figure 5b). In this case, however, the most closely related subspecies could not be determined.

DISCUSSION

Taken together, the results of this study point to a hybrid origin of *S. diegensis*. The SSR-based neighbor-joining tree shows a split between *S. exigua* and *S. virgata*, with *S. diegensis* clustering with *S. exigua* (Figure 3, see star). The chloroplast data, however, clearly show that *S. diegensis* carries an *S. virgata*-like cpDNA haplotype. This pattern of non-concordance between nuclear and cytoplasmic data is one of the hallmarks of reticulate evolution (Arnold 1997) and provides strong support for the hypothesis of a hybrid origin of *S. diegensis*. Moreover, subsequent analyses (discussed in detail below) revealed that the *S. diegensis* nuclear genome is a mosaic of the *S. exigua* and *S. virgata* genomes, which is consistent with a hybrid origin, potentially via recombinational

speciation. Finally, assuming maternal transmission, the cpDNA data indicate that *S. virgata* likely served as the seed parent in the initial hybridization event(s).

In terms of genomic composition, the PCO plot revealed that *S. diegensis* is most similar to *S. exigua* along the first coordinate, and to *S. virgata* along the second coordinate, suggesting a stronger affinity for the former as compared to the latter (Figure 2). Consistent with this finding, the STRUCTURE analysis (Figure 4) revealed that *S. diegensis* individuals exhibit ca. 65% identity with *S. exigua* and ca. 35% identity with *S. virgata*, and HINDEX classified 11 of 17 of the SSR loci (64.7%) as being *S. exigua*-derived and 4 of the 17 SSR loci (23.5%) as *S. virgata*-derived. These results were found to be largely consistent across populations, suggesting that the *S. diegensis* populations surveyed trace back to a common origin. Interestingly, despite showing a stronger affinity for *S. exigua* in terms of nuclear genome composition, *S. diegensis* exhibits the *S. virgata* karyotype for the two chromosomes (out of a haploid number of n = 8) that can be visibly distinguished based on banding differences between *S. exigua* and *S. virgata* (Gottlieb 1971). Overall, these data accord well with the earlier suggestions that *S. diegensis* is a homoploid hybrid species (Gottlieb 1971, Gallez & Gottlieb 1982), and further suggest that its genome is disproportionately derived from *S. exigua.* It is noteworthy that Gallez and Gottlieb (1982) also found *S. diegensis* to be more closely allied with *S. exigua* based on allozyme data.

Beyond providing evidence of a hybrid origin, our data are suggestive of a single origin of *S. diegensis*. All *S. diegensis* individuals exhibited similar genomic composition based on the STRUCTURE analysis regardless of their population of origin, and all also carried the *S. virgata* chloroplast haplotype. Moreover, *S. diegensis* formed a single

cluster in all of the neighbor-joining analyses, including those in which the markers were subdivided by presumptive species of origin (Figures 3 and 5). We cannot, however, entirely rule out the possibility of multiple origins, particularly if the same parental subspecies were involved in each case. While the HINDEX results were, as noted above, largely consistent across populations, there were a small number of instances in which locus assignments differed between populations. While this finding might be superficially consistent with multiple origins, there was no apparent pattern in terms of the population/locus combinations that gave conflicting results. It is thus more likely that these inconsistencies reflect the challenges associated with reliably assigning individual loci to a particular parental species (Gross et al. 2003, Gross & Rieseberg 2007). While the lack of cpDNA polymorphism limited our ability to use those data to test for multiple origins, as has previously been done in studies of hybrid speciation in *Helianthus* (Gross et al. 2003, Schwarzbach & Rieseberg 2002, and Welch & Rieseberg 2002), the balance of our data were fully consistent with a single origin with *S. virgata* having served as the seed parent.

It appears from the hybrid speciation literature that instances of singly-derived, homoploid hybrid plant species are relatively rare. This conclusion is, however, based on a rather small number of well-documented cases of homoploid hybrid speciation under natural conditions. In four of these five cases (*Helianthus anomalus*, *H. deserticola*, *Pinus densata*, and *Argyranthemum sundingii*) the hybrid species was found to have been multiply-derived (Brochmann et al. 2000, Schwarzbach & Rieseberg 2002, Wang et al. 2001, Gross et al. 2003, 2007, Song et al. 2003). In the fifth case, *Helianthus paradoxus* was found to trace back to a single origin, perhaps because genetic constraints related to

its adaptation to a unique salt marsh habitat have limited the potential for multiple origins (Welch & Rieseberg 2002). While *Senecio squalidus* has likewise been found to have had a single origin, the stabilization of this species appears to have required long distance, human-mediated dispersal of hybrids from Italy to the British Isles (James & Abbott 2005).

Given the apparent tendency for homoploid hybrid species to be multiply-derived, the observation that *S. diegensis* likely traces back to a single origin requires explanation. One factor that might reduce the likelihood of multiple origins is a lack of opportunity for hybrid speciation. However, *S. exigua* and *S. virgata* overlap broadly, and hybridization occurs throughout the region of overlap (Gottlieb 1971, Sherman pers. obs.), making this an unlikely explanation. Alternatively, as noted above, Welch & Rieseberg (2002) have argued that genetic constraints related to the adaptation of a hybrid neo-species to an extreme habitat might decrease the likelihood of multiple origins. However, the habitat preferences of *S. diegensis* appear to overlap with those of its parents, which are likewise relatively similar to each other (Gottlieb 2006, N. Sherman, pers. obs.). Conversely, it has been argued that ecological differentiation resulting in spatial isolation dramatically increases the likelihood of homoploid hybrid speciation (Buerkle et al. 2000). Perhaps the relative lack of ecological divergence between these species has limited the potential for the stabilization of multiple hybrid lineages in this case.

In terms of the parentage of *S. diegensis*, the *S. exigua*-derived portion of the genome appears most closely related to either *S. exigua* ssp. *exigua* (which, like *S. exigua* ssp. *macrocarpa*, shows evidence of possible past admixture based on the STRUCTURE analysis) or *S. exigua* ssp. *deanei* (Figure 5a). In concordance with our findings,

sequence analysis of the ITS region has revealed that *S. diegensis* is most similar to *S. exigua* ssp. *deanei* and *S. exigua* ssp. *exigua* (Lee et al. 2002), and a recent analysis of sequence diversity at the *PgiC* locus places *S. diegensis* with *S. exigua* ssp. *deanei*, to the exclusion of all other subspecies of *S. exigua* (Ford et al. 2006). With regard to the *S. virgata* parental subspecies, there was insufficient resolution to make any clear inferences. This may be due, at least in part, to the low number of *S. virgata*-derived loci in our data. Furthermore, *S. virgata* ssp. *virgata* and *S. virgata* ssp. *pleurocarpa* are very similar to each other, perhaps due to relatively weak isolation between these subspecies as compared to the barriers between *S. exigua* subspecies (Gottlieb 1969, 1971). It is also possible that the formation of *S. diegensis* predated the divergence of the subspecies within *S. exigua* and *S. virgata*.

Conclusions and Future Directions

Our data provide strong support for a hybrid origin of *S. diegensis*. The nuclear genome of this species is a mosaic of the *S. exigua* and *S. virgata* genomes, with a greater proportion derived from the former as compared to the latter. Despite this closer alliance with *S. exigua*, *S. diegensis* is karyotypically more similar to *S. virgata*, and all individuals also carry the *S. virgata* chloroplast haplotype. When the nuclear and chloroplast results are combined, the picture that emerges is one in which pollen flow from *S. exigua* to *S. virgata* gave rise to the hybrid neospecies, and that subsequent backcrossing likewise involved pollen flow from *S. exigua*. The fact that *S. diegensis* appears to be singly-derived places it in the minority of homoploid hybrid species that have been analyzed to date. While it has been suggested that ecological divergence plays

a major role in determining the likelihood of homoploid hybrid speciation, the role of ecological divergence in promoting or limiting hybrid speciation in *Stephanomeria* requires further investigation. A better understanding of the timing of the origin of *S. diegensis*, as well as of the divergence of subspecies within each of the parental species, will also provide important insights into the origin and parentage of this hybrid species.

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Table 2.1 Locus names, primer sequences, and repeat motifs for each SSR marker.

Locus	Primer sequences (5'-3')	Repeat Motif
Steph_0279	F: TGGTGAGTATGGTGGTGGTC	GAT
	R: TCTGCCATACCTGGTTCTCC	
Steph_0227	F: CCCAGTTCGATTCCTCTTCC	GCA
	R: AAATTTAAACACGCGGATCG	
Steph_0283	F: TTCATTCACGACTTTGATCATTC	CGG
	R: AATTCTGCACACCCATGTTG	
Steph_0196	F: GACAACTGAGATGAACATTGAAGATGG	GAT
	R: ACAATCGGAGCCTTGAAATG	
Steph_0094	F: ACCCGGATCAAACGAAATAC	GGC
	R: GCACACTCCACCCTATCTCC	
Steph_0024	F: GGGAGGAGAGAGAGAAGAGAGAG	GA
	R: TTCATCGTCAAATCCAGGTTC	
Steph_0103	F: GTCCACCACCCATGAACAAG	TC
	R: AGCAACATACTCAAACCACAAAG	
Steph_0140	F: AATATTCACCCACGCTGACG	CGA
	R: TTGCACCGTGTGGTCTTTAG	
Steph_0202	F: AGAACGGAGGAGGATGCAAG	TG
	R: TTCATCAAGTTCAGTCGCTATC	
Steph_0004	F: ACAGAGGCATGTGGTTTTCC	TAT
	R: TTCAGTAGATGAAAATGGTTCAAAG	
Steph_0226	F: CCCACTTGAAGAACCCTACC	AAG
	R: TAATGCTACCTGCGGAAACC	
Steph_0237	F: GAGTAACCGTGCAGCATTCC	CAT
	R: GAATCTCCAGAGCAGCAACC	
Steph_0231	F: CGTACCAATTTCCACCAACC	TGG
	R: ACGCATTCGTCTTCTTGGAG	
Steph_0288	F: GCCTTGACCTTGTTCATGTG	AAG
	R: TCCGCCCATCAGTATATTCC	
Steph_0078	F: CCGAGTTTCTGCAAATTTCTC	GA
	R: CCTGGAGACACCTGAACTGG	
Steph_0195	F: AACCATGGAGAACGAGAACG	CAC
	R: CGACCGAATTTGCATAACAC	
Steph_0072	F: ATGCAGGTGCTGCTACTGTG	TG
	R: TTTCAAGATTTGGGCAGAATG	

Table 2.2 Summary of sample sizes and the results of the chloroplast/nuclear genotyping.

	Subspecies Designation*	**# of inds. (pops.)**	**% with cpDNA restriction site**	**Mean alleles per locus**	**Mean H_e****	**% Polymorphic Loci**
S. exigua	**SEM**	13 (3)	100.0%	3.1 (0.4)	0.42 (0.07)	82.4%
	SECO	82 (10)	100.0%	13.1 (1.3)	0.69 (0.04)	100.0%
	SECA	41 (5)	100.0%	9.6 (1.1)	0.71 (0.03)	100.0%
	SEE	18 (2)	100.0%	6.0 (0.7)	0.57 (0.07)	100.0%
	SED	62 (6)	100.0%	12.7 (1.7)	0.69 (0.05)	100.0%
S. virgata	**SVV**	49 (8)	0.0%	9.6 (1.1)	0.68 (0.05)	100.0%
	SVP	30 (4)	0.0%	9.1 (0.9)	0.72 (0.05)	100.0%
S. diegensis	**SDI**	79 (9)	0.0%	11.5 (1.5)	0.64 (0.05)	100.0%
Grand Total		**374 (47)**				

*See Figure 1 legend for definition of subspecies designations.
**Refers to Nei's (1978) unbiased expected heterozygosity averaged across loci.

