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CONSERVATION GENETICS OF NABALUS SERPENTARIUS AND ITS COMMON CONGENER, NABALUS TRIFOLIOLATUS (ASTERACEAE) WITH IMPLICATIONS ON CONSERVATION

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by

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Abstract

Nabalus serpentarius (Pursh) Hooker is a species of concern to conservation management on the island of Nantucket, MA where four populations have been recently observed. A comparative population genetic study was carried out on *N. serpentarius* and a common congener, *N. trifoliolatus* using genetic markers developed from *Taraxacum kok-saghyz* and *Nabalus serpentarius*. Evaluations of genetic diversity and population structure of both *Nabalus* species on Nantucket were conducted. *Nabalus serpentarius* showed no reduction in population genetic variation as compared to the common congener, and the two species demonstrated similar and very low levels of population genetic structure. These data indicate that genetic threats to the rare species are not an immediate concern. However, given the small numbers of populations and the potential for stochastic demographic threats such as disease and weather phenomena, we recommend protection of all populations and encourage any programs that bolster population number.

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Introduction

Conservation biology is the practice of maintaining or restoring parts of ecosystems to prevent extinction or extirpation of species, reestablish endangered organisms, or to maintain or increase biodiversity. The implementation of conservation practices fluctuates depending on the specific intentions of the organization involved, however the overarching theme is preventing the loss of biodiversity in response to anthropogenic stressors (Soulé, 1985). These stressors can be relatively small like localized pollution or anthropogenic deforestation, or they can be as grand as climate change. Conservation biologists inform conservation management groups to improve the effectiveness of their action.

Conservation management often attempts to increase or maintain diversity in ecosystems, species, or genetic variation. It is important to first identify the appropriate units for protection and management (Allendorf, Hohenlohe, & Luikart, 2010). The unit on which management is focused is referred to as a management unit (MU). Conservation genetics is one of many tools used to effectively manage the conservation of any MU (taxa, population, etc.) within a region. Conducting a population genetic study helps to better understand the variation within and among divisions of that MU which can provide insights into appropriate management strategies. Genetic variation can be seen as the smallest unit of biodiversity (Kyrkjeeide, et al., 2020)and is used as a proxy for determining the level of risk a population may be under. It is thought that the greater the variation is, the greater potential a species might have to adapt to environmental stressors (Bruford, Davies, Dulloo, Faith, & Walters, 2017). Given that low levels of genetic variation can limit adaptive and evolutionary potential in the long-term (Sgrò, Lowe, & Hoffmann, 2011), a central goal of conservation biology and, consequently, conservation genetics is to understand and preserve population and species genetic variation (Soulé, 1985). Understanding the genetic

variation within and among populations allows conservation managers to determine a species' susceptibility to certain threats and which individuals or groups of individuals are necessary for the maintenance of high levels of genetic variation in the defined region. This is especially important in rare species.

Rarity of an organism can arise under various circumstances and can be described based on different aspects including geographic range, habitat specificity, or local population size (Rabinowitz, 1981), but rarity can generally be defined as having low abundance and/or small ranges (Gaston, 1994). The understanding and protection of genetic diversity is particularly important for rare and threatened species which often exhibit small population sizes (Kyrkjeeide, et al., 2020), low genetic variation (Gitzedanner & Soltis, 2000), and reduced connectivity or higher population genetic structure (Kyrkjeeide, et al., 2020). Reductions in population size often lead to a reduction in mating opportunities putting these species at greater risk of loss of alleles through genetic drift and increased homozygosity. Increased homozygosity could lead to reduced fitness from inbreeding depression and the fixation of deleterious alleles (Charlesworth & Charlesworth, 1999; Wright, 1931). Genetic drift in small populations leads to a progressive loss of genetic variation (genetic erosion) that will reduce fitness of these populations (Soulé, 1985). If the species incurs an extended period of genetic erosion, it may enter a logarithmic decline known as an extinction vortex (Aguilar, et al., 2019; Fagan & Holmes, 2006; Gilpin & Soulé, 1986). Numerous efforts and interventions can be put in place when informed by conservation genetic data, including *in situ* (protection of the species in their natural habitats) and *ex situ* strategies (preservation of the species by housing genetic variation out of their natural habitat e.g., in a germplasm or seed bank). Therefore, data from population genetic studies of endangered species can provide information relevant to conservation planning. While studies of

this kind often find evidence for reduced levels of genetic diversity in rare populations or species, a common approach is to compare population genetic parameters of the rare species with those of a closely related and common congener species (Ellis, et al., 2006; Gitzedanner & Soltis, 2000; Purdy & Bayer, 1996). This approach is useful in providing a comparative framework in which to evaluate the measures of genetic diversity for the rare species. With the intention of informing conservation management groups, I carried out a population genetic analysis of a rare and threatened plant species, *Nabalus serpentarius*, and compared these metrics to a related species *N. trifoliolatus*.

