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Genetic Diversity among Populations of Endemic Hawaiian *Portulaca sclerocarpa* and *P. villosa* (Portulacaceae) Assessed Using SRAP Markers[1]

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Abstract: Populations of the Hawaiian endemic *Portulaca sclerocarpa* and *P. villosa* are becoming rare throughout their distribution. *Portulaca sclerocarpa* (endemic to Hawai'i Island) and *P. villosa* (populations from Nihoa to Hawai'i Island) are federally listed endangered species. Questions have frequently arisen if they are distinct species. Populations were investigated using sequence-related amplified polymorphism (SRAP), Principal coordinates analysis (PCO), and STRUCTURE analyses to detect a species boundary and genetic diversity among populations. Nuclear (ITS) and chloroplast (*atpI–atpH*, *trnT–psbD*, and *rpl14–rpl36*) regions were compared for plants collected from natural populations or from greenhouse nursery material representing natural populations. SRAP analysis clarified the taxonomic uncertainty of *Portulaca sclerocarpa* and *P. villosa* as distinct species. PCO graphs and STRUCTURE analysis results of $K = 2$ suggested a species boundary between *P. sclerocarpa* and *P. villosa*, and definite population structures among islands. Plants of *P. villosa* from Nihoa were genetically distinct and may represent a new taxon. The taxonomic identity of a collection from Olowalu, Maui, had been previously questioned and was here determined to represent a *P. villosa* form that should be recognized as a distinct variety. Conservation recommendations include regularly monitoring and maintaining the existing populations and individual, and seed collection or vegetative cuttings for propagation. Reintroduction and translocation should be done within the island of population origin in order to preserve unique genetic variations of existing populations.

Keywords: genetic diversity, Hawai'i, population genetics, *Portulaca sclerocarpa*, *Portulaca villosa*, Portulacaceae, SRAP

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The native and introduced species of *Portulaca* (Portulacaceae), known in Hawaiian as *'ihi*, are prostrate to weakly ascending annual and perennial herbs with succulent stems and leaves (Wagner et al. 1999). At present, seven species of *Portulaca* are recognized in Hawai'i, including one indigenous (*P. lutea* Sol. ex G. Forster), three endemic (*P. molokiniensis* Hobdy, *P. villosa* Cham., and *P. sclerocarpa* A. Gray), and three naturalized aliens (*P. oleracea* L., *P. pilosa* L., and *P.* sp. A collected in 1986) (Wagner et al. 1999). The native Hawaiian *Portulaca* species are the result of two colonization events, with *P. villosa* and *P. sclerocarpa* resulting from one, and *P. lutea* and *P. molokinensis* from the other (Ocampo and Columbus 2012).

Ocampo and Columbus (2012) investigated the phylogeny of 59 *Portulaca* species, including the four Hawaiian natives (*P. lutea*, *P. molokiniensis*, *P. sclerocarpa*, and *P. villosa*). Their analysis resulted in a well-supported monophyly of *Portulaca* with an age of the most recent common ancestor of 23 Myr. Their study revealed that two major lineages existed within *Portulaca*: the OL clade (comprising opposite-leaved species) distributed in Africa, Asia, and Australia, and the AL clade (comprising alternate to subopposite-leaved species) that are more widespread and originated in the New World. The Hawaiian species belong to the new world AL clade. Within the AL clade, there are two subclades: the Pilosa clade with terete leaves that includes *P. sclerocarpa* and *P. villosa* as sister species, and the Oleracea clade with flattened leaves that includes *P. lutea* and *P. molokiniensis*. Sequence data for *P. sclerocarpa* and *P. villosa* were identical for ITS, *ndhA* intron, and *ndhF*, and single polymorphisms in *atpI-atpH*, *rpl14-rps8-infA-rpl36*, and *trnT-psbD* (Ocampo and Columbus 2010, 2012).

Portulaca sclerocarpa is closely related to *P. villosa*, sharing a diploid chromosome number of $2n = 18$ (Kim and Carr 1990*a*), and morphologically differ only in capsule characteristics. Kim-Yun (1989) examined morphological traits of several *Portulaca* species, including that of *P. sclerocarpa* grown from seeds collected from a single population and of *P. villosa* from seeds of five populations across the species range. She measured the capsule thickness among species and found that *P. sclerocarpa* did have very thick ovary walls averaging 369.5 μm (range: 240–560 μm), largely due to the average 25 cell layers of sclerenchyma in the capsule wall. *Portulaca villosa* was much thinner, averaging 53.5 μm (range: 20–100 μm); sclerenchyma was also present in the capsule wall but was only one cell layer thick. Capsules of *P. sclerocarpa* are indehiscent or tardily dehiscent as opposed to the capsules of *P. villosa* that are circumscissile near the base. *Portulaca sclerocarpa* is also geographically distinct, occurring only at higher elevations (1,000–1,650 m) at Hawai'i Volcanoes National Park (HAVO) near Puhimau Crater and now largely within the Pōhakuloa Training Area (PTA) between Mauna Loa and Mauna Kea, Hawai'i Island (Wagner et al. 1999). There was a single specimen of *P. sclerocarpa* from Po'opo'o, an islet of Lāna'i, collected in 1982 by (*R. Hobdy 1356*, BISH460483). A botanical survey of Lāna'i offshore islets was unsuccessful in relocating this population (Starr et al. 2006). *Portulaca villosa* is widespread, being reported from Nihoa in the Northwest Hawaiian Islands, Ka'ula (a small islet 35 km SE of Ni'ihau), and all the main islands except Kaua'i and Ni'ihau. It was reported to occur at lower elevations from sea level to 490 m (Wagner et al. 1999), although collections at 1600 m have been made at PTA for this study. Populations of *P. villosa* have been declining and are currently only found on the islands of Nihoa, Maui, Moloka'i, and Hawai'i. Both species are listed as Endangered by the US Fish and Wildlife Service (*P. sclerocarpa* since 1994, *P. villosa* since 2016), and *P. sclerocarpa* has also been listed as Endangered on the IUCN Red List since 2016.