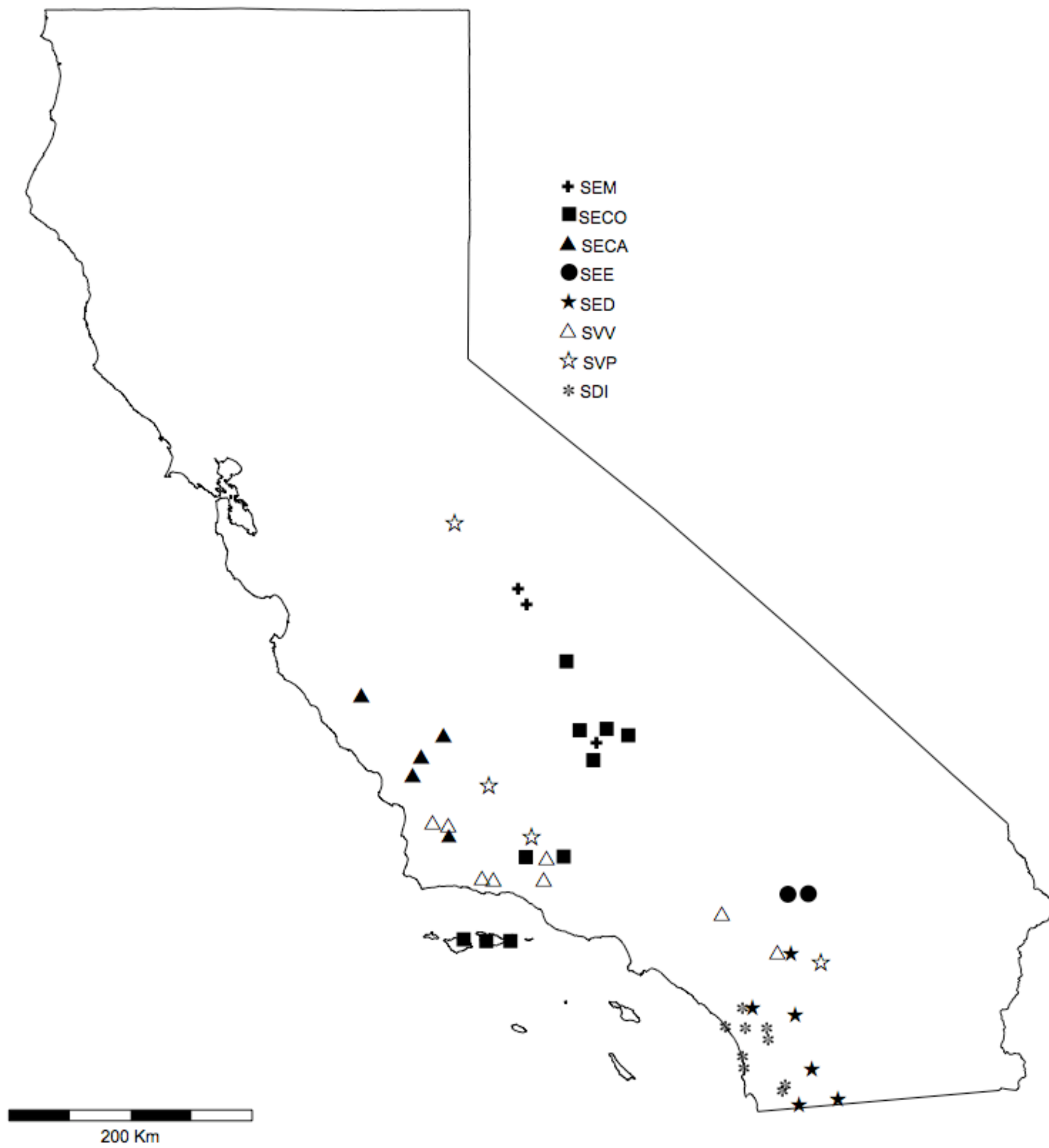

Figure 2.1 Map of California, USA, showing the locations of *Stephanomeria* populations sampled for this study. SEM, *S. exigua* ssp. *macrocarpa*; SECO, *S. exigua* ssp. *coronaria*; SECA, *S. exigua* ssp. *carotifera*; SEE, *S. exigua* ssp. *exigua*; SED, *S. exigua* ssp. *deanei*; SDI, *S. digenesis*; SVV, *S. virgata* ssp. *virgata*; SVP, *S. virgata* ssp. *pleurocarpa.*

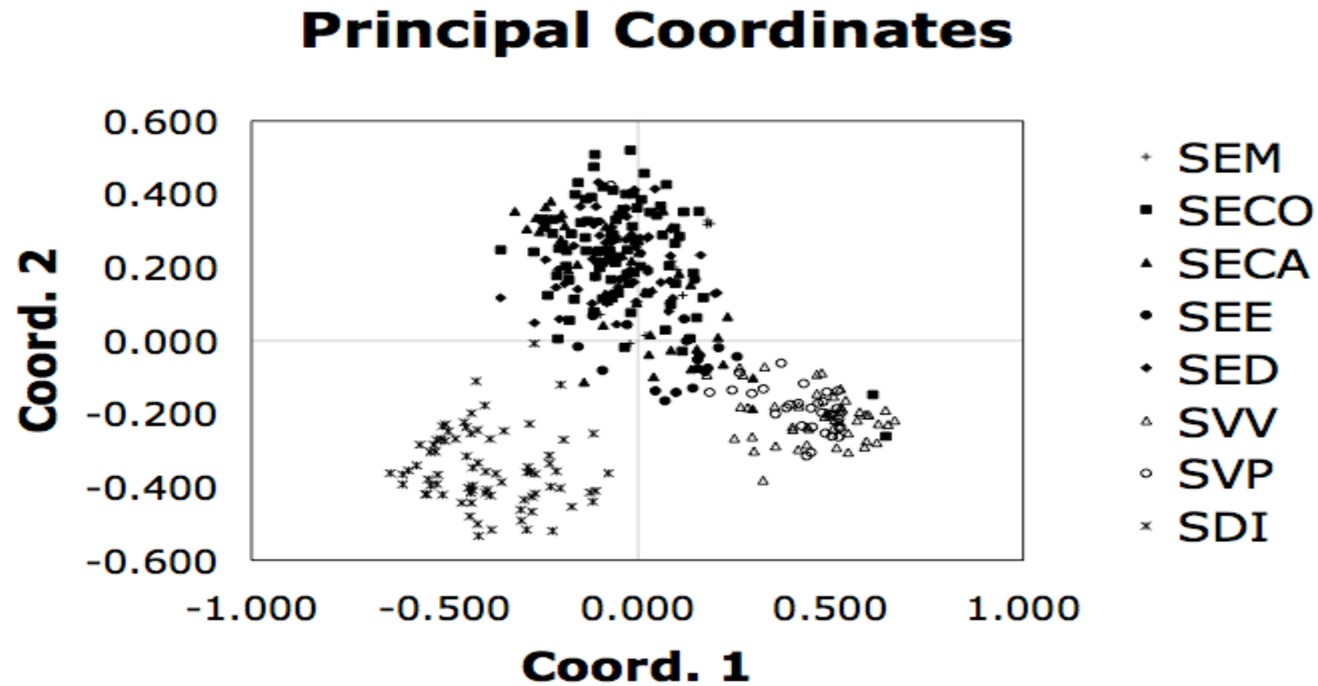

Figure 2.2 Results of the principal coordinate (PCO) analysis of *S. exigua*, *S. virgata*, and *S. diegensis* individuals used in this study based on genotypic data from seventeen SSR loci.

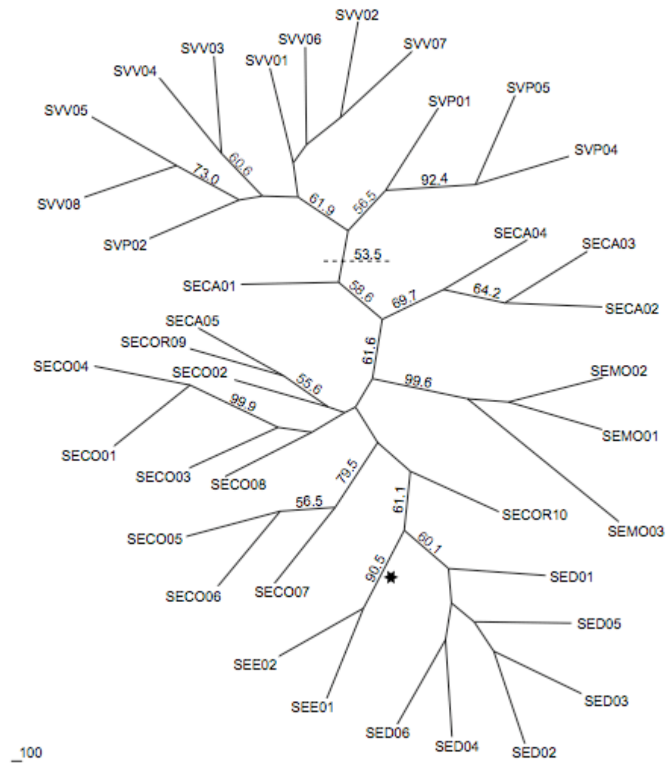

Figure 2.3 Unrooted neighbor-joining dendrogram of *S. exigua* and *S. virgata* constructed using genotypic data from all seventeen SSR loci. The asterisk (*) indicates the location of the *S. diegensis* cluster when populations of this species are included. Numbers along branches represent bootstrap support after 1000 replicates. Only bootstrap values greater than 50 are shown.

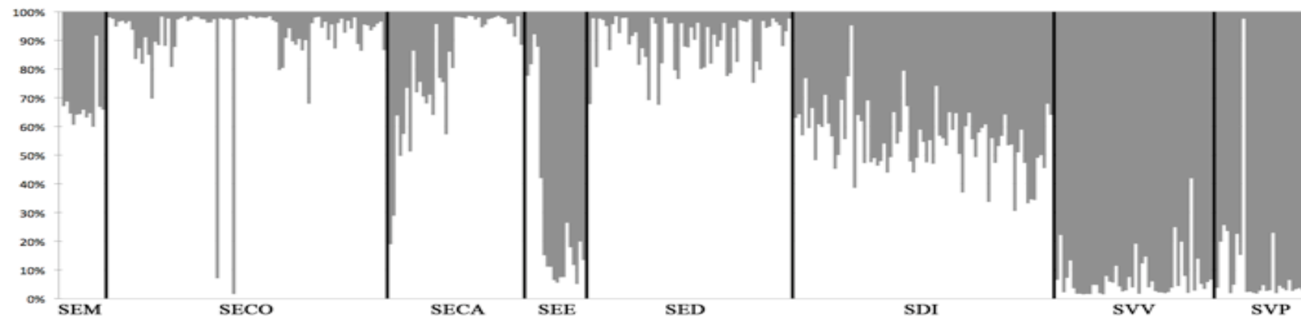

Figure 2.4 Results of the STRUCTURE analysis with $K = 2$ corresponding to *S. exigua* and *S. virgata* and *S. diegensis* treated as unknowns. Bars for each individual reflect the average result across five independent runs.