Study System

Nabalus, once a subgenus of *Prenanthes* (Milstead, 1964)was originally described by Cassini (1825). In Cassini's description of North American of *Nabalus*, he recognized the similarities between *Nabalus* and *Prenanthes* (Milstead, 1964). New species of *Nabalus* are still being described and corrected taxonomically (Chen, et al., 2019; Wang, et al., 2020) but, however, the genus currently consists of 17 generally recognized species and one hybrid (ICN portal, 2021). Some species within this genus are globally listed as critically imperiled, imperiled, or vulnerable within their respective ranges (G1-G3; NatureServe, 2021) and many rely on disturbances, often anthropogenic, to persist.

Nabalus serpentarius (Pursh) Hooker (2n = 8; Babcock, et al., 1937), originally *Prenanthes serpentaria* Pursh, is a perennial, herbaceous plant that occupies diverse habitats throughout its native range but commonly thrives on disturbed land (roadsides, powerline right of way, etc.) (Everett & Lepley, 2002; Haines, 2011; Horn, et al., 2005; Ballou, personal observation). The species grows to about 5 to 6 feet tall and has coarse, purple stems that are

filled with latex. Its leaves are alternately arranged and generally irregularly lobed, especially towards the base of the plant. One of the unique characteristics of some *Nabalus* species, as seen with *N. serpentarius*, is that there is typically considerable variability in leaf shape within an individual plant (Johnson, 1980). The upper third of the mature plant has a panicle-like cyme of capitula. Each capitulum has a subtending involucre that consists of two whorls of green phyllaries. Each capitulum consists of 9 to 13 cream or yellow-colored corollas. Each flower is 8 to 14 mm long with the petals fused into a tube on the lower portion and develops into a single cypsela (a single seeded, dry, indehiscent fruit). Extending from each achene is pappus that generally aids in wind dispersal. The means of pollination are uncertain; however, insects have been observed to visit these plants (Everett & Lepley, 2002).

Colloquially known as Lion's Foot, the native range of *N. serpentarius* is in the Eastern United States of America (from Florida to Vermont and New Hampshire and west to Ohio, south through Kentucky to Mississippi) (Everett & Lepley, 2002). Throughout most of this range *N. serpentarius* is listed secure (N1(U.S.) & G1), however, the global status has not been reviewed since 1984 (NatureServe, 2021). In its Northern range it is listed as vulnerable (New York and Pennsylvania(S3)), critically imperiled (Massachusetts(S1)), or possibly extirpated (New Hampshire (SH)) (NatureServe, 2021).

In Massachusetts, *N. serpentarius* has lost much of its original habitat likely due to human population development and reforestation after abandonment of agricultural land (Everett & Lepley, 2002). Rabinowitz (1981) established seven forms of rarity based on geographic range, habitat specificity, and local population size. Considering the rarity of this species in the context of these standards, the whole species maintains a large range, spanning about a quarter of the United States. It has a narrow habitat specificity due to its dependence on disturbance and, in

Massachusetts, it appears to be small and non-dominant having few known sites with few individuals per site (pers. obs.). Four collection sites of *N. serpentarius* have been identified on the island of Nantucket, Massachusetts (Jennifer Karberg, Kelly Omand, pers. comm.) and at each of these sites 20 or fewer individuals were able to be found.

When evaluating a rare species, it is important to consider what controls might be used as points of comparisons for measures of genetic diversity. Related, common congeners are often used as controls by which to compare genetic variation and population structure in rare species (Baskauf, et al., 1994; Ellis, et al., 2006; Gitzendanner & Soltis 2000). This reference species, that is believed to share an evolutionary history, allows for the assignment of "higher" or "lower" levels of genetic variation as calculated from various population genetic statistics. The common congener chosen for this project is *Nabalus trifoliolatus* Cassini, the three-leaved rattlesnake-root, because it inhabits the island, is known from five current sites (pers. comm. Kelly Omand) and shares a generic identity with *N. serpentarius*. Although a full phylogeny for *Nabalus* is not known to have been published, it is accepted, with confidence, that species within the same genus share a recent common ancestor allowing for congeneric comparisons within genus in the absence of a robust phylogeny (Gitzedanner & Soltis, 2000). *Nabalus trifoliolatus* (2n = 8; Babcock, et al., 1937), originally *Prenanthes trifoliolata* (Cass.) Fernald, is the closest known relatives to *N. serpentarius* on the island of Nantucket, MA making it the ideal option as a congener species for population genetic comparisons. The species has the similar habit to *N. serpentarius* of occupying regularly disturbed areas but is associated with damp, shaded areas within forests (personal observation). The species was described by Cassini (1825) as an herbaceous plant that is about 1.5 feet tall (~ 0.457m). The leaves are alternate, divided until the base into three lanceolate leaflets, and unequally and irregularly lobed with ciliate margins