In addition to the capsule thickness trait used to distinguish the species (Kim-Yun 1989), she further found that *P. sclerocarpa* differs significantly in reproductive characters from all other (native and alien) species of *Portulaca* occurring in Hawai'i (Kim and Carr 1990*b*). Time to capsule/seed maturity differed greatly among these species. *Portulaca villosa* matured in 14.9 days, which was similar to all other species tested except *P. sclerocarpa* that was nearly double this at 28.6 days. The number of seeds in *P. sclerocarpa* was also much higher,

with an average of 232.3 seeds per capsule, compared to *P. villosa*, and the other species tested ranged from 22.9 to 76.4 seeds per capsule. The average pollen viability (85–99%) was high among all species and did not show noteworthy differences. Kim and Carr (1990*b*) suggested the unusual volcanic fumarole habitat may have favored the modification/adaptation of the thick-walled indehiscent fruits by *P. sclerocarpa*. Having numerous, small seeds in a thick capsular ovary, such as in *P. sclerocarpa*, has also been found in other small-seeded species and is considered to be one of the adaptation techniques to increase the probability of finding an appropriate habitat for growth (Plaza et al. 2004). This leads to further questions about genetic variations among species and among populations that may reflect these reproductive characters.

The capsule thickness trait has been widely questioned as a character that actually defines a distinction among the two species. Because of their similarities, Geesink (1969) reduced *P. sclerocarpa* to a synonym of *P. villosa*. Wagner et al. (1999) questioned their distinction but maintained them as separate species until further evidence was available.

To clarify taxonomic uncertainty between *Portulaca sclerocarpa* and *P. villosa*, natural populations across each species range were investigated using sequence-related amplified polymorphism (SRAP) analysis (Li and Quiros 2001) to detect a species boundary and genetic diversity among populations. SRAP analysis has been an efficient tool to study closely related plant species, including *Capparis* and *Cleome* species (El zayat et al. 2020), *Portulaca* species with highly diverse morphological variations and varieties (Jia et al. 2017), *Cyclamen* varieties (Qiao et al. 2022), *Eremochloa ophiuroides* populations (Wang et al. 2023), and *Lavandula* varieties (Zagorcheva et al. 2020). Additional sequence data for ITS (White et al. 1990), *atpI-atpH*, *trnT-psbD*, and *rpl14-rps8-infA-rpl36* (Shaw et al. 2007) regions were obtained for each species from all populations across islands to determine if island-specific signals were evident and further investigate the relationship between the two species and among their populations.

MATERIALS AND METHODS

Plant Material and DNA Extraction

A total of 68 plants were collected from natural populations or from greenhouse nursery plants grown *ex situ* from natural populations. Thirty-six individuals of *P. villosa* from 11 different collection sites (including Hawai‘i, Maui, Moloka‘i, and Nihoa Islands) and 32 individuals of *P. sclerocarpa* from six different collection sites (all Hawai‘i Island) were sampled (Table 1). Most plants were collected from natural populations. Plants from the natural populations at Mahana Bay, Pu‘u Anahulu, Pu‘u Papapa, Puhimau thermal areas, and South Point, Hawai‘i, were grown from cuttings at the Volcano Rare Plant Facility of HAVO. Two samples were obtained from the Hui Ku Maoli Ola (HKMO) nursery on O‘ahu and represent *P. sclerocarpa* from Hawai‘i Island (HKMO-s) and *P. villosa* from Maui Island (HKMO-v), both from unknown specific population locations. Two herbarium specimens were used in analyses: *P. sclerocarpa* (*S. Evans 474*, CEMML) from Pu‘u Nohona-O-Hae, Waiki‘i, Hawai‘i, and *P. villosa* (*T. Wager*, *R.B. Shaw & B. Close 4961*, CEMML) from Pu‘u Ke‘eke‘e, Hawai‘i. Nihoa samples were from two natural populations found in West Palm Valley and at Miller’s Peak. Two Maui specimens from now extirpated populations were grown *ex situ*. One was described in Wagner et al. (1999) from near Olowalu, Maui, as morphologically distinctive and possibly a recently naturalized species; it has been maintained in cultivation at ‘Ulupalakua Ranch by Diana Crow (HPDL 12630). The other sample is from Kanaio Beach and was grown at the Maui Nui Botanical Garden nursery (HPDL 12629). All Moloka‘i specimens are from the Kalaupapa National Historic Park nursery and were collected from a single wild population that is suspected to be from Kauhakō Crater, Kalaupapa, although detailed records are unclear. A map indicating population locations is provided (Figure 1). Because both species are federally listed as endangered species, the project was coordinated with the U.S. Fish and Wildlife Service, and all sample collections were made per guidelines in federal permits for

TABLE 1

Portulaca Species and Populations Examined for SRAP and Sequence Analysis with Collection Locality, Voucher Information, Number of Samples, HPDL Accession, and Map Location

Species/Location	Island	V/A	N	HPDL	Map
P. sclerocarpa					
PTA-1	Hawai'i	Unavailable[a]	4	12598–12601	10
PTA-2	Hawai'i	Unavailable[a]	6	12611–12616	11
PTA-3	Hawai'i	Unavailable[a]	1	12621	11
PTA-4	Hawai'i	Unavailable[a]	4	12617–12620	12
PTA-5	Hawai'i	Unavailable[a]	7	12602–12608	13
Puhimau[b]	Hawai'i	BISH 469877	5	12622–12626	14
Pu'u Papapa	Hawai'i	*VanDeMark 110634*	2	12638, 12639	7
Nohona-O-Hae	Hawai'i	*Evans 474*[c]	1	12722[c,d]	6
Pu'u Anahulu	Hawai'i	BISH 616258	1	12725[e]	8
HKMO—s[b]	Hawai'i	*Keir* s.n.	1	12627	
		Subtotal	32		
P. villosa					
Miller's Peak	Nihoa	BISH 436136	5	12592–12596	1
West Palm Valley	Nihoa	BISH 676709	5	12578–12591	2
Kalaupapa	Moloka'i	BISH 718538	5	12631–12635	3
HKMO—v[b]	Maui	BISH 715114	1	12628	
Kanaio Beach	Maui	HAW 44727	1	12629	4
Olowalu[b]	Maui	HAW 44715	1	12630	5
PTA-6	Hawai'i	Unavailable[a]	2	12609[f],12738	10
PTA-7	Hawai'i	Unavailable[a]	5	12610[f],12739–12742	10
Pu'u Anahulu	Hawai'i	*VanDeMark 140218*	2	12636, 12637	8
Mahana Bay	Hawai'i	*VanDeMark 094435*	4	12640–12641	15
		VanDeMark 140557		12642–12643	15
South Point	Hawai'i	*VanDeMark 150105*	4	12644, 12645, 12723, 12724	16
Pu'u Ke'eke'e	Hawai'i	*Wager 4961*[c]	1	12674[c]	9
		Subtotal	36		
		Total	68		