Figure 2.5a

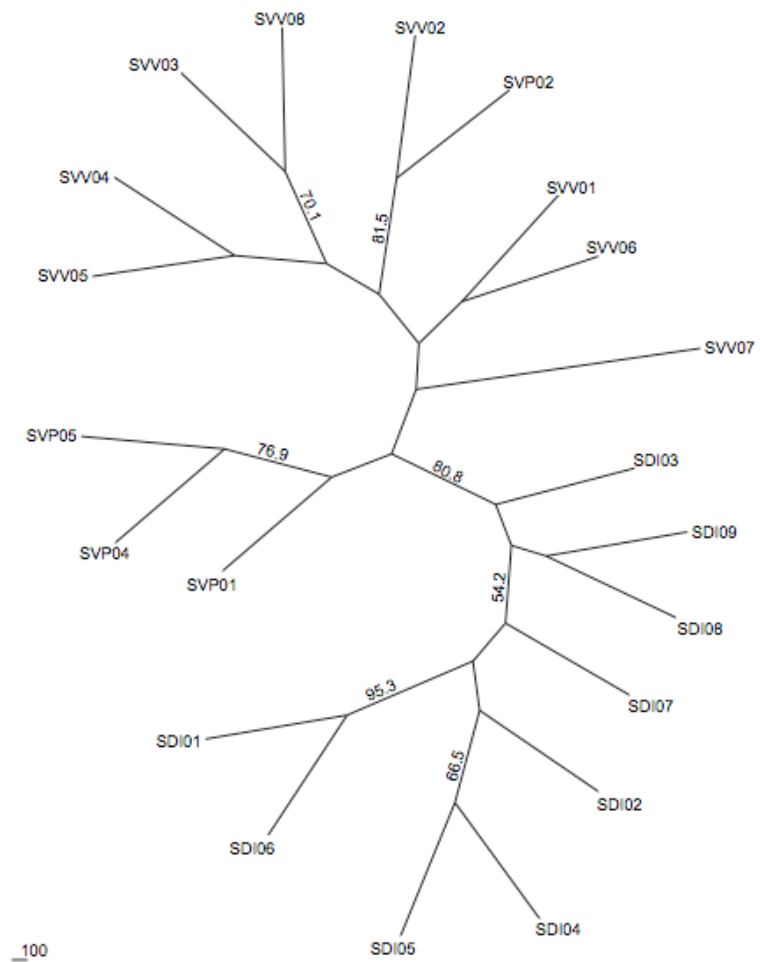

Figure 2.5b

Figure 2.5 continued Unrooted neighbor-joining dendogram of (a) *S. exigua* and *S. diegensis* using genotypic data from the eleven *S. exigua*-derived SSR loci, and (b) *S. virgata* and *S. diegensis* using genotypic data from the four *S. virgata*-derived SSR loci. Numbers along branches represent bootstrap support based on 1000 replicates. Only bootstrap values over 50 are shown.

CHAPTER 3

POPULATION GENETIC ANALYSIS REVEALS MULTIPLE ORIGINS OF THE ALLOPOLYPLOID *STEPHANOMERIA ELATA* (ASTERACEAE)[2]

[2] SHERMAN, N.A. and J.M. BURKE. Population genetic analysis reveals multiple origin of the allopolyploid *Stephanomeria elata*. To be submitted to *Evolution*.

Abstract

Allopolyploidy has been historically viewed as an evolutionary dead end, in part to to the presumed lack of genetic variation in polyploid lineages following the bottleneck that led to their formation. However, more recent work has revealed that allopolyploids can have multiple origins, and that crossing between independently-derived individuals and/or populations can result in a genetically diverse species with high evolutionary potential. Here I describe an investigation of the origin of the allopolyploid *Stephanomeria elata* utilizing based on chloroplast and nuclear DNA variation. The issue of single vs. multiple origins was investigated, as was the level of differentiation between two recognizable morphotypes, termed the Northern and Southern morphotypes. This work identified three likely origins, with the Southern morphotype having arisen twice and the Northern morphotype having arisen just once. In addition, a high level of variation in genome size in each morphotype was observed.

Introduction

Natural hybridization is a relatively widespread and important process in plant evolution. While hybridization has historically been viewed as little more than an impediment to evolutionary divergence (Mayr 1970; but see Anderson & Stebbins 1954), recent decades have seen a growing appreciation for the potentially creative outcomes of hybridization, including adaptive trait introgression (reviewed in Rieseberg & Wendel 1993; see also Bailey *et al.* 2009, Currat *et al.* 2008, Gagnaire *et al.* 2009, Kawakimi *et al.* 2009, Kim *et al.* 2008, Lepis *et al.* 2009, Wood & Nakazato 2009), the origin of novel adaptations (reviewed in Arnold 2004), and even the formation of new species (reviewed in

Rieseberg 1997, Soltis & Soltis 2009). Hybrid speciation can occur via one of two main pathways. Homoploid hybrid speciation occurs when a new species with the same chromosome number as its parental taxa arises following hybridization (reviewed in Rieseberg 1997; see also Rieseberg *et al.* 2003, Gross & Rieseberg 2005). Alternatively, allopolyploid species contain a full chromosome complement from each parent, thereby resulting in reproductive isolation from its parental taxa (reviewed in Ramsey & Schemske 1998, Otto & Whitton 2000, Soltis & Soltis 2009).

In early work, it was presumed that allopolyploids would be genetically depauperate due to the population bottleneck resulting from the presumed rarity of genome doubling following hybridization (Soltis & Soltis 2009). Allopolyploidy was thus typically viewed as an evolutionary dead end (Wagner 1970). Further work has shown, however, that allopolyploids can be multiply-derived (e.g., Soltis & Soltis 1993, 1995, Soltis 1995), such that crosses between independently-derived individuals/populations can result in a highly diverse allopolyploid species with high evolutionary potential (Soltis & Soltis 1999). Here we investigate the number of origins of *Stephanomeria elata* Nutall (Asteraceae), an allopolyploid species that is distributed from Southern California north through Oregon in the western USA.

The genus *Stephanomeria*, which is one of ca. 1100 genera within the sunflower family (Compositae or Asteraceae; Jeffrey 1993), is composed of six annual and ten perennial species. These species are predominantly self-incompatible, and have a base chromosome number of $n = 8$. Within the annual taxa a single, self-compatible tetraploid species, *S. elata* (n=16; Gottlieb 1969), has been identified. *Stephanomeria elata* exhibits regular chromosome pairing and disjunction, with no multivalents or other meiotic

abnormalities being observed, and pollen fertility is generally greater than 98% (Gottlieb 1969). The apparently disomic inheritance of individuals within this species is most consistent with an allopolyploid origin. Interestingly, there are two identifiable morphotypes of *S. elata* (Northern and Southern; Figure 1). These morphotypes, which could reflect either differentiation following polyploidization or multiple independent origins of *S. elata*, can be distinguished on the basis of their pappus bristles as well as the length of their achenes. Based on overall morphological similarity as well as species distributions, Gottlieb (1969) suggested that the parents of *S. elata* are most likely the annual, diploid species *S. exigua* and *S. virgata*. He could not, however, rule out the possibility of independent origins, and he further recognized the possibility that the Southern morphotype may have arisen following hybridization between *S. virgata* and the homoploid hybrid species *S. diegensis*, which itself arose as a result of hybridization between *S. exigua* and *S. virgata* (Sherman & Burke 2009).

In the present study, we used chloroplast and nuclear DNA markers to investigate the possibility of multiple origins of *S. elata*, and to further investigate population-level differentiation within and among the Northern and Southern morphotypes. We also report on the genome size of both morphotypes of *S. elata* and its presumptive parental species.

MATERIALS AND METHODS

Study material

Achenes were collected from four populations each of the Northern and Southern morphotypes of *S. elata* (Figure 1). Voucher specimens were collected for two

individuals per population and deposited at the University of Georgia Herbarium (GA). Seeds were then germinated and grown in the greenhouse at the University of Georgia, and total genomic DNA was extracted using a modified CTAB-based protocol (Doyle & Doyle 1990) from buds of 53 and 51 plants of the Northern and Southern morphotypes, respectively. Sample sizes for each population ranged from ten to eighteen.

Genome Size Estimates

Genome size was estimated via flow cytometry for one plant per *S. elata* population (n = 8 total). In addition, genome size estimates were done for three plants from different populations of each of the five and two subspecies of *S. exigua* and *S. virgata*, respectively. Plants were grown to the rosette stage and 0.5 grams of tissue was collected and sent to the Flow Cytometry and Imaging Core Laboratory at the Benaroya Research Institute at Virginia Mason, Seattle, WA. Four genome size estimates were made for each sample and then averaged.

Chloroplast Genotyping

In a previous study, a species-specific sequence difference resulting in presence/absence of an *Rsa*I cut site that differentiates *S. exigua* from *S. virgata* was identified in the chloroplast *trn*L(UAA)5'exon-*trn*F(GAA) region (Sherman & Burke 2009). This region was thus PCR amplified from all sampled *S. elata* individuals and digested with *Rsa*I following the previously established protocol. Total PCR volume was 20 µl and included 20 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl, 100 µM each deoxynucleotide triphosphate, 0.1 µM each of the forward and reverse primers (see Taberlet et al. 1991), and one unit of *Taq* DNA polymerase. Cycling conditions followed a touchdown protocol to reduce non-specific binding (Don *et al.* 1991) as

follows: initial denaturation at 95°C for 3 min; followed by 10 cycles of 30 s at 94°C, 30 s at 65°C (annealing temperature was reduced by 1° per cycle), and 45 s at 72°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C; and a final extension time of 20 min at 72°C. Restriction digestions were conducted following the manufacturer's protocol (Promega Corp. Madison, WI). Restriction fragments were then separated via gel electrophoresis using 2% agarose gels and visualized by staining with ethidium bromide.

Nuclear Genotyping

Seventeen simple-sequence repeat (SSR) primers that reliably amplify across the diploid annual *Stephanomeria* species were selected from a previously published set of markers (see Table 1 in Sherman & Burke, 2009).

All seventeen loci were amplified using a modification of the three-primer PCR protocol outlined by Schuelke (2000; Wills et al. 2005). Total PCR volumes were 14 μl, and each reaction contained 10 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl, 100 μM each deoxynucleotide triphosphate, 0.02 μM forward primer (with an M13 -29 sequence tail [5'-CACGACGTTGTAAAACGACA-3']), 0.1 μM reverse primer, 0.1 μM fluorescently-labeled M13 -29 primer (labelled with either HEX or TET), and one unit of *Taq* DNA polymerase. Cycling conditions followed a touchdown protocol as described above.. Amplicons were then diluted 1:50 or 1:150 (depending on product intensity) and visualized on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA) with MapMarker 1000 ROX size standards (BioVentures, Murfreesboro, TN) included in each lane to allow for accurate fragment size determination and peaks were identified using the software package GeneMarker v.

1.70 (SoftGenetics, State College, PA). Although the SSR primers employed here are known to amplify a single locus in diploid individuals, their use on polyploids results in multi-locus banding patterns. As such, “genotypes” were scored as binary characters based on presence/absence of all peaks in each individual.

Data Analysis

The Bayesian clustering algorithm implemented in the program STRUCTURE 2.2 (Pritchard et al. 2000) was used to investigate genetic differentiation across *S. elata* populations. All STRUCTURE runs were performed using the admixture model with non-correlated allele frequencies, no prior population information, and default parameter settings with a burn in period of 20,000 iterations followed by 50,000 MCMC repetitions, with 5 runs per k value (where k = the number of inferred clusters/subgroups). These analyses were run from k = 1 to 9, and the methods of Evanno et al. (2005) was used to determine the optimal number of clusters. Relationships amongst *S. elata* populations were also graphically assessed via principal coordinate analysis (PCO) using GenAlEx v. 6.69 (Peakall & Smouse 2006) using the covariance matrix with data standardization of genetic distance.

To test for the robustness of the recoded data, those loci that exhibited no more than two peaks per locus for over 90% of all *S. elata* individuals were re-analyzed as if they were diploid loci. Individuals with three or more peaks were treated as missing date. Relationships amongst populations were then re-assessed utilizing GenAlEX, as outlined above.

RESULTS

Genome Size Estimates

Genome size estimates ranged from 2.05 to 3.0 pg/C across populations/subspecies of *S. exigua* (Table 1). For *S. virgata*, the range was from 1.29 to 2.51 pg/C. In *S. elata* the Northern morphotype ranged from 3.80 to 6.37 pg/C, whereas the Southern morphotype ranged from 3.24 to 5.88 pg/C.

Chloroplast Genotyping

All individuals of the Northern morphotype exhibited only one *Rsa*I restriction site within the *trn*L(UAA)5'exon-*trn*F(GAA) chloroplast region, which is characteristic *S. virgata*. Individuals of the Southern morphotype exhibited both restriction profiles, with all individuals of population SLS1 exhibiting the additional restriction site that is characteristic of *S. exigua*. Two individuals of SLS2 exhibited the *S. virgata* restriction profile, and the remainder exhibited the *S. exigua* profile. Finally, all individuals of populations SLS3 and SLS4 exhibited the *S. virgata* restriction profile.