(Bogler, 2006; Cassini, 1825 (roughly translated)). The inflorescence forms a panicle with numerous pedunculated, pendant heads with pale yellowish ray florets with a pale yellow pappus (Bogler, 2006).

Rationale for the Study

Nabalus serpentarius is a regionally threatened species, known at the start of this study from only four sites on Nantucket Island. Understanding the variation and distribution of alleles within and among these sites will allow conservation agencies to better protect the greatest level of variation present and to delegate attention to unique variation such as private alleles (alleles with that occur within a single or few populations), rare genotypes, and sites with greater variation. I hypothesize that there will be lower levels of variation in *N. serpentarius* compared to the congener on the island of Nantucket due, in part, to the local rarity of the species seen in the relatively low number of individuals found on the island. With fewer individuals, there are fewer reservoirs of genetic material increasing susceptibility to genetic drift. Considering that the species is located on an island, there is a potential for noticeable levels of inbreeding (Jennings, et al., 2016). There is a potential that it experiences gene flow from the mainland but this has not yet been investigated. If the species arrived in one colonization event, then it might be expected that the species experienced a bottleneck, or founder effect, and would have low levels of genetic variation. Even if the sites are a result of multiple colonization events, only four are known with few individuals and they may not retain high levels of variation over time as it is unlikely that substantial gene flow is occurring between mainland and island sites. It is commonly believed that large effective population sizes are needed to maintain diversity (Sgrò, Lowe, & Hoffmann, 2011; Soulé, 1985). These sites will likely suffer greater risk of loss of genetic variation as genetic drift erodes the present variation (Soulé, 1985). The common congener may also exhibit

low variation given similar island isolation affects as above, but the species has the potential to harbor greater variation due to having more individuals present on the island (pers. obs.). I also hypothesize that the structure of the variation on the island will be low and resemble that of a single population for both species. *Nabalus serpentarius* is reported to be wind dispersed (Everett & Lepley, 2002) and the sites are close together allowing for the possibility of unhindered mixing of geographical sites. If wind dispersed, *N. trifoliolatus* may experience a slight reproductive barrier in the more densely forested sites where it is located on the island. Low population structure may warrant less concern of the loss of any single population (Ellis, et al., 2006) considering the genetic variation that is lost may be represented in the remaining populations. However, if population genetic structure is high, the most distinct populations will be of greatest concern.

To elucidate allelic diversity of these populations, I used existing population genetic markers from a related genus, *Taraxacum* (*Taraxacum* and *Nabalus* both are in tribe Cichorieae) and new markers developed using previously generated genomic data to genotype individuals. Genotypic data from *N. serpentarius* was compared to similar data for *N. trifoliolatus*. Analyses of the resulting data allowed me to understand the levels and structuring of genetic diversity across the rare populations, as well as understand if the overall variation in the species is relatively low. The results will be used to inform conservation management on the island of Nantucket.

Materials and Methods

Sampling and Extraction of DNA

In July of 2016, 76 total tissue samples (one leaf from at most 20 individuals per collection site) were collected from 4 known locations of *N. serpentarius* from Nantucket, Massachusetts by Drs. Jennifer Karberg and Jennifer Mandel. When plants were clustered in groups (rarely), care was taken to sample plants a minimum of 10-15 centimeters apart to minimize the potential of collecting very close relatives. For each collection, tissue was placed in a plastic bag with dry silica crystals and GPS data were collected. After all tissue was collected, it was mailed to the Mandel Lab at the University of Memphis. The collection sites were named as follows: *Nabalus serpentarius* Airport (NSAP), *Nabalus serpentarius* Airport South (NSAS), *Nabalus serpentarius* Middle Moors (NSMM), and *Nabalus serpentarius* Raceway (NSRW). Exact coordinates are being withheld for the security of the collection sites.