Abbreviations: PTA, Pōhakuloa Training Area; HKMO, Hui Ku Maoli Ola Nursery.
[a]Only a few leaves for DNA analysis of each plant (no vouchers) were collected from populations at PTA. UTM coordinates are available upon request.
[b]Nursery grown
[c]From herbarium specimen
[d]Did not amplify for *atpI–atpH* in sequence analysis.
[e]Did not amplify in SRAP or sequence analysis and was excluded.
[f]Did not amplify in SRAP analysis and excluded from population analysis.

each facility. A permit to receive endangered plants, extract their DNA, and maintain in collection at the University of Hawai'i at Mānoa (UHM) is provided by Hawai'i State DLNR permit I6591.

Fresh or silica-dried leaves from each individual (except the herbarium specimens indicated above) were extracted for DNA using the CTAB method (Doyle and Doyle 1987) with some modifications (Morden et al. 1996).

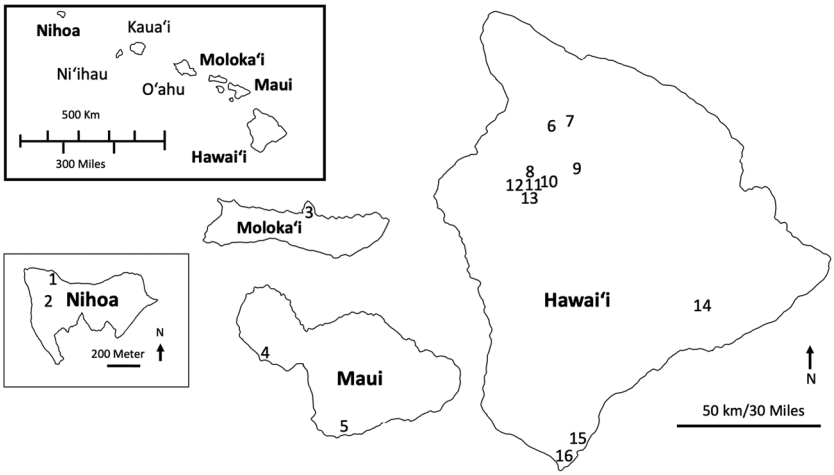

FIGURE 1. Map of Hawaiian Islands with collection locations of *P. sclerocarpa* and *P. villosa*. Numbers correspond to populations indicated in Table 1. Inset: islands where plant collections were made are shown in bold.

The concentration and quality of DNA were determined using a NanoDrop Spectrophotometer (ND-1000, v 3.6.0, Thermo Scientific). All DNA materials were accessioned into the Hawaiian Plant DNA Library (HPDL) at the UHM (Morden et al. 1996, Randell and Morden 1999) (Table 1).

SRAP Analysis

Samples were examined for variation among individuals by the SRAP method (Li and Quiros 2001). Samples were diluted to 10–15 ng/μl and stored at −20 °C until used. Five individuals from different populations were selected to screen 104 SRAP primer combinations (using 11 forward and 20 reverse primers) (Table 2) (Budak et al. 2004, Liao et al. 2016). Sixteen primer-pair combinations from six forward and 12 reverse primers produced clear and reproducible bands and were selected for further study of all samples.

SRAP analyses were conducted using the 20 μl PCR reaction mixture. Cocktails for reactions included 5x Green GoTaq PCR Reaction Buffer [50 mM Tris-HCL (pH 9.0), 50 mM NaCl, 5 mM MgCl2, Promega], 0.25 mg BSA, 0.2 mM dNTPs, 0.5 mM of each forward and reverse primers (IDT, Coralville, IA, USA), 1 unit of GoTaq G2 DNA polymerase (Promega, Madison, WI, USA), and approximately 15–30 ng of DNA. All reactions were carried out using an MJ Research DNA Thermocycler (MJ Research, Waltham, MA, USA) or Eppendorf Thermal Cycler (Eppendorf, Hamburg, Germany). Reaction conditions followed Li and Quiros (2001). Amplified PCR products were separated on a 2% agarose gel, stained with EtBr, and visualized with a UV light source. Negative control reactions (without DNA) were run for all PCR amplifications to ensure reaction components were uncontaminated. Amplifications were repeated if needed with selected samples to confirm the reproducibility of the genetic markers. The marker size was estimated using a 100 bp ladder (Promega, Madison, WI, USA). Final gel images were digitally recorded.

Data Analyses

SRAP markers were scored as either present (1) or absent (0). The data were entered into a binary matrix, assessed for the level of polymorphism and expected heterozygosity (assumption made that populations were in Hardy–Weinberg equilibrium) across individuals within each population, and then averaged across all markers. Expected heterozygosity

TABLE 2

Forward (me) and Reverse (em) SRAP Primers and Selected Primer Combinations

Name	Forward Sequence (5′–3′)	Name	Reverse Sequence (5′–3′)	Primer Combination
me20	TGA GTC CAA ACC GCT GT	em1	GAC TGC GTA CGA ATT AAT	20/2
me21	TGA GTC CAA ACC GGT CA	em2	GAC TGC GTA CGA ATT TGC	20/11
me22	TGA GTC CAA ACC GGG CA	em3	GAC TGC GTA CGA ATT GAC	20/13
me23	TGA GTC CAA ACC GGA TG	em4	GAC TGC GTA CGA ATT TGA	20/15
me25	TGA GTC CAA ACC GGG CT	em5	GAC TGC GTA CGA ATT AAC	20/16
me26	TTC AGG GTG GCC GGA TG	em8	GAC TGC GTA CGA ATT CAC	20/17
		em11	GAC TGC GTA CGA ATT GCA	20/18
		em13	GAC TGC GTA CGA ATT CTA	21/2
		em15	GAC TGC GTA CGA ATT CTT	22/4
		em16	GAC TGC GTA CGA ATT GAT	22/5
		em17	GAC TGC GTA CGA ATT ATG	23/3
		em18	GAC TGC GTA CGA ATT AGC	23/8
				23/11
				25/5
				26/3
				26/7

was calculated for each population in total for each marker as follows:

$$H = 1 - (p^2 + q^2)$$

where p is the frequency of the dominant allele and q is the frequency of the null allele. Genetic relationships within and among populations were estimated using the similarity coefficients of Nei and Li (1979). Principal coordinates analysis (PCO) using Gower general similarity coefficients (Gower 1971) was calculated and plotted using MVSP 3.0 (Kovach 2007). Pairwise similarities were averaged for individuals within and among populations.