Nuclear Genotyping

Utilizing the binary data set, the STRUCTURE analysis identified two clusters of individuals (k = 2) corresponding to the Northern and Southern morphotypes of *S. elata* (Figure 3.2). Also utilizing the binary data, the PCO analysis revealed that the Northern morphotype formed a tight cluster that is largely distinct from the Southern morphotype (Figure 3.3). Within the Southern morphotype, two primary clusters are apparent. One cluster is comprised of SLS3 and SLS4. As noted above, these two populations are characterized by the *S. virgata* chloroplast haplotype. The other cluster consists of SLS1

and SLS2. As noted above, with the exception of two SLS2 individuals, these two populations are characterized by the *S. exigua* chloroplast haplotype. Interestingly, the two individuals of SLS2 that exhibit the *S. virgata* chloroplast haplotype grouped with the Northern morphoptype in the PCO analysis (Figure 3.3, labeled with arrows) and also showed majority membership with the Northern morphotype in the STRUCTURE analysis. When the data were recoded as diploid loci (see above), the same general patterns were observed (data not shown).

DISCUSSION

The data presented herein provide evidence of a minimum of two origins of *S. elata*, with the Northern morphotype having been formed on the *S. virgata* chloroplast background, and two populations of the Southern morphotype (SLS3 and SLS4) likewise carrying the *S. virgata* chloroplast haplotype. The other two Southern populations (SLS1 and SLS2) were primarily comprised of individuals carrying the *S. exigua* chloroplast haplotype. Assuming maternal chloroplast inheritance (e.g., Reboud & Zeyl 1994; Mogensen 1996), the presence of both the *S. virgata* and *S. exigua* chloroplast haplotypes within *S. elata* indicates reciprocal origins of this allopolyploid species. In other words, the Northern and some Southern populations (SLS3 and SLS4) appear to have arisen as a result of hybridization with *S. virgata* serving as the maternal parent, whereas the remaining Southern populations (SLS1 and SLS2) likely arose as a result of hybridization with *S. exigua* as the maternal parent. The fact that SLS3 and SLS4 are phenotypically Southern populations, but are carrying the *S. virgata* chloroplast haplotype that is characteristic of

the Northern morphotype suggests the possibility of a third origin, though the chloroplast data alone are not enough to support this view. Note that the possibility of an *S. virgata* × *S. diegensis* origin in the south can be ruled out, at least in part, by that fact that *S. diegensis* (which is itself a homoploid hybrid species) is known to carry the *S. virgata* chloroplast haplotype (Sherman and Burke 2009). As such, this species pair could not have given rise to populations SLS1 and SLS2.

The STRUCTURE analysis of the nuclear SSR data revealed that *S. elata* forms two groups corresponding to the Northern and Southern morphotypes, which suggests that there is insufficient gene flow amongst regions to homogenize these populations. Despite the apparently strong differentiation amongst regions, there were two individuals within SLS2 that appeared to be a product of long distance dispersal from the north (i.e., they carried the *S. virgata* chloroplast haplotype and showed strong nuclear similarity with the Northern populations). The genetic distinctiveness of these individuals could be due to recent dispersal, or perhaps to earlier dispersal accompanied by a high level of self-pollination or reproductive incompatibility amongst origins (Halliburton 200). The PCO analysis revealed a slightly more complex situation, with coordinate 1 separating the Northern and Southern morphotypes, as observed in the STRUCTURE analysis, and coordinate 2 separating SLS1/SLS2 from SLS3/SLS4. This pattern of differentiation lends further support to the possibility of a third origin, with the Southern morphotype having arisen twice. Assuming this to be the case, the moprhological similarity of these populations may be due to selection favoring the Southern morphology in the southern portion of the distribution.

In terms of genome size, there is almost two-fold variation amongst *S. elata* individuals (3.24-6.37 pg/C). The lowest value was observed in the SLS4 individual, though it is noteworthy that this individual exhibited the *S. virgata* chloroplast haplotype, and its genome size estimate was outside the range of either of the parental species. Moreover, individiuals from this population exhibited multi-locus banding patterns, as expected of polyploids. It thus seems unlikely that this individual is a mis-identified diploid. Rather, it may be the product of hybridization between *S. virgata* and *S. exigua* individuals from the low end of their respective genome size distributions. Alternatively, the wide range of genome size estimates in *S. elata* could be reflective of differential gene loss across lineages following polyploidization. Gene loss in polyploids has been well documented (e.g., Lukens et al. 2006; Grover et al 2004), and it is possible that the origin of *S. elata* is even more complex than outlined above, with multiple independent lineages of different ages having experienced different amounts of gene loss.

Taken together, the results of this study indicate a complex origin of *S. elata*, as has been observed in a several other allopolyploid species (e.g., Fortune et al. 2008 Soltis et al. 1995;). Beyond documenting a minimum of two or three origins of this species, it seems clear from this work that *S. elata* is the product of reciprocal parentage. Interestingly, the direction of the cross does not appear to correspond to the distinctive morphological differences, as has been observed in *Tragopogon miscellus* (Soltis & Soltis 1995). As noted above, this lack of correspondence between cross type and morphlogy may be the result of selection, with populations at the northern and southern ends of the distribution having experienced unique selective pressures. Finally, it remains

to be seen whether or not the different origins of *S. elata* are fully reproductively-compatible, and thus whether or not the current taxonomy reflects biological reality.

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Table 3.1 Genomic size for *S. exigua*, *S. virgata*, and *S. elata*

Species	subspecies	population	pg/C
S. exigua			
	carotifera		2.66
	coronaria		2.35
	deanei		2.53
	exigua		3.49
	macrocarpa		2.76
S. virgata			
	pleurocarpa		1.37
	virgata		1.76
S. elata			
	Northern morphotype	SLN1	3.80
		SLN2	4.61
		SLN3	5.88
		SLN4	3.24
	Southern morphotype	SLS1	5.12
		SLS2	5.46
		SLS3	5.88
		SLS4	3.24

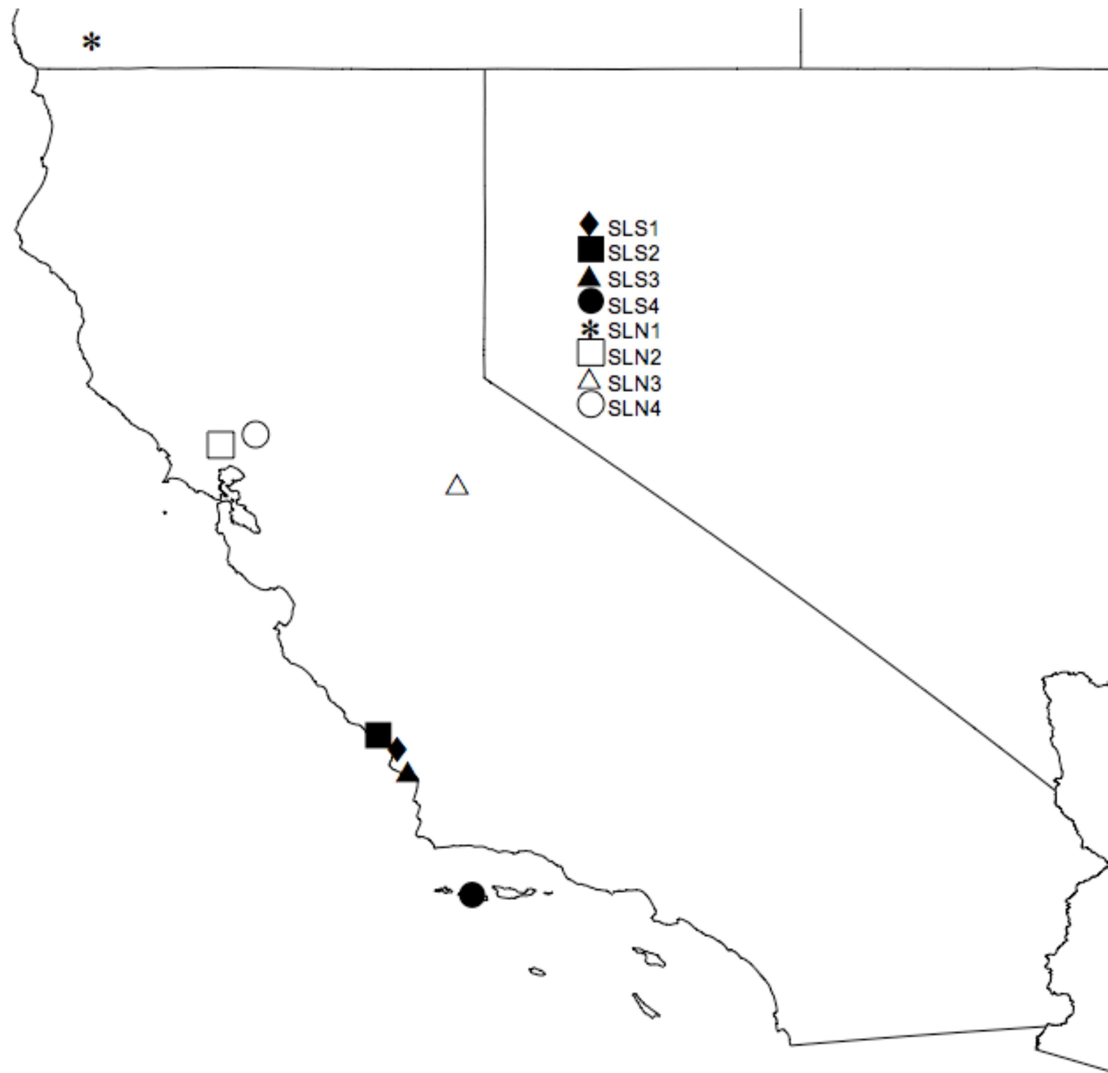

Figure 3.1 Map of population locations. SLN, *Stephanomeria elata* Northern morphotype; SLS, *Stephanomeria elata* Southern morphotype

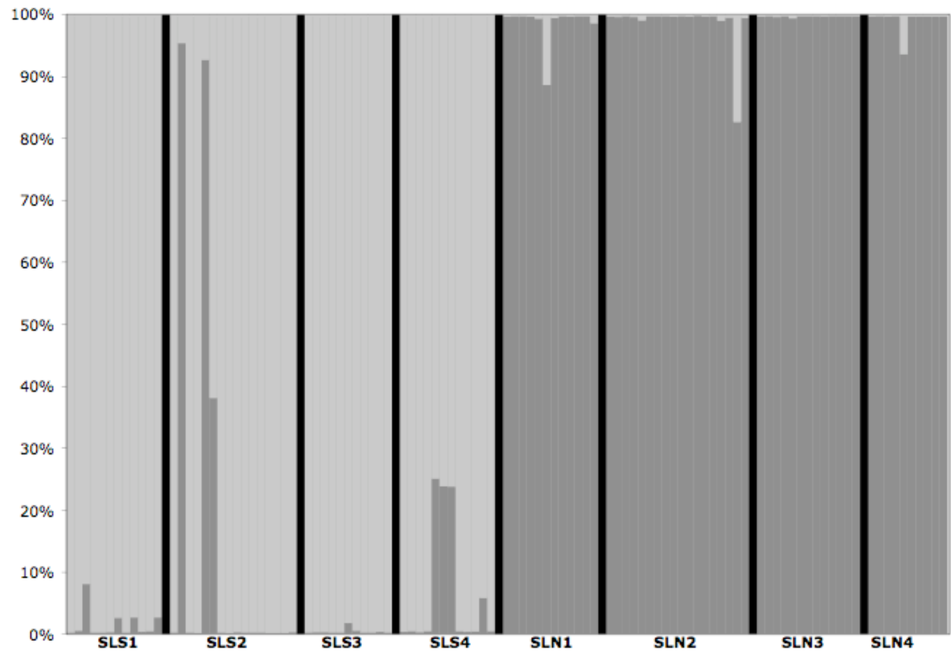

Figure 3.2 STRUCTURE results of the STRUCTURE analysis with k=2 which correspond to the Northern and Southern morphotypes. Bars for each individual reflects the average result across five independent runs.