In July of 2017, I collected one to two leaves from 19 to 21 *N. trifoliolatus* individuals per collection site from five locations on Nantucket island based on information from Kelly Omand. The number of individual samples collected within each site is variable due to the lack of spacing between individuals, i.e., numerous individuals were found but many were clustered within centimeters of each other. Only one individual from each cluster was collected to avoid collecting sibling plants and the plants from which tissue was taken were a minimum of 15 cm apart. Most collections were estimated two or more meters apart. Leaf tissue, once collected, was stored and shipped in the same manner as the *N. serpentarius* samples. The populations were named as follows: *Nabalus trifoliolatus* Squam Farm (NTSF), *Nabalus trifoliolatus* Norwood

Farm (NTNF), *Nabalus trifoliolatus* Masquetuck (NTMT), *Nabalus trifoliolatus* Squam Swamp (NTSS), and *Nabalus trifoliolatus* Windswept (NTWS).

The Squam Farm property was once agricultural land and is now largely populated by hardwood forests, freshwater bogs, shrub swamps, and grassy meadows. Upon observation of the forested areas where *N. trifoliolatus* was found, the forests were damp and the meadow areas were relatively dry. Adjacent to this property was the more heavily forested Squam Swamp which is characterized by dense vegetation, including but not limited to: ferns, trees, and low areas filled with a *Sphagnum* species. The Norwood property is similar to the Squam Farm property with the additions of shrublands and rolling hills. The Masquetuck property is a forest that is bordered by marshlands. The windswept cranberry bog property has a series of cranberry bogs surrounded by grassland and dirt trails with few forested areas between the bogs. In every population, *N. trifoliolatus* was found in the forest understory on the side of trails. Although all sites were thoroughly searched, no individuals were found beyond the edge of the trail.

DNA was extracted from all tissue samples collected in 2016 and 2017. Leaf tissue was either ground for 3 minutes in the Geno/Grinder 2000 (SPEX Sample Prep, Metuchen, NJ) or at 3.1 speed for 4, 30 second cycles in a Fisherbrand™ Bead Mill 24 Homogenizer (Thermo Fisher Scientific, Waltham, MA). An OMEGA Bio-tek E.N.Z.A. SQ Plant DNA kit (Omega Bio-tek, Inc., Norcross, GA) was used to extract DNA from the tissue with the SQ1 buffer modified by adding 7.5 mg of PVP and 6.75 mg of 120 Ascorbic acid per 750µL of SQ1 buffer. The kit protocol was otherwise unchanged. DNA quantity and purity were assessed using a Thermo Scientific Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Transferability of Taraxacum *markers to* Nabalus serpentarius

Taraxacum kok-saghyz L.E.Rodin (Russian dandelion; hereafter abbreviated as TKS) primers that target microsatellite, or Simple Sequence Repeat (SSR), loci were chosen due to the genetic relatedness of the genus *Taraxacum* to *Nabalus* (Mandel, et al., 2017) and the availability of primers (McAssey, et al., 2016) and TKS DNA. All seventeen primer pairs from McAssey, et al. (2016) were ordered from Integrated DNA Technologies (IDT; Integrated DNA Technologies, Coralville, IA). These primers were tested for efficacy with a sample set of 8 DNA samples (4 *N. serpentarius*, 3 *N. trifoliolatus*, and 1 TKS control). Polymerase Chain Reaction (PCR) (protocols and reagents list in Table S1) was carried out in a BioRad T100 Thermocycler using a program with the following specifications: initial denaturation step of 3 min at 95ºC, followed for 40 cycles with 30 sec denaturation at 94ºC, 30 sec annealing at 55ºC and 1 min extension at 72ºC, with a final 10 min extension period at 72ºC. The products of the PCR were separated via electrophoresis in a 1% agarose gel. The voltage on the electrophoresis apparatus was set to 120V. To visualize DNA fragments, 4.5μL of each sample was mixed with a gel red/loading buffer mix (1ml of 3x LD and add 2ul of gel red 10,000X). Once the fragments had migrated roughly 4/5 the distance across the gel, the gel was imaged in a Benchtop 2UV® transilluminator. Eight primers produced consistent bands between 100 and 500bp.

Development of Nabalus *specific nuclear primers*

A *Nabalus serpentarius* genomic library was generated using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, Massachusetts, USA) following to the manufacturer's protocol. The sample was enriched with the Comp-1061 Bait Kit of Mandel, et al. (2014) following established protocols in the Mandel Lab (e.g., Mandel, et al., 2019).