STRUCTURE version 2.3.4 (Pritchard et al. 2000, Falush et al. 2007) analyses were used to investigate differences in the distribution of genetic variations among populations. A Bayesian algorithm was employed by placing samples into groups whose members share similar patterns of variation. This algorithm infers genetic discontinuities from individual multilocus genotypes without *a priori* knowledge of geographic location or taxonomy. The program's default settings were used with an admixture model. To determine the most likely number of groups (K) in the data, a series of analyses were performed from K = 1–7 (upper limit determined by the number of islands plus three following Evanno et al. [2005]; island collections included Nihoa, Maui, Moloka'i, and Hawai'i), using a burn-in period and Markov Chain Monte Carlo (MCMC) both set at 200,000 repetitions, with ten iterations per K (Porras-Hurtado et al. 2013). STRUCTURE results were uploaded to Structure Harvester (Earl and vonHoldt 2012) to estimate K (Evanno et al. 2005) and to obtain the graphical representation of the results.

Sanger Sequence Alignments

A previous study (Ocampo and Columbus 2012) investigated *Portulaca* species sequence variation. Based on their results, we sampled 20 individuals representing each population location for four DNA regions: nuclear ribosomal ITS (White et al. 1990) and chloroplast $trnT^{(GGU)}$–*psbD* intergenic spacer (Shaw et al. 2014; hereafter referred to as *trnT–psbD*),

rpl14–rps8–infA–rpl36 (Shaw et al. 2007; hereafter referred to as *rpl14–rpl36*), and *atpI-atpH* intergenic spacer (Shaw et al. 2007).

PCR amplifications were carried out in 25 µl reaction volumes under the following conditions: ca. 25 ng of DNA, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1X *Taq* Polymerase buffer, 1.5 mM MgCl2, 0.50 mg bovine serum albumin (BSA), 0.2 µM forward and reverse primers, and ca. 1 unit of GoTaq G2 DNA Polymerase (Promega, Madison, WI, USA). PCR was performed in a DNA thermocycler (MJ Research) or Eppendorf Thermal Cycler (Eppendorf, Hamburg, Germany). For both nuclear and chloroplast markers, amplification was initiated by a denaturation of 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 3 min. Negative control reactions were run for all PCR amplifications to ensure reaction components were uncontaminated. Amplified products were examined on 1.5% agarose gels, stained with EtBr, and visualized with a UV light source. DNA fragment size was confirmed using the 100 bp ladder (Promega, Madison, WI, USA). The successful PCR products were cleaned using the ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. All samples were bi-directionally sequenced using forward and reverse primers. Samples were sequenced at the University of Hawai'i Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) facility (http://hawaii.edu/microbiology/asgpb) or GENEWIZ, LLC (South Plainfield, NJ), with PCR products undergoing a cycle sequencing reaction using BigDye terminator chemistry (Applied Biosystems, Foster City, CA). Sequences were aligned using the aligner function in Geneious Prime (ver. 2022.0.1; Biomatters, New Zealand) with default parameters. Final adjustments were done by visual inspection.

RESULTS

SRAP Analysis

A total of 65 samples were ultimately analyzed using the selected 16 primer combinations. Three of the 68 samples were removed from the data due to their poor quality and inconsistent PCR results (12725: *P. sclerocarpa* Pu'u Analuhu, Hawai'i; 12609 and 12610: *P. villosa*, PTA, Hawai'i). There was a range of 7–21 markers per primer pair (average: 12.2). Notable genetic diversity was found in both *P. sclerocarpa* and *P. villosa* among Hawaiian Islands. A total of 195 loci were scored, of which 142 loci (72.8%) were polymorphic (Table 3). SRAP analysis identified 31 unique markers for *P. sclerocarpa* and 29 unique markers for *P. villosa* (Table 3), although these markers were variable among populations for these species. Polymorphism among islands ranged widely. *Portulaca villosa* varied from 13.2% on Maui (n = 3), 24.2% on Moloka'i (n = 5), 38.5% on Nihoa (n = 10), and 63.9% on Hawai'i (n = 16). Variation for *P. sclerocarpa* on Hawai'i was the highest at 65.2%, but also had the most samples analyzed (n = 31). The overall expected heterozygosity was 0.183 across all samples and ranged from 0.021 (Maui: *P. villosa*, n = 3) to 0.138 (Hawai'i: *P. villosa*, n = 16).

Genetic similarity (Gower 1971) between species and among islands was compared (Table 4). A value of 0.0 indicates complete dissociation, and 1.0 indicates complete genetic identity. Similarity among species and islands ranged from 0.792 (*P. sclerocarpa* and Nihoa *P. villosa*) to 0.936 (Maui and

TABLE 3

SRAP Analysis Data Summary

Species and Island	*N*	*A*	PA	*P*	%*P*	He
P. sclerocarpa						
Hawai'i	31	135	31	88	65.2	0.161
P. villosa						
Nihoa	10	109	8	42	38.5	0.073
Moloka'i	5	91	1	22	24.2	0.043
Maui	3	91	2	12	13.2	0.021
Hawai'i	16	133	11	85	63.9	0.138
All Islands	34	154	29	119	61.0	0.167
Combined Data	65	195	—	142	72.8	0.183

N = number of samples; *A* = number of loci observed; PA = number of private alleles; *P* = polymorphic markers; %*P* = percentage polymorphism; He = expected heterozygosity.