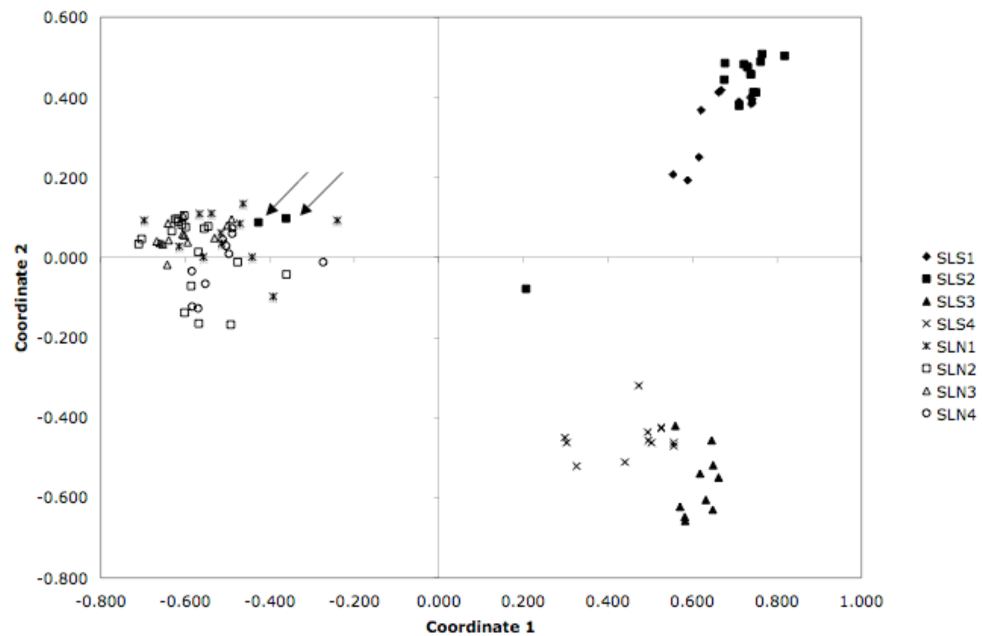

Figure 3.3 Results of the principal coordinate (PCO) analysis of *S. elata* individuals used in this study based on genotypic data from seventeen SSR loci. Coordinate 1 explains 51.94 % and Coordinate 2 15.51% of the observed variation.

CHAPTER 4

POPULATION GENETIC STUDIES OF A PERIPHERAL ISOLATE POPULATION OF *STEPHANOMERIA EXIGUA* SSP. *CORONARIA* (ASTERACEAE)[3]

[3] SHERMAN, and J.M. BURKE. Population genetic studies of a peripheral isolate population of *Stephanomeria exigua* ssp. *coronaria* (Asteraceae)
To be submitted to *Conservation Genetics*.

ABSTRACT

When evaluating conservation priorities, an important consideration is that contemporary population sizes and the apparent degree of spatial isolation are not necessarily predictive of the genetic composition of a population. In order to make informed management decisions, a formal population genetic analysis should be carried out. Here I report the results of a population genetic analysis of the flowering plant species *Stephanomeria exigua* and a closely related, endangered congener, *S. malheurensis*. Of particular interest is a disjunct population of *S. exigua* ssp. *coronaria* (termed the Frenchglen population), which is thought to have given rise to *S. malheurensis* outside of the contiguous distribution of the *S. exigua*. Utilizing simple sequence repeat (SSR) markers, I found that the Frenchglen population and S. malheurensis are genetically disctinct from the balance of populations/subspecies elsewhere in the range of *S. exigua*, but very similar to one another. Moreover, S. *malheurensis* exhibits drastically reduced genetic variability, whereas the Frenchglen population of *S. exigua* ssp. *coronaria* is quite diverse. Thus, management efforts should focus on habitat preservation to protect the French Glen complex rather than focusing on the re-establishment of *S. malheurensis*.

INTRODUCTION

Populations near the edge of a species' range are often smaller and more spatially isolated as compared to centrally-located populations (Lawton 1993; Sagarin & Gaines 2002). Such peripheral populations are typically expected to harbor reduced levels of genetic diversity and to exhibit increased levels of genetic differentiation relative to populations

elsewhere in the range (Lesica & Allendorf 1995). By extension, disjunct populations are expected to exhibit an even greater reduction in genetic diversity and an accompanying increase in genetic differentiation (Hamilton & Eckert 2007). While a reduction in standing genetic variation can reduce the evolutionary potential of populations (e.g., Hoffman & Blows 1994; Vuctetich & Waite 2003), peripheral and/or disjunct populations often occur in marginal habitats, potentially resulting in unique selective pressures that can produce rapid evolutionary divergence (e.g., Mayr 1982; Davis & Shaw 2001; Lenormand 2002; Hendry & Taylor 2004).

Peripherally-isolated populations are often the focus of conservation efforts, though there has been considerable debate as to the value of such efforts (Millar & Libby 1991; Lesica & Allensdorf 1992, 1995; Hunter & Hutchinson 1994). For example, some have argued against the use of limited resources to protect species that are locally rare, but globally common. Beyond this, range limits are in a constant state of flux, such that attempts to maintain peripheral populations might prove futile in cases where a range is naturally shifting away from a particular region. In contrast, arguments in favor of protecting such populations are often based on the premise that the successful long-term conservation of species is likely to depend on the protection of genetically distinct populations. In this light, Ehrlich and Pringle (2008) went so far as to argue that the loss of genetically distinct populations within species is at least as important a problem as the loss of entire species.

When evaluating conservation priorities, an important consideration is that contemporary population sizes and the apparent degree of spatial isolation are not necessarily predictive of the genetic composition of a population. This is because

population genetic parameters can be strongly influenced by recent historical events such as population bottlenecks and/or range expansion/contraction (Pamilo & Savolainen 1999; Hewitt 2000; Vucetich & Waite 2003; Hamilton & Eckert 2007). Thus, in order to make informed management decisions, a formal population genetic analysis should be carried out. Here we report the results of a population genetic analysis of the flowering plant species *Stephanomeria exigua* and a closely related, endangered congener, *S. malheurensis*. Of particular interest is a disjunct population of *S. exigua* ssp. *coronaria*, which is thought to have given rise to *S. malheurensis* outside of the contiguous distribution of the *S. exigua* (Gottlieb 1973).

The genus *Stephanomeria*, which is one of ca. 1100 genera within the sunflower family (i.e., the Compositae or Asteraceae; Jeffrey 1993), is composed of six annual and ten perennial species (Gottlieb 2006). Of these, *Stephanomeria exigua* is the most widespread species, with a range encompassing much of western North America. This species is composed of five subspecies (Figure 4.1), the most common of which (*S. exigua* ssp. *coronaria*) has a distribution spanning northern Mexico through Southern Oregon, and from the Channel Islands east through Utah. Along the northern margin of this subspecies is a single disjunct population (known as the “Frenchglen” population) that is isolated from the balance of the species range by ca. 150 km. Throughout it’s range, *S. exigua* ssp. *coronaria* exhibits substantial phenotypic variation. For example, the seeds are non-dormant and require cool conditions for germination in coastal California, whereas overwintering is required to break dormancy in seeds from plants growing in the Sierra Nevada, as well as to the east and north of the Sierra Nevada. There are also differences in growth period, with coastal plants germinating with the first rains

of the wet season, but not flowering until late summer. In contrast, populations on the eastern side of the Sierra Nevada exhibit a much shorter growing season, with spring germination and flowering before the summer heat begins (Gottlieb 2006).

The Frenchglen population of *S. exigua* ssp. *coronaria* is phenotypically most similar to the eastern populations, exhibiting seed dormancy, a shorter growing season, and smaller stature than populations from the western portion of the range (Gottlieb 1969). This population occurs in a sage brush desert near Burns, Oregon (Gottlieb, 1973), and is sympatric with the only known population of the endangered endemic *S. malheurensis*. Following its discovery in 1969 and description by Gottlieb in 1973, *S. malheurensis* obtained species status in 1978 (Gottlieb 1978). Unfortunately, the community structure of this site was dramatically altered by a human-caused fire in 1972 and the subsequent invasion of *Brumus tectorum* (cheatgrass) in 1975 (Bruaner 1988; Gottlieb 1991). *Stephanomeria malheurensis* was given federal protection under the Endangered Species Act in 1982, and was extinct in the wild by 1985. While multiple reintroduction efforts have been made for *S. malheurensis*, they have been met with limited success, and no efforts have been made to protect/restore the Frenchglen population of *S. exigua* ssp. *coronaria*.

Gottlieb (1973) argued that *S. malheurensis* is derived from, and arose in sympatry with, the Frenchglen population of *S. exigua* ssp. *coronaria* as a result of a change in mating system (i.e., a transition to selfing) and a temporal shift in floral receptivity (Gottlieb 1973). Phenotypically, *S. malheurensis* is differentiated from the Frenchglen population of *S. exigua* ssp. *coronaria* by an increase in achene size, an increase in taproot size, and a loss of seed dormancy. Reproductive isolation results from

reduced seed set in interspecific crosses, a reciprocal translocation resulting in reduced F_1 hybrid fertility, and additional genetic incompatibilities that result in further breakdown in pollen fertility in segregating F_2 individuals (Gottlieb 2003).

In contrast to Gottlieb's (1973) view, Grant (1981) argued that *S. malheurensis* likely arose in a peripheral allopatric site, and subsequently came back into contact with the Frenchglen population of *S. exigua* ssp. *coronaria*. Under this scenario, it seems possible that *S. exigua* ssp. *coronaria* originally exhibited a wider distribution at the north end of its range, as it would indicate the presence of suitable habitat outside of the current local. The spatial isolation of the Frenchglen population as well as *S. malheurensis* from the balance of the *S. exigua* ssp. *coronaria* range could thus be the result of a subsequent range contraction. Regardless of its evolutionary history, this site is of special concern, as *S. malheurensis* is currently listed as an endangered species.

An intial allozyme survey revealed a close relationship between *S. malheurensis* and the Frenchglen population, though diversity was markedly reduced in the former as compared to the latter (Gottlieb 1976). To date, however, there has been no investigation of these populations in the context of the broader range of *S. exigua* ssp. *coronaria*, much less the other four subspecies of *S. exigua*. Here we further investigate the Frenchglen population, *S. malheurensis* (using pre-extinction seed collections), and populations from throughout the rest of the range of *S. exigua* using simple sequence repeat (SSR) markers.

MATERIALS AND METHODS

Study Material

The primary focus of this study is a geographically isolated population of *S. exigua* ssp. *coronaria* (termed the Frenchglen population), which co-occurs with the endangered *S. malheurensis*. This population is located in Harney County, Oregon, USA, approximately 25.5 miles south of Burns in a 70-acre Bureau of Land management (BLM) site which has been designated as the Narrows Area of Critical Environmental Concern (ACEC). As *S. malheurensis* no longer occurs in the site, seeds were acquired from Dr. Leslie Gottlieb (University of California, Davis). These seeds were collected in the field in June 1973 (prior to the extinction of this species in the wild) and were grown out and selfed for a single generation in the greenhouse at the University of California, Davis in 1985. In 2009 seeds from each of 42 maternal lineages were germinated and grown in the greenhouse at University of Georgia. Buds from one individual per line were then collected for DNA extractions.