Sequencing was performed on an Illumina MiSeq sequencer (paired end, sequencing chemistry V2, 150 bp; Illumina, San Diego, California, USA) at the Feinstone Center for Genomic Research (University of Memphis, Memphis, Tennessee, USA).

Data for primer development was obtained by quality trimming *N. serpentarius* raw FASTQ data and assembling cleaned reads following the approach outlined in Mandel, et al. (2014). Contigs in the form of FASTA data were exported and subsequently imported to Geneious v 6.5 (Biomatters, Ltd., Auckland, New Zealand) where the Phobos v3.3.12 tandem repeat finder plugin (Mayer, 2006-2010) was used to determine potential primer pairs from microsatellite repeats within the sampled contigs. Primers were designed produce amplicons at the length of 100 to 500 base pairs to match the range of the DNA ladder that was available. Many possible primer pairs were predicted. From the these, thirty primer pairs were selected based on expected repeat motif of the microsatellite and expected amplicon length to maximize diversity of possible lengths between 100 and 500 base pairs and repeat motifs (di- through penta-). Each potential primer was assessed via tblastx to determine if the targeted sequences were genic or non-coding.

Twenty primer pairs (Table 1) were chosen and ordered from IDT. Eight samples separate from those collected in 2016 and 2017 were used to test these newly designed primers using the same methods observed in the TKS trials. Twelve of these twenty primers produce reliable amplicons within the 100 to 500 bp range.

Table 1. Pairs of microsatellite primers developed from *N. serpentarius* FASTA data using the Phobos tandem repeat finder within Geneious.

Primer Name	Reverse Primer	Forward Primer
NS01	TTTGCGCTCTAACTTGCCAC	TTCATTGATGATACCGAGCTGGT
NS02	AAGAAGAACCTTTAAACTAGCCAAA	ATCAATCCCTTGCCGATGCT
NS03	AGTCGGTGAACGGATCGAAC	AAGCTGAGAGGGAGGCAGTA
NS04	CGCAATCCGCCTCTTTCATG	CCGGCCACAAACATTAAGCA
NS05	ACAAGGAGGTGCAAAGGGAG	GTGGAGGCTGCAAAGGGTAT
NS06	CCAACATCATCGGCAAACCC	GGGCACAGATCCCTTCAACA
NS08	AACCACCACCATGAAGTCGT	CACTGCCCTGCCAGAAAAAC
NS11	GTCGATCGAGAAGGAGGCAG	ACCCTTGCATGGTGGTCAT
NS12	AGTTCTAACCTCCATCCAACACT	TAAGGTATTGGGCCCGCATG
NS14	AGCAGAGGAGAGCCGAGTTA	GTGTCGGAGAAATGGCCTGA
NS15	TCAAGGTAGCAGCAGCCAAA	AGCCCGATGAGCTGTTGAAA
NS17	GGCTTCCAGCATCCATCTGT	ACATCATTCATCATGTATTGGCAA
NS18	TGCCTACCAACCCAGCAAAA	GATGGCGGTAGATGTGGAGG
NS20	GATTGCCAAAAAGCATGAGGC	AGTTGCGGATACAGTGGGTG
NS23	GGTCTTGAACTCCTGGGCTC	GGATGGAGAGAGGGAGGGAA
NS24	CATGACCGCTTCAAGCATCG	GTCGGTTTAGTGCGGTCAGA
NS26	GGTCCTGCAGCACTGAATTG	GTTACAAGCCACCTAAGCGC
NS27	AGGTGAGAGGTAGGATGCGT	GTCTCAAACTCCTGGTCCCTG
NS28	ACTGTTTTTGACTTGTTTGGTATTGT	TCAAAAAGCGGTCCCAAACG
NS29	ATGACCAAGTCAGGACCCTG	ACTGGGTGATGCAGGTAAGA

Genotyping and analysis

The eight TKS and twelve *Nabalus* primers were used to amplify a subset of the DNA. Genotyping was performed at the Molecular Resource Center at UTHSC by Thom Cunningham on an ABI 3130XL Sequencer and data were output in fragment analysis data (FSA) format. FSA files were uploaded to GeneMarker v 2.6.3 for allelic scoring.

The potential for genotyping errors due to null alleles, short allele dominance (large allele dropout) and scoring errors due to stuttering were assessed using Micro-Checker (Van

Oosterhout, et al., 2004). The results of this analysis were used to guide the removal of spurious loci prior to population genetic analysis. Any monomorphic loci and loci out of Hardy-Weinberg Equilibrium were removed from downstream analyses.