TABLE 4

Pairwise Population Genetic Identity Matrix (Nei and Li 1979) between *Portulaca sclerocarpa* and *P. villosa* among Islands Based on SRAP Data

	Species		1	2	3	4	5
1	*P. sclerocarpa*	Hawai'i	0.875				
2	*P. villosa*	Nihoa	0.792	0.926			
3	*P. villosa*	Maui	0.807	0.815	0.959		
4	*P. villosa*	Moloka'i	0.803	0.793	0.936	0.949	
5	*P. villosa*	Hawai'i	0.795	0.797	0.833	0.821	0.853

Moloka'i *P. villosa*). Variation among individuals on the same island was typically higher and ranged from 0.853 for Hawai'i *P. villosa* to 0.959 for Maui *P. villosa*.

Genetic relationships for *P. sclerocarpa* and *P. villosa* within and among populations and islands were visualized using PCO. Samples from all islands examined together indicated a notable genetic boundary between the two species along axis 1 (Figure 2). There was also a notable grouping of individuals among islands. For *P. villosa*, Nihoa individuals were very distinct from those of other islands along axis 2. Individuals from Maui and Moloka'i grouped closely together, and the plant from Olowalu overlapped slightly with those from Hawai'i. Hawai'i individuals showed considerable variation along axis 1.

Populations of *P. sclerocarpa* and *P. villosa* from Hawai'i Island were analyzed by PCO separately (Figure 3). The two species are clearly separated along axis 1. For *P. villosa* (triangles), individuals from each of the four populations with multiple collections (Mahana Bay, PTA, Pu'u Anahulu, and South Point) grouped more closely together. Similarly,

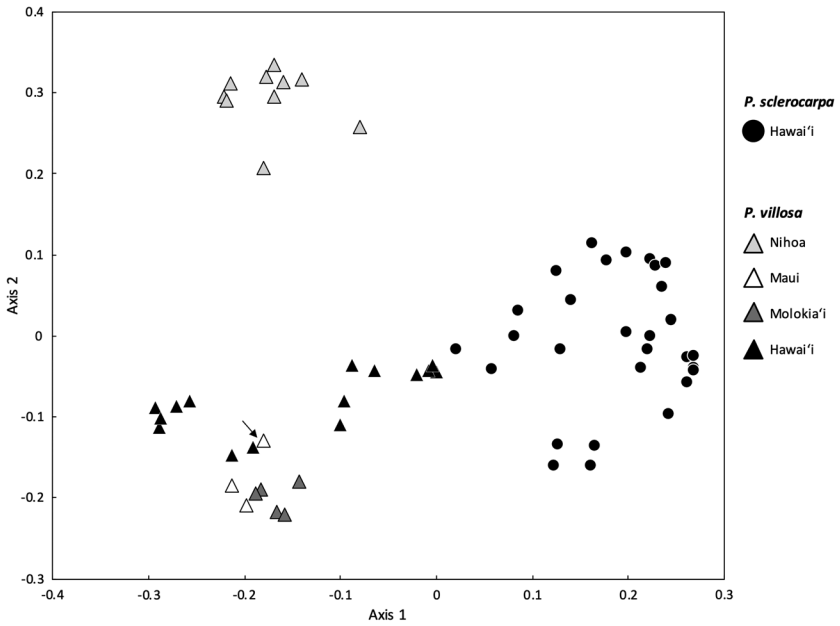

FIGURE 2. PCO graph based on SRAP data showing the relationship among *P. sclerocarpa* and *P. villosa* individuals collected from all islands. The first two principal coordinates accounted for 33.9% of the variation among samples (axis 1, 20.7%; axis 2, 13.2%). Arrow indicates the collection from Olowalu, Maui.

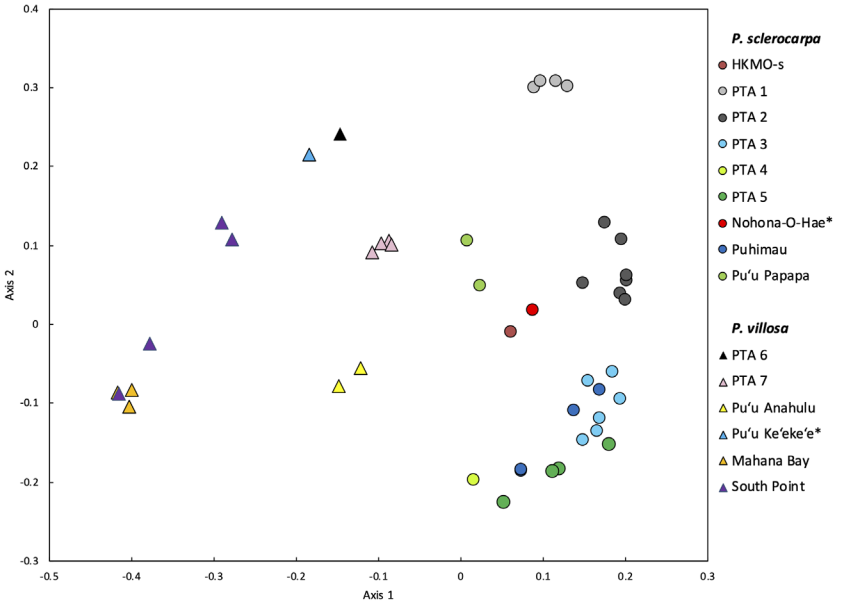

FIGURE 3. PCO graph based on SRAP data showing relationships among *Portulaca sclerocarpa* and *P. villosa* individuals collected on Hawai'i Island. The first two principal coordinates accounted for 38.4% of the variation among samples (axis 1, 24.8%; axis 2, 13.6%). Pōhakuloa numbers represent different field locations within this geographic area. Asterisks (*) indicate samples from herbarium specimens.

individuals of *P. sclerocarpa* (circles) from the same population grouped closely together.