Seeds of *S. exigua* ssp. *coronaria* from the Frenchglen population were also acquired from Dr. Gottlieb. In this case, seeds were originally field-collected from one hundred wild individuals in 1984. These seeds were subsequently germinated and plants were grown at the University of California, Davis in an isolated field with natural pollinators. All seeds were subsequently collected and combined. For the present study, 200 seeds were nicked, germinated, and reared in the greenhouse at the University of Georgia. As above, buds were collected from 42 individuals for DNA extractions. All other samples included in the analyses below have been previously described (Sherman

& Burke 2009). Briefly, buds and/or achenes (i.e., single-seeded fruits) were collected from ten populations of *S. exigua* ssp. *coronaria*, and sixteen populations of the other four subspecies of *S. exigua.* In a previous analysis (Sherman & Burke 2009) *S. exigua* ssp. *coronaria* did not form a distinct group and appears intermixed with the other *S. exigua* subspecies, thus all subspecies are being included in this study. Care was taken to include samples from all subspecies throughout the range. Buds collected in the field were preserved in a saturated NaCl, 30% CTAB solution, shipped back to the lab, and stored at -20° C until DNA could be extracted (Rogstad 1992). Voucher specimens were collected for one or two individuals per population and were deposited at the University of Georgia herbarium. For all samples, DNA was isolated from either the preserved buds or from buds of greenhouse-grown plants (see above) using a modified CTAB-based protocol (Doyle & Doyle 1990).

Genotyping

Simple sequence repeat (SSR) primers were designed from 288 SSR-bearing *Stephanomeria* expressed-sequence tags that were developed and screened for a previous study (Sherman and Burke, 2009). Fourteen of the markers used in the present study were also included in the previous study (Steph_0296, Steph_0227, Steph_0283, Steph_0196, Steph_0094, Steph_0024, Steph_0103,Steph_0004, Steph_0237, Steph_0231, Steph_0288, Steph_0078, Stephy_0195, and Steph_0072), whereas one marker (Steph_0104; forward primer: 5’-CTTGGCAACGTACACACACTC-3’; reverse primer: 5’-TTAGGCGGCAATAACTCCAC-3’; repeat motif GA) was a new addition. Three of the primer pairs used in the previous study (Steph_0140, Steph_0202, Steph_0226) did

not amplifying reliably in *S. malheurensis* or the Frenchglen population, and were thus not analyzed in this study.

All fifteen loci were amplified using a modification of the three-primer PCR protocol outlined by Schuelke (2000; see Wills et al. 2005). Total PCR reaction volumes were 14 μl. Each reaction contained 10 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl, 100 μM each deoxynucleotide triphosphate, 0.02 μM forward primer (with an M13 -29 sequence tail [5'-CACGACGTTGTAAAACGACA-3']), 0.1 μM reverse primer, 0.1 μM fluorescently-labeled M13 -29 primer (labeled with either HEX or TET), and one unit of *Taq* DNA polymerase. Cycling conditions followed a touchdown protocol to reduce non-specific binding (Don *et al.* 1991), as follows: initial denaturation at 95°C for 3 min; followed by 10 cycles of 30 s at 94°C, 30 s at 65°C (annealing temperature was reduced by 1° per cycle), and 45 s at 72°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C; and a final extension time of 20 min at 72°C. Amplicons were then diluted 1:50 or 1:150 (depending on product intensity) and visualized on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA) with MapMarker 1000 ROX size standards (BioVentures, Murfreesboro, TN) included in each lane to allow for accurate fragment size determination. Alleles were called using the software package GeneMarker v. 1.70 (SoftGenetics, State College, PA).

Data Analysis

Descriptive population genetic statistics were calculated on a population and taxon level using GenAlEx v. 6.1 (Peakall & Smouse 2006) (Table 1). These values included percentage of polymorphic loci, mean number of alleles per locus, and gene diversity (calculated as Nei's [1978] unbiased expected heterozygosity; H_e). Relationships

amongst taxa were then graphically assessed via principal coordinate analysis (PCO; GenAlEx) using the covariance matrix with data standardization of genetic distance. Neighbor-joining trees were constructed in PHYLIP 3.67 (Felsenstein 2007) using a distance matrix from MSAnalyzer (Dieringer & Schlotterer 2003) based on Nei et al.'s (1983) genetic distance (calculated from the allele frequency data). Nodal support was calculated with 1000 bootstrap replicates using the CONSENSE program in PHYLIP 3.67 (Felsenstein 2007). Trees were constructed with two data sets, one that included all populations of *S. exigua* ssp. *coronaria* and *S. malheurensis*, and another that also included the remaining *S. exigua* subspecies: *S. exigua* ssp. *carotifera*, *S. exigua* ssp. *exigua*, *S. exigua* ssp. *deanei*, and *S. exigua* ssp. *macrocarpa*. Differences in levels of genetic diversity were investigated by conducting a two-factor analysis of variance (ANOVA) with population and locus as main effects. A least square means differences were conducted using a Tukey HSD test. These analyses were performed using JMP Version 8 (SAS Institute, Cary, NC).

The Bayesian clustering algorithm implemented in the program STRUCTURE 3.0 (Pritchard et al. 2000) was then used to investigate the partitioning of genetic variation across populations/taxa. All STRUCTURE runs were performed using the admixture model with non-correlated allele frequencies, no prior population information, and default parameter settings with a burn in period of 20,000 iterations followed by 100,000 MCMC repetitions, with 5 runs per k value (where k = the number of inferred clusters/subgroups). The method of Evanno et al. (2005) was used to determine the optimal number of clusters. Data were analyzed with three different partitionings of the data set, as follows: (1) *S. malheurensis* was analyzed with Frenchglen (k = 1-4), (2) *S.*

malheurensis, Frenchglen, and *S. exigua* ssp. *coronaria* were analyzed together (k = 1-7), and (3) *S. malheurensis*, Frenchglen, and *S. exigua* ssp. *coronaria* were analyzed along with the other four subspecies of *S. exigua* (k = 1-10).

The demographic history of the Frenchglen population was investigated using the program Bottleneck (Piry et al. 1999). This program tests for evidence of a recent population bottleneck by comparing the observed heterozygosity to that expected at mutation-drift equilibrium. More specifically, population bottlenecks are expected to reduce both allele numbers and heterozygosity, but allelic diversity is reduced more rapidly than observed heterozygosity, due to a transient deficiency in allele number. In other words, the observed heterozygosity will be higher than the expected heterozygosity given the observed allele frequencies and assuming mutation-drift equilibrium. Recent bottlenecks are thus evidenced by an excess of observed heterozygosity Unfortunately, *S. malheurensis* could not be analyzed in the same manner, as evidence indicates it is a primarily selfing species (1973, 1979), and Bottleneck assumes outcrossing.

RESULTS

Stephanomeria malheurensis exhibited a reduction in the percentage of polymorphic loci as compared to the Frenchglen population (66.7% vs. 93.3% respectively) (Table 1). Notably, the predominantly selfing taxon *S. exigua* ssp. *macrocarpa* exhibited reduced levels of polymorphism, on par with that seen in *S. malheurensis*. The mean number of alleles per locus in *S. exigua* ssp. *coronaria* populations ranged from 4.3 (range 2-7) in SECO05 to 6.2 (range 4-10) in SECO08. The Frenchglen population exhibited a slightly

lower value with 4.1 (range 1-11), and *S. malheurensis* was even lower, with just 2.3 alleles per locus (range 1-6). Looking across other subspecies, the highest number of alleles per locus (8.3, range 3-16) occurred in an *S. exigua* ssp. *deanei* population (SED04). While these numbers must be interpreted with caution due to differences in sampling depth, the deepest sampling occurred in Frenchglen and *S. malheurensis*. Interestingly, there were four alleles present in *S. malheurensis* and the Frenchglen population that were not observed in any other *S. exigua* populations. Beyond this, Frenchglen had seven unique alleles, and *S. malheurensis* had three unique alleles. In addition *S. malheurensis* had 5 alleles that were not observed in Frenchglen, but were present in other *S. exigua* populations. In terms of overall levels of genetic diversity, H_e in *S. exigua* ssp. *coronaria* ranged from a low of 0.59 ± 0.05 (mean ± SE) in SECO05 to a high of 0.75 ± 0.04 in SECO08. The value of H_e in Frenchglen was lower than for all other *S. exigua* populations (0.44 ± 0.08), and *S. malheurensis* was lower yet, with a value of 0.2 ± 0.07. When comparisons of mean expected heterozygosity are made at the population level, *S. malheurensis* was significantly lower than other populations, but the Frenchglen population was on par with other populations of *S. exigua* ssp. *coronaria* (and other *S. exigua* subspecies).

The principal coordinate analysis (PCO) revealed that *S. malheurensis* and the Frenchglen population each form independent clusters regardless of the other taxa included (Figure 4.2a). Indeed, *Stephanomeria exigua* ssp. *coronaria* formed a cluster independent of both *S. malheurensis* and Frenchglen, and the inclusion of the remaining *S. exigua* subspecies reveal that, outside of the Frenchglen population, all subspecies exhibited a high degree of overlap (Coordinate 1: 38.74% of the variation, Coordinate 2:

18.89% of variation, and Coordinate 3: 14.82% of the variation), thus only the PCO of all taxa is included (Figure 4.2 a &b) . The neighbor-joining tree based on data from all fifteen loci likewise revealed that Frenchglen and *S. malheurensis* are unique, placing them together on a common, well-supported branch with no other populations grouping with them (with 98 % bootstrap support; Figure 4.3). This same general topology was observed whether or not the additional subspecies of S. exigua were included in the analysis.

Similar to the PCO and neighbor-joining analyses, the STRUCTURE analysis revealed that *S. malheurensis* and Frenchglen form distinct clusters relative to one another (Figure 4.4a). When they are analyzed alongside the balance of *S. exigua* ssp. *coronaria*, the most likely number of clusters stabilizes at k = 3 (Figure 4.4b). When the four additional subspecies of *S. exigua* are added to the analysis, the most likely number of clusters stabilizes at k = 2. In this case, *S. malheurensis* and the Frenchglen population collapse into a single cluster with all other S. exigua populations (including the balance of *S. exigua* ssp. *coronaria*) forming a single cluster (Figure 4.4c).

Finally, the Bottleneck analysis of the Frenchglen failed to detect evidence of a recent bottleneck in this population. This analysis was based on 14 loci, as one locus was monomorphic and thus removed from the dataset prior to analysis.

DISCUSSION

Taken together, the results of this study indicate that the peripherally-isolated Frenchglen population of *Stephanomeria exigua* ssp. *coronaria* is genetically distinctive from

populations in the contiguous range of this taxon, and that it is not genetically depauperate relative to other *S. exigua* populations. *Stephanomeria malheurensis*, on the other hand appears to be genetically depauperate, and shares most of its alleles with the Frenchglen population. This work also revealed that the Frenchglen and *S. malheurensis* populations are quite similar to one another. Indeed, these two populations grouped together in the neighbor-joining analysis, and the STRUCTURE analysis on the full dataset (i.e., including all *S. exigua* subspecies) likewise resulted in the Frenchglen population and *S. malheurensis* being grouped together. This is not, however, to say that the two populations are identical, as the PCO analyses clearly showed that they can be distinguished.

The results of the Bottleneck analysis are particularly interesting, as they suggest that the Frenchglen population did not suffer a recent decline in effective population size between the invasion of cheatgrass following the anthropomorphic disturbance of the early 1970s and the mid-1980s when the collections were made. The lack of a detectable bottleneck following the invasion of cheat grass may be due to the presence of a large and long-lived seed bank. Prior to disturbance, population size estimates were in the range of thousands of individuals (Currin et al. 2007). With the required seed dormancy and apparent longevity of seeds (N. Sherman, personal observation), one might expect the Frenchglen population to have a substantial seed bank. The relatively high levels of diversity in the Frenchglen population are also somewhat surprising in light of its peripheral location. Meta-analyses have suggested that peripheral populations should exhibit reduced genetic diversity and elevated genetic differentiation (Eckert et al. 2008), but the expected loss of diversity is not seen in this case. One possible explanation for

this observation is that *S. exigua* ssp. *coronaria* previously had a broader distribution, and the Frenchglen population is a relic of this former distribution. An alternative explanation is that fluctuating environmental conditions can maintain genetic variation (Eckert et al. 2008; Hoffmann & Hercus 2000). Given the apparently large historical size of the Frenchglen population and the highly variable climactic conditions of this region, this second scenario is quite plausible.