The GenAlEx 6.5 (Peakall & Smouse 2006, 2012) plugin in Microsoft Excel was used to estimate private alleles, mean number of alleles at a locus (N_a), mean number of effective alleles (N_e), expected and observed heterozygosity (H_E and H_O, respectively), inbreeding coefficient (F). Population genetic structure was calculated by Wrights's F statistics (Wright, 1949) and estimated through an analysis of molecular variance (AMOVA) framework (Excoffier, et al., 1992; Weir & Cockerham, 1984) in GenAlEx. The program was set to partition observed variance into within and among population categories. P-values for H_E, H_O, F, and AMOVA data were generated using the Analysis Toolpak add-in found in Microsoft Excel. Nei's genetic distance (Nei, 1972) and pairwise F_{ST} were calculated in GenAlEx. With a similar approach to what was done within each of the study species, AMOVA was also analyzed between the two species using the shared set of loci. Unbiased expected heterozygosity (uHe) was assessed for each species on a per locus basis to better understand differences in variation seen between each primer set. P-values for each species were generated by comparison between primer sets using the Analysis Toolpak add-in found in Microsoft Excel.

Population structure in *N. serpentarius* and *N. trifoliolatus* was investigated using the Bayesian, model-based clustering algorithm implemented in the software STRUCTURE v 2.3.4 (Pritchard, et al., 2000). Individuals were assigned to K population genetic clusters based on their multi-locus genotypes. The *N. serpentarius* and *N. trifoliolatus* datasets including both TKS and NS primers were run separately using the program testing potential K values 1 through 8 with 5 replications of each K. The burn-in period was set to 200,000 iteration with 1,000,000 posterior

iterations. STRUCTURE results were saved then compressed to a zip file format to be uploaded to Structure Harvester (Earl & vonHoldt, 2012) to determine the most likely K value via the Evanno method (Evanno, et al., 2005). Subsequently, five permutations of each run result for the best K value were performed in CLUMPP (Jakobsson & Rosenberg, 2007) and plots were generated using distruct (Rosenberg, 2004). A three principal coordinate analyses (PCoA; one for each species and one between species) were conducted on pairwise genetic distances (Nei's) in GenAlEx in Microsoft Excel.

Results

MicroChecker Analysis

Bonferroni analysis using MicroChecker (Van Oosterhout, et al., 2004) provided evidence of scoring error due to stuttering, large allele dropout, or null alleles via homozygote excess in primers NS11, NS26, TKS25, and TKS123 of the *N. serpentarius* samples and NS5, NS11, NS26, TKS25, and TKS97 of the *N. trifoliolatus* samples.

Assessment of Genetic Variation

Using the GenAlEx plugin for Microsoft Excel, mean number of alleles (N_a), the number of effective alleles (N_e), heterozygosity values (H_O and H_E), inbreeding (F), and private alleles were found for *N. serpentarius* and *N. trifoliolatus* separately (Table 2).

Table 2. Comparison of mean values and ±SE for number of alleles at a locus (Na), number of effective alleles (Ne), observed and expected heterozygosity (Ho and He, respectively), and inbreeding coefficient (F) between primer sets calculated using GenAlEx 6.5 in Microsoft Excel (P- value: HO: 0.907, HE: 0.994, F: 0.979).

Species	Population	N	Na	Ne	Ho	uHe	F
N. serpentarius	MM	14.58 (0.84)	4.33 (0.70)	2.95 (0.45)	0.57 (0.08)	0.59 (0.06)	-0.01 (0.09)
	NA	15.50 (1.01)	4.42 (0.82)	2.97 (0.592)	0.58 (0.09)	0.56 (0.07)	-0.04 (0.11)
	NAS	17.5 (0.79)	4.50 (0.93)	3.10 (0.59)	0.55 (0.10)	0.58 (0.07)	-0.004 (0.13)
	NR	17.25 (1.84)	4.25 (0.81)	2.47 (0.50)	0.46 (0.09)	0.49 (0.08)	0.04 (0.10)
N. trifoliolatus	MT	17.63 (0.80)	4.63 (0.71)	2.73 (0.46)	0.55 (0.12)	0.54 (0.10)	-0.04 (0.09)
	NW	18.75 (0.53)	5.00 (0.89)	2.80 (0.53)	0.55 (0.13)	0.53 (0.11)	-0.08 (0.09)
	SF	17.88 (0.95)	4.50 (0.80)	2.91 (0.66)	0.57 (0.11)	0.54 (0.10)	-0.10 (0.06)
	SS	19.13 (0.74)	4.88 (0.79)	3.12 (0.68)	0.61 (0.12)	0.56 (0.10)	-0.12 (0.07)
	WS	19.5 (0.68)	5.13 (0.85)	3.24 (0.62)	0.60 (0.14)	0.57 (0.11)	0.04 (0.16)
N. serpentarius	**Total**	**16.21 (0.61)**	**4.38 (0.40)**	**2.87 (0.26)**	**0.54 (0.04)**	**0.55 (0.03)**	**-0.003 (0.05)**
N. trifoliolatus	**Total**	**18.58 (0.34)**	**4.83 (0.35)**	**2.96 (0.25)**	**0.58 (0.05)**	**0.55 (0.04)**	**-0.06 (0.043)**