STRUCTURE analyses were carried out at two different levels (Figure 4). First, all samples of *P. sclerocarpa* and *P. villosa* were examined across all islands of population origin and for multiple values of *K* (Figure 4*A*,*B*). Second, samples of *P. sclerocarpa* and *P. villosa* from only Hawai'i Island were examined, also for multiple values of *K* (Figure 4*C*,*D*). Both line graphs generated by STRUCTURE Harvester indicate $K = 2$ as the uppermost level of structure for both analyses (Figure 4*A*,*C*). Genetic groups are indicated by bar graphs with the proportion of color within bars representing each group from $K = 2$ to $K = 4$. Across all islands (Figure 4*B*), Nihoa populations were distinct from those of other islands at $K = 3$ and $K = 4$. Plants of *P. sclerocarpa* were largely distinct from those of *P. villosa*, although there was some admixture of the genetic groups found in some individuals of *P. villosa* on Hawai'i Island. For $K = 2$ and $K = 3$, Maui and Moloka'i (and Nihoa individuals at $K = 2$) were genetically similar to *P. villosa* on Hawai'i Island, although they were distinct at $K = 4$. On Hawai'i Island (Figure 4*D*), the species are clearly distinct at $K = 2$, although admixture is also evident in *P. villosa* individuals from PTA, Pu'u Anahulu, Pu'u Ke'eke'e, and South Point.

DNA Sequence Analysis

One individual from each population/location (20 individuals in total) was represented in the initial sequence analysis of the four DNA regions examined (Table 5). Amplified products from two individuals would not sequence properly for two gene regions with repeated attempts (HPDL 12725 for ITS and HPDL 12727 for *atpI-atpH*). A total of 3,702 bp were sequenced across the four gene regions (636 bp in ITS, 1355 bp in *trnT–psbD*, 1,041 in *rpl14–rpl36*, and 670 bp in *atpI–atpH*). Aligned sequences of *P. sclerocarpa* and *P. villosa* were identical for *rpl14–rpl36*. A 5-bp insertion and a 1-bp indel were found among *trnT–psbD* sequences, and a 1-bp SNP was found among

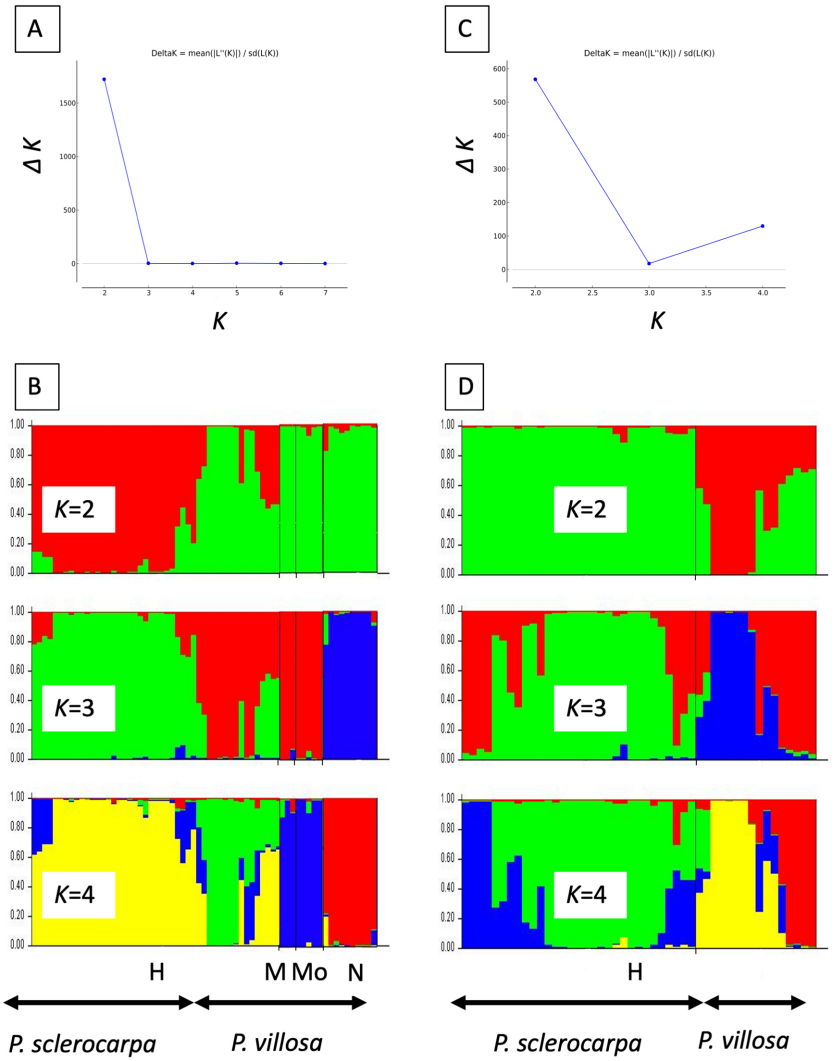

FIGURE 4. *K* graphs and bar plots for *Portulaca sclerocarpa* and *P. villosa* based on STRUCTURE analysis. (*A*) $K = 2$ identified for collections from all islands. (*B*) STRUCTURE bar plot for collections from all islands based on $K = 2$, $K = 3$, and $K = 4$. Vertical lines separate collections from each island (H = Hawai'i, M = Maui, Mo = Moloka'i, N = Nihoa). (*C*) $K = 2$ identified for collections of *P. sclerocarpa* and *P. villosa* from Hawai'i Island. (*D*) STRUCTURE bar plot for collections from Hawai'i Island based on $K = 2$, $K = 3$, and $K = 4$. Vertical lines separate collections of *P. sclerocarpa* and *P. villosa*.

atpI–atpH sequences (Table 6). The 5-bp insertion was found only in the *P. villosa* population from Pu'u Ke'eke'e, and the 1-bp indel and SNP were both variable across species and populations. The ITS region was also identical in sequence with the exception of a single 3-bp insertion-deletion (indel) found at positions 611–613 of the aligned sequence (Table 7). Two individuals of *P. villosa* (both from Nihoa) shared this 3-bp segment. To confirm the extent of this indel, ITS was sequenced for all individuals (62 in total). The 3-bp segment was present in all 10 Nihoa individuals of *P. villosa*. None of the *P. villosa* or *P. sclerocarpa* from Hawai'i, Maui, or Moloka'i Islands shared this 3-bp segment.