It is difficult to make inferences about the origin of *S. malheurensis* from the current data set, as different analyses showed somewhat different patterns of relatedness. Both the neighbor-joining tree and the STRUCTURE analysis grouped Frenchglen and *S. malheurensis* together, but the PCO analysis separates them from one another (as well as from the balance of *S. exigua* populations). However, the fact that *S. malheurensis* harbors alleles not present in the Frenchglen population indicates the possibility of a non-sympatric origin. *S. malheurensis* harbors eight alleles that are unique from the Frenchglen population, of which only three are unique from all *S. exigua* samples included in the study. The alleles that are unique to *S. malheurensis* may be de novo mutations, alleles that were once present in Frenchglen but have been lost, or alleles that *S. malheurensis* obtained from outside sources. Possible explanations include a sympatric origin of *S. malheurensis* from Frenchglen, with de novo mutations and or subsequent loss of alleles in Frenchglen. This hypothesis is supported by earlier allozyme work showing that *S. malheurensis* has just one unique allele relative to the Frenchglen population (Gottlieb 1976; Gottlieb 2003). Alternatively, *S. malheurensis* may have originated in allopatry, perhaps being derived from another and/or experiencing gene

with another (now extinct) *S. exigua* population, only recently having come into contact with the Frenchglen population.

In making management decisions for the Frenchglen/*S. malheurensis* complex, it appears the best strategy would be to focus on preserving the habitat and reducing the impact from invasive species such as cheatgrass. Although *S. malheurensis* exhibits several unique life history characteristics, it has never accounted for more than ca. 2% of the total Stephanomeria population at the Narrows ACEC (Gottlieb 2003). It also exhibits a number of potentially maladaptive traits, such as a loss of germination dormancy, and thus may be predisposed to extinction, as is the case with many neospecies. In contrast, the Frenchglen population is genetically distinctive and captures much of the genetic diversity present within *S. malheurensis*. Moreover, this population has been very robust in the past. As such, management efforts might be best directed at protecting the peripherally-isolated Frenchglen population as opposed to attempting to re-establish *S. malheurensis* in this area. Genetically unique peripheral isolates, such as the Frenchglen population are likely to be particularly valuable for the future conservation of species in the face of an uncertain climate.

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Table 4.1 Table of mean alleles per locus, expected heterozygosity, and % polymorphic loci per species, subspecies, and population.

Species	Subspecies designation*	Pop	Mean alleles per locus	Mean H_e**	% Polymorphic Loci
S. malheurensis	**SMAL**		2.3(0.4)	0.200(0.067)	66.67%
S. exigua	**SECO (FG)**	**FG**	4.1(0.7)	0.438(0.078)	93.33%
	SECO		14.0(1.6)	0.736(0.036)	100.00%
		SECO002	4.7(0.4)	0.643(0.050)	100.00%
		SECO004	5.3(0.4)	0.602(0.041)	100.00%
		SECO005	4.3(0.4)	0.585(0.054)	100.00%
		SECO006	4.6(0.5)	0.655(0.050)	100.00%
		SECO007	6.0(0.7)	0.652(0.065)	100.00%
		SECO008	6.2(0.6)	0.745(0.043)	100.00%
	SECA		10.4(1.2)	0.721(0.033)	100.00%
		SECA004	6.3(0.7)	0.745(0.035)	100.00%
		SECA005	5.8(0.6)	0.576(0.060)	93.33%

Table 4.1 continued

Species	**Subspecies designation***	**Pop**	**Mean alleles per locus**	**Mean H_e****	**% Polymorphic Loci**
S. exigua	**SEM**		2.9(0.4)	0.379(0.076)	80.00%
	SEE		6.7(0.8)	0.589(0.071)	100.00%
		SEE002	4.9(0.7)	0.484(0.072)	93.33%
	SED		14.3(1.9)	0.733(0.049)	100.00%
		SED002	4.5(0.5)	0.594(0.072)	93.33%
		SED003	5.2(0.6)	0.692(0.037)	100.00%

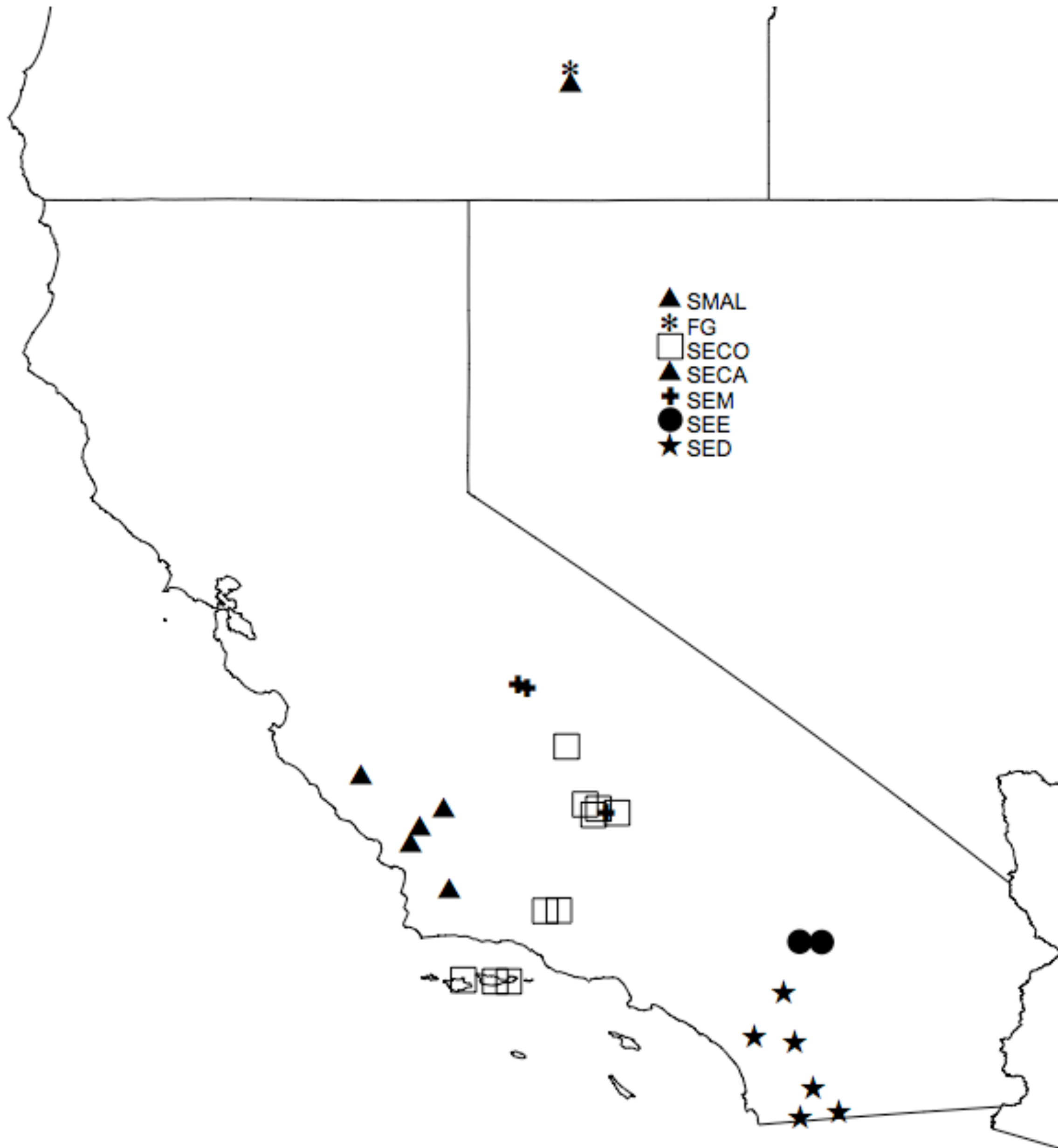

Figure 4.1 Map of California and Oregon, USA, showing the locations of *Stephanomeria* populations sampled for this study. SMAL, S. *malheurensis*; FG, Frenchglen population of *S. exigua* ssp. *coronaria*; SECO, *S. exigua* ssp. *coronaria*; SECA, *S. exigua* ssp. *carotifera*; SEM, *S. exigua* ssp. *macrocarpa*; SEE, *S. exigua* ssp. *exigua*; SED, *S. exigua* ssp. *deanei*

Figure 4.2

Figure 4.2 continued. Unrooted neighbor-joining dendrogram of *S. malheurensis*, the Frenchglen population of *S. exigua* ssp. *coronaria*, and all other *S. exigua* subspecies, constructed using genotypic data from all fifteen SSR loci. Numbers along branches represent bootstrap support after 1000 replicates. Only bootstrap values greater than 50 are shown.