Table 3. Description of loci for *Taraxacum kok-saghyz* (TKS) and *Nabalus serpentarius* (NS) based primers and mean values for unbiased expected heterozygosity for *N. serpentarius* and *N. trifoliolatus*

Locus	Repeat motif	Location	*N. serpentarius*	*N. trifoliolatus*
TKS03*	Di-	5' UTR	0.47 (0.07)	0.56 (0.04)
TKS25**	Tri-	5' UTR	0.43 (0.09)	0.07 (0.02)
TKS97	Di-	3' UTR	0.51 (0.001)	0.62 (0.07)
TKS105**	Tri-	3' UTR	0.40 (0.05)	0.33 (0.03)
TKS111	Tri-	CDS	0	0
TKS113	Tri-	CDS	0	0
TKS123	Tri-	CDS	0.28 (0.16)	0.18 (0.11)
TKS138*	Tri-	CDS	0.36 (0.06)	0
TKS177**	Tri-	CDS	0.21 (0.02)	0.26 (0.04)
NS1**	Di-	Unknown	0.87 (0.02)	0.84 (0.01)
NS4**	Di-	Unknown	0.76 (0.02)	0.80 (0.02)
NS5	Di-	Unknown	0.51 (0.0004)	0.31 (0.19)
NS6*	Di-	Unknown	0.86 (0.02)	0.82 (0.04)
NS11**	Tri-	CDS	0.64 (0.04)	0.69 (0.04)
NS14	Tri-	CDS	0.58 (0.02)	0.52 (0.004)
NS15**	Tri-	CDS	0.67 (0.05)	0.70 (0.02)
NS18	Tri-	Unknown	0.51 (0.0008)	0.52 (0.009)
NS20**	Tri-	Unknown	0.60 (0.03)	0.69 (0.02)
NS26*	Tetra-	Unknown	0.38 (0.16)	0.16 (0.10)
NS28	Tetra-	Unknown	0	0
NS29	Tetra-	Unknown	0	0
TKS Mean			**0.30 (0.04)**	**0.22 (0.04)**
NS Mean			**0.53 (0.04)**	**0.50 (0.04)**

*Primers that were used for analysis of only *N. serpentarius*

**Primers that were used for analysis of both *N. serpentarius* and *N. trifoliolatus*

P-value: *N. serpentarius* (0.046) and *N. trifoliolatus* (0.038)

Assessment of Population Structure

An analysis of molecular variance (AMOVA) was performed to determine the percent of variance within populations compared to among populations (Figure 1). F_{ST} in *N. serpentarius* was 0.066 and 93% of the observed variance was within populations. F_{ST} in *N. trifoliolatus* was

0.016 and 98% of the observed variance was within populations. The AMOVA between the two species showed that F_{ST} was 0.14 and 86% of the observed variance was within species.