TABLE 5

Portulaca sclerocarpa and *P. villosa* Samples Sequenced for this Study and GenBank Accessions for Chloroplast *psbD–trnT*, *rpl14–rp36*, *atpI–atpH*, and Nuclear ITS

Species	HPDL	*trnT–psbD*	*rpl14–rp36*	*atpI–atpH*	ITS[a]
P. sclerocarpa	12602	OR820939	OR840900	OR840916	OR873568
P. sclerocarpa	12623	OR820940	OR840895	OR840917	OR873585
P. sclerocarpa	12627	OR820941	OR840899	OR840920	OR873589
P. sclerocarpa	12638	OR820942	OR840898	OR840918	OR873612
P. sclerocarpa	12722	OR820943	OR840897	[b]	OR875236
P. sclerocarpa	12725	OR820944	OR840896	OR840919	[b]
P. villosa	12587	OR840881	OR840904	OR840922	OR873591
P. villosa	12588	OR840882	OR840905	OR840923	OR873592
P. villosa	12609	OR840883	OR840906	OR840924	OR873601
P. villosa	12610	OR840884	OR840907	OR840925	OR873602
P. villosa	12738	OR840894	OR840915	OR840935	OR873621
P. villosa	12628	OR840885	OR840908	OR840926	OR873603
P. villosa	12629	OR840886	OR840909	OR840927	OR875235
P. villosa	12630	OR840887	OR840902	OR840928	OR873604
P. villosa	12631	OR840888	OR840910	OR840929	OR873605
P. villosa	12636	OR840889	OR840911	OR840930	OR873610
P. villosa	12640	OR840890	OR840912	OR840931	OR873614
P. villosa	12674	OR840891	OR840901	OR840932	OR873620
P. villosa	12723	OR840892	OR840913	OR840933	OR875237
P. villosa	12724	OR840893	OR840914	OR840934	OR873590

[a]GenBank ID for additional 43 *Portulaca* ITS sequences are among accessions OR873564 to OR873622.
[b]Sequences not available due to repeated poor PCR or Sanger sequence results.

No other variation was found among samples for the ITS region.

DISCUSSION

Hawaiian plant species are often found with substantial morphological and molecular-based genetic variation yet little or no genetic variations at the DNA sequence level. Examples include *Sesbania tomentosa* (Fabaceae; Cole and Morden 2021), *Acacia koa* (Fabaceae; Daehler et al. 1999, Adamski et al. 2012, LeRoux et al. 2014), *Sida fallax* (Malvaceae; Pejhanmehr et al. 2022, Pejhanmehr et al. 2024), and *Metrosideros polymorpha* (Myrtaceae; Aradhya et al. 1991, James et al. 2004, Stacy and Sakishima 2019). Each of these studies represented species with their distributions across the high islands of Hawai'i and demonstrated distinct morphological, ecological, and population genetic divergence among populations. This indicates that it is critical to study Hawaiian taxa further with special attention to morphological variation along with genetic markers that can detect signals for population-level differences where extensive sampling across the species genome is possible.

Portulaca sclerocarpa and *P. villosa* were genetically distinct based on SRAP analysis and, as such, do represent separate species. There was also considerable variation found for each species, both within and among populations and islands. Although sequence variation was found among populations of both species, there were no apparent differences distinguishing the two species based on the sequences examined. Indel and SNP variation was present among collections of both species and not identified with any specific geographical location except for ITS in Nihoa populations.

TABLE 6

Indel or SNP in Sequences of *P. sclerocarpa* and *P. villosa* for Chloroplast *trnT-psbD*, *atpI–atpH*

Species	HPDL	*trnT–psbD* (960–964)[a]	*trnT–psbD* (1157)[a]	*atpI–atpH* (393)[a]
P. sclerocarpa	12602	GC-----TT	GA-TTTTTTTTTTCG	TT**C**GA
P. sclerocarpa	12623	GC-----TT	GA-TTTTTTTTTTCG	TT**C**GA
P. sclerocarpa	12627	GC-----TT	GA**T**TTTTTTTTTTCG	TT**G**GA
P. sclerocarpa	12638	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. sclerocarpa	12722	GC-----TT	GA**T**TTTTTTTTTTCG	[b]
P. sclerocarpa	12725	GC-----TT	GA-TTTTTTTTTTCG	TT**C**GA
P. villosa	12587	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12588	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12609	GC-----TT	GA-TTTTTTTTTTCG	TT**C**GA
P. villosa	12610	GC-----TT	GA-TTTTTTTTTTCG	TT**C**GA
P. villosa	12738	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12628	GC-----TT	GA**T**TTTTTTTTTTCG	TT**G**GA
P. villosa	12629	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12630	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12631	GC-----TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12636	GC-----TT	GA-TTTTTTTTTTCG	TT**C**GA
P. villosa	12640	GC-----TT	GA**T**TTTTTTTTTTCG	TT**G**GA
P. villosa	12674	GC**TTAAT**TT	GA**T**TTTTTTTTTTCG	TT**C**GA
P. villosa	12723	GC-----TT	GA**T**TTTTTTTTTTCG	TT**G**GA
P. villosa	12724	GC-----TT	GA**T**TTTTTTTTTTCG	TT**G**GA

[a]Numbers represent the position of indel or SNP (in bold font) in the aligned sequence.
[b]Sequence not available due to repeated poor PCR results.

TABLE 7

Alignment of ITS Sequences Surrounding the Insertion-Deletion Position and the Number of Collections Assessed on Each Island

Species	*N*	Island	Portion of ITS DNA Sequence
P. sclerocarpa	31	Hawai'i	CGGACCCTTA---GGTGGTATGTCACGCAAAACTATGCGA
P. villosa	18	Hawai'i	CGGACCCTTA---GGTGGTATGTCACGCAAAACTATGCGA
P. villosa	3	Maui	CGGACCCTTA---GGTGGTATGTCACGCAAAACTATGCGA
P. villosa	5	Moloka'i	CGGACCCTTA---GGTGGTATGTCACGCAAAACTATGCGA
P. villosa	10	Nihoa	CGGACCCTTAGGTGGTGGTATGTCACGCAAAACTATGCGA

Sequence starts at position 601 and ends at position 640 of the aligned ITS sequence. Dashes indicate the location of the sequence indel (positions 611–613).