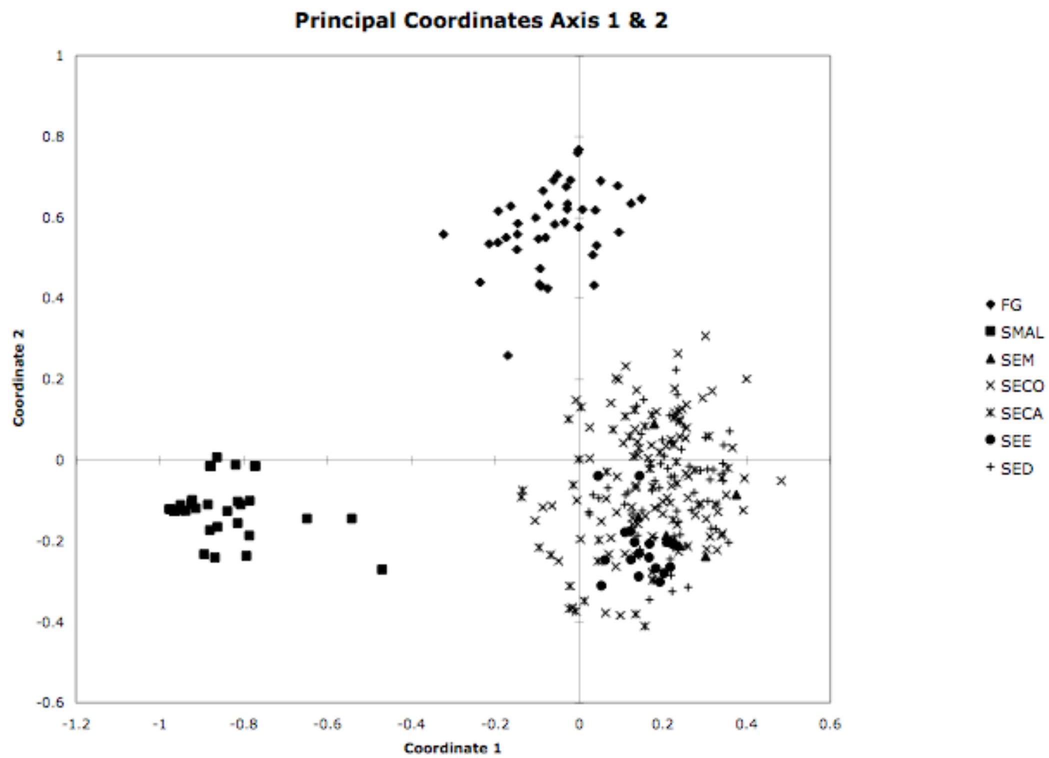

Figure 4.3a

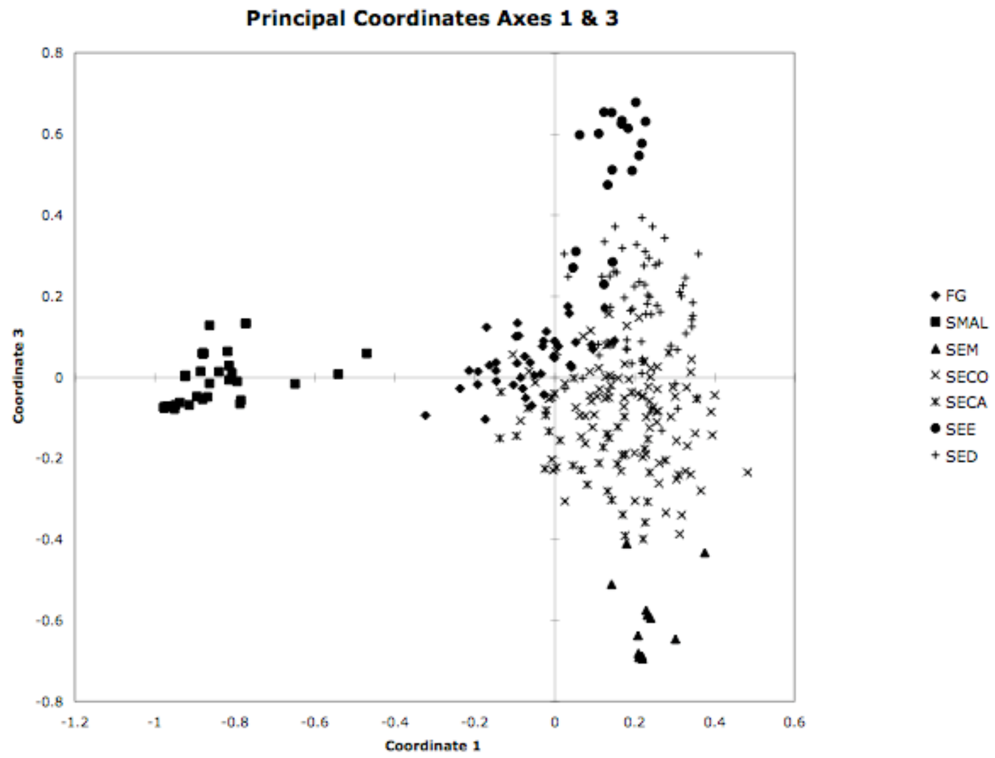

Figure 4.3b

Figure 4.3 a &b continued Results of the principal coordinate (PCO) analysis of *S. malheurensis*, Frenchglen population of *S. exigua* ssp. *coronaria*, and all other *S. exigua* individuals used in this study based on genotypic data from fifteen SSR loci. (a) coordinates 1 & 2, (b) coordinates 1 & 3.

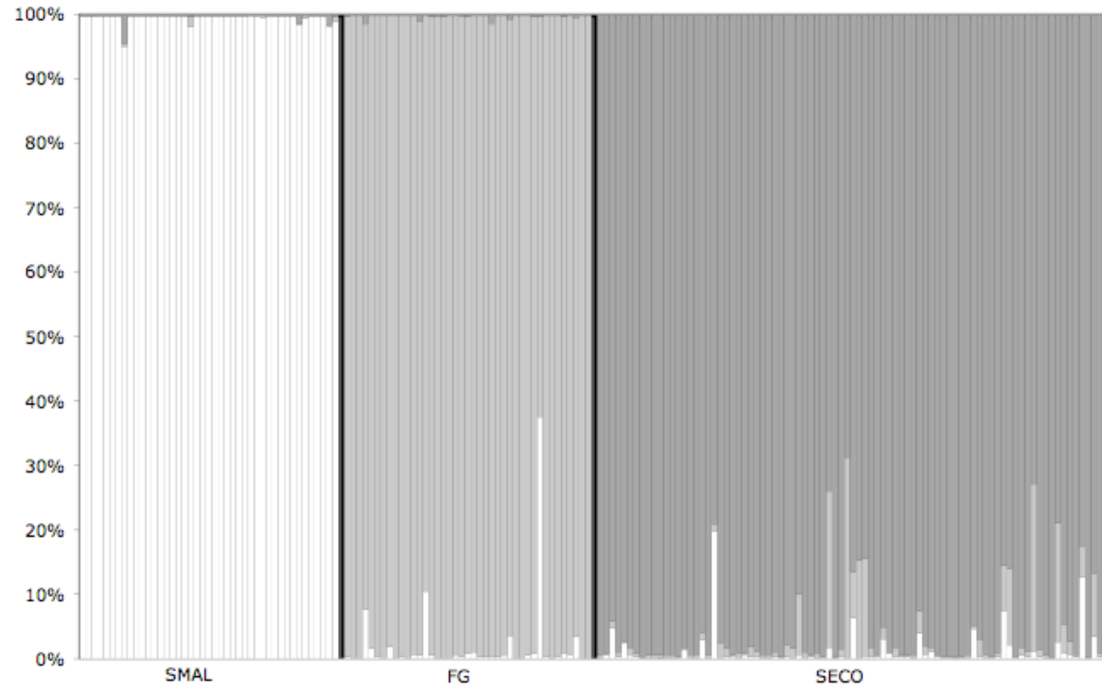

Figure 4.4a

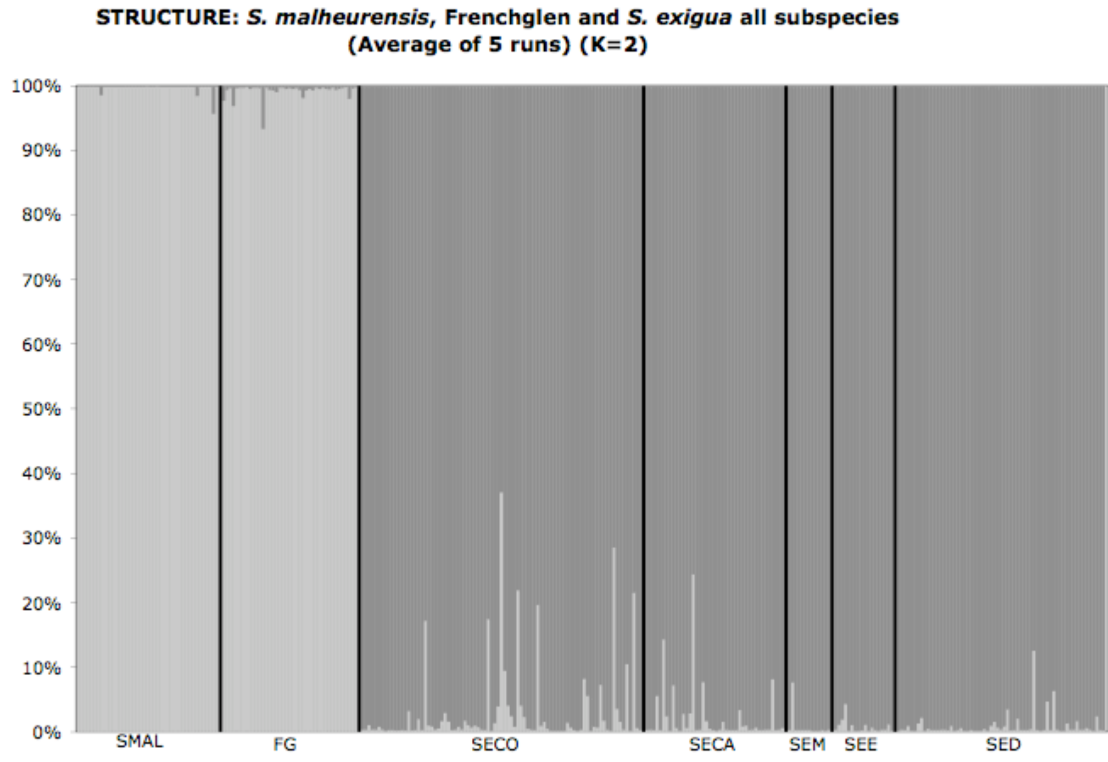

Figure 4.4b

Figure 4.4 a & b continued Results of the STRUCTURE analysis with (a) K = 3 for the analysis of *S. malheurensis*, the Frenchglen population of *S. exigua* ssp. *coronaria*, and *S. exigua* ssp. *coronaria* (b) k=2 for the analysis of S. malheurensis, the Frenglen population of *S. exigua* ssp. *coronaria*, and all subspecies of *S. exigua.* Bars for each individual reflect the average result across five independent runs.

CHAPTER 5

CONCLUSIONS

The chapters of this dissertation provide unique insights into evolutionary divergence within the genus *Stephanomeria*. Briefly, the first study provided clear evidence of a homoploid hybrid origin of *S. diegensis*, the second study identified three likely origins of the allopolyploid *S. elata*, and the third study revealed that the Frenchglen population of *S. exigua* ssp. *coronaria* harbors a level of genetic diversity on par with populations of *S. exigua* ssp. *coronaria* that are within the contiguous range of the taxon, whereas the endangered endemic *S. malheurensis* is genetically quite similar to the Frenchglen population, albeit with greatly reduced levels of variation.

The results of the first study provide strong support for a hybrid origin of *S. diegensis*. The nuclear genome of this species appears to be a mosaic of the *S. exigua* and *S. virgata* genomes, with approximately two thirds being derived from *S. exigua*. Despite the similarity with the nuclear genome of *S. exigua*, *S. diegensis* is karyotypically more similar to *S. virgata* (Gottlieb1969, 1971), and all individuals also carry the *S. virgata* chloroplast haplotype. When the nuclear and chloroplast results are combined, the picture that emerges is one in which pollen flow from *S. exigua* to *S. virgata* gave rise to the hybrid neospecies, and that subsequent backcrossing likewise involved pollen flow from *S. exigua*. The fact that *S. diegensis* appears to be singly-derived places it in the minority

of homoploid hybrid species that have been analyzed to date. While it has been suggested that ecological divergence plays a major role in determining the likelihood of homoploid hybrid speciation, the role of ecological divergence in promoting or limiting hybrid speciation in *Stephanomeria* requires further investigation. A better understanding of the timing of the origin of *S. diegensis*, as well as that of the divergence of the various subspecies within each of the parental species, would also provide interesting additional insights into the origin of this hybrid species, as it remains unclear whether *S. diegensis* arose before or after the divergence of the subspecies within its parental species.

In the second study, it was shown that the allopolyploid *S. elata* has multiple origins, with the chloroplast and nuclear DNA evidence combining to suggest that there may have been at least three independent origins of this species. As such, *S. elata* appears to have had a relatively complex origin, with the original hybrid crosses having occurred in different directions in different instances. Notably, the direction of the cross does not appear to have influenced the morphology of the descendant populations, as has been observed in *Tragopogon miscellus* (Soltis *et al.* 2004). The observed lack of correspondence between cross type and morphology may be the result of selection, with populations at the northern and southern ends of the species range having experienced unique selective pressures, or perhaps it could relate to the specific subspecies involved in the hybrid crosses that gave rise to the various polyploid lineages. Regardless of the cause of the morphological differentition, it remains to be seen whether or not the different origins of *S. elata* are fully reproductively compatible, and thus whether or not the current taxonomy, with a single named species, reflects biological reality.

Given that *S. diegensis* and *S. elata* are byproducts of hybrid crosses between the same pair of parental species, the genus *Stephanomeria* appears to be an ideal system for studying genomic stabilization following hybridization. Indeed, the study of these species in tandem will allow for an analysis of the genomic impact of homoploid vs. polyploid hybridization while controlling for parental identity.

Finally, the results of the third study indicate that the peripherally-isolated Frenchglen population of *Stephanomeria exigua* ssp. *coronaria* is genetically distinct from populations throughout the contiguous range of this taxon, and that it is not genetically depauperate relative to other *S. exigua* populations. In contrast, *S. malheurensis* appears to be genetically depauperate, primarily harboring a subset of the alleles present within the Frenchglen population. This close similarity of the Frenchglen and *S. malheurensis* populations resulted in these two populations being grouped together in both the neighbor-joining and STRUCTURE analyses. This is not to say that these two populations are identical, as the PCO analyses clearly showed that they can be distinguished.Overall, these results are consistent with the view that peripherally-isolated populations can be as important as taxonomically-independent species in a conservation context. Care should thus be taken to account for this fact when making management decisions, as such populations might harbor valuable alleles that could facilitate future adaptation, particularly along the margin of the species' range.

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