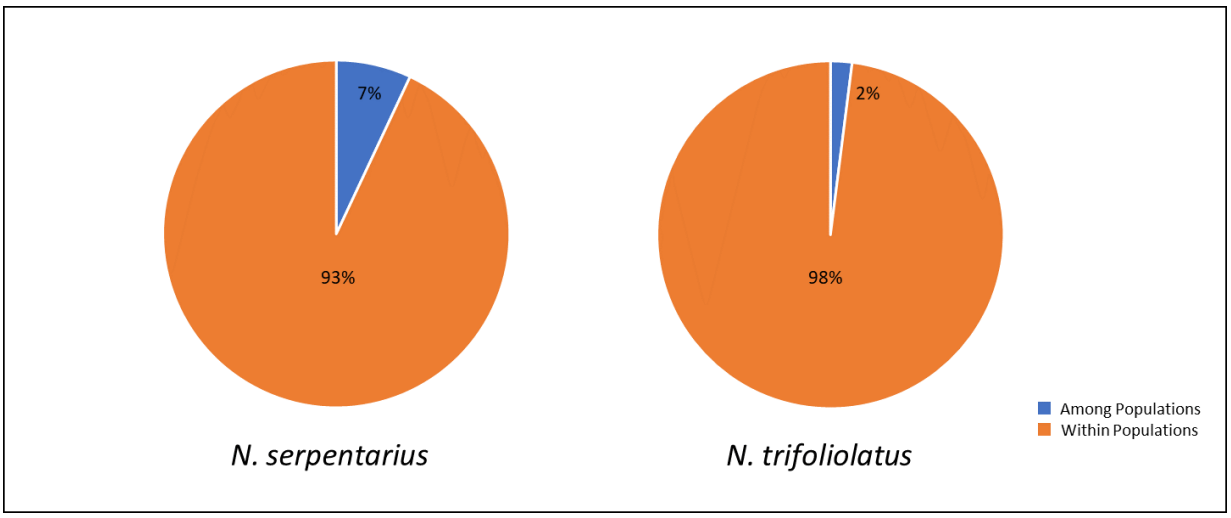

Figure 1. Analysis of Molecular Variance in *Nabalus serpentarius* and *Nabalus trifoliolatus* using both *Taraxacum* and *Nabalus* primers. (P- value: 0.061)

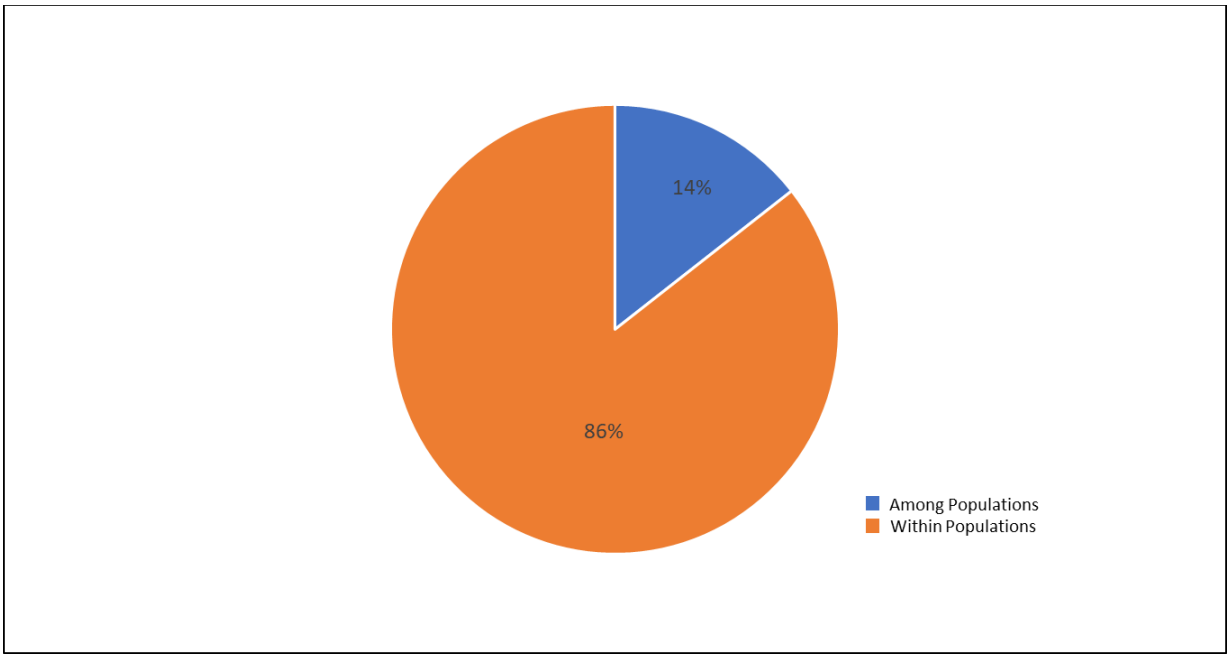

Figure 2. Analysis of Molecular Variance between both *Nabalus* species among shared loci.

To estimate population genetic structure between collection sites, pairwise F_{ST} and Nei's genetic distance (Table 4) were calculated in GenAlEx. PCoAs (