Earlier studies based on morphology and anatomy did find variation present among the characters studied (Kim-Yun 1989, Kim and Carr 1990*b*). However, the source material for *P. sclerocarpa* was from a single population on Hawai'i Island (Puhimau, HAVO), while that of *P. villosa* was from Nihoa, Hawai'i Island (South Point) and Maui (Manawainui Gulch and Kaupo). The collections for their study of both *P. sclerocarpa* and *P. villosa* were not representative of the diversity for either species across Hawai'i Island. Further investigation of the correlation between plant locations, ecological information, and morphological

characters may help to explain the variations and make meaningful identifications between *P. sclerocarpa* and *P. villosa* on Hawai'i Island.

Natural hybridization among the two species has not been documented but is highly possible. Artificially produced hybrids have been generated (Kim and Carr 1990*a*), and F_1 plants were found to have pollen fertility ranging from 45% to 95% with apparent fertile seed production of 22 to 33 seeds per capsule. This is fewer than normally found in either parental species, where *P. sclerocarpa* averaged 232.3/capsule and *P. villosa* averaged 66.8/capsule (Kim and Carr 1990*b*). Wide variations in the thickness of the ovary wall and fruits were also noted among hybrids. Although hybrids were not fully fertile, there exists a strong likelihood of natural hybrids occurring where species are sympatric that could lead to introgression among the species. This would be consistent with the finding of admixture of genotypes in individuals identified as *P. villosa* in this study.

An alternative interpretation to admixture through hybridization is that *P. sclerocarpa* is derived from *P. villosa* populations of west Hawai'i, where the species occurred from near sea level to over 1500 m elevation, and the variation found here is a remnant of that transition. A gradation of genetic diversity from one species to the other is consistent with the diversification of morphological forms at these higher elevations. Future studies should investigate the origin of this admixture within these populations using genomic studies that can better assess the chromosomal diversity.

The Nihoa populations of *P. villosa* were genetically distinct from those on Hawai'i, Maui, and Moloka'i based on both population markers and DNA sequence. There are no known collections of *P. villosa* from Kauai or Ni'ihau, but it had been previously collected on Ka'ula and each of the other main Hawaiian Islands. It is possible that the Nihoa populations should be recognized as taxonomically distinct from the remainder of the species, either as a new species or subspecific taxon. However, there are no identified morphological differences of Nihoa *P. villosa* plants that have been documented, and this should be examined more fully.

A plant collected near Olowalu (west Maui) was included as a novel form of *P. villosa*, although it was suspected to be either a previously unknown introduced species or an as-yet undescribed endemic taxon (Wagner et al. 1999). These distinctive plants differed from other *P. villosa* by their erect, subshrub habit (vs. fleshy and prostrate), dark burgundy stems (vs. gray-green), dark green leaves (vs. gray-green) that are 1–2 mm (vs. 1.5–3 mm) wide with tufts of dingy white hairs (vs. yellowish-brown) in the leaf axils, and flowers with 14 stamens (vs. 18–50) and 3-branched styles (vs. 5- to 7-branched). Although the population is no longer present, plants have been maintained in cultivation at 'Ulupalakua Ranch and were made available for study by horticulturalist Diana Crow (H. Oppenheimer, personal communication). Our results place this closely to other plants in the Hawai'i-Maui-Moloka'i group. There were only three Maui plants of *P. villosa* available for this study (due to declining numbers of individuals and the loss of populations), and the Olowalu plant does not cluster closely with the other two but instead is more closely affiliated with plants from Hawai'i Island. As such, there is support to include the Olowalu plant within *P. villosa*, where it should be recognized as a distinct variety based on its unique morphological traits.

Conservation Recommendations

There has frequently been confusion regarding the differentiation of *P. sclerocarpa* from *P. villosa*. Following its recognition as a species by Asa Gray (1854), the status of *P. sclerocarpa* has been questioned and was placed in synonymy with *P. villosa* by Geesink (1969). The two species were later recognized as distinct by Wagner et al. (1999) based on the capsule thickness with recognition that further study of the species was needed. In preparation for this study, our preliminary hypothesis was that the two species would be genetically indistinguishable and should be placed as synonyms. However, our study using SRAP analysis clarified this taxonomic uncertainty, and they should be recognized as distinct species. Further, it is our recommendation that the Nihoa population is genetically distinct from

the remainder of the Hawaiian populations of *P. villosa*, and a study should be made to determine if there are morphological distinctions to allow it to be recognized separately.

Conservation efforts are needed for both *P. sclerocarpa* and *P. villosa*. Both are federally listed endangered species, and *P. villosa* is rare on the islands of Hawai'i, Maui, and Moloka'i, and extirpated from Lāna'i and O'ahu. Hawaiian portulacas are known to be readily vegetatively propagated (Bornhorst and Rauch 2003), and efforts to expand the collections of the Olowalu, Maui plant with potential goals to reintroduce it to natural environments should be pursued. Plants being grown in nurseries regularly produce seedling volunteers (personal observation), and source plants for outplanting should be readily obtainable. Outplanting efforts of the Olowalu form of *P. villosa* are presently underway by the Hawai'i Invertebrate Program (Division of Forestry and Wildlife) in the vicinity of its original habitat in an effort to provide nectar for yellow-faced bees (*Hylaeus* spp.) (K. Bustamente, Hawai'i Invertebrate Program, personal communication). Management activities for these species currently include seed collection, propagation for reintroduction, and translocation within the island population origin in order to preserve unique genetic variations of existing populations. Over 35,000 seeds representing more than 100 founder individuals of *P. sclerocarpa* are in seed storage at the PTA Seed Storage Facility, and over 75,000 seeds of *P. villosa* from Maui, Nihoa, and O'ahu are maintained in seed storage on O'ahu (N. Kingsley, Seed Conservation Laboratory, Lyon Arboretum, University of Hawai'i, personal communication). Efforts to outplant *P. sclerocarpa* at HAVO (Pratt et al. 2011) and PTA on Hawai'i Island have yielded mixed results warranting additional research to optimize outplant and seedling establishment. To better conserve these species, additional research is needed to understand and manage other factors that may be limiting population recovery, such as seed depredation by rodents (Pratt et al. 2011). Because HAVO and PTA are home to extant populations and individuals of these species, regular monitoring and management are essential for their protection.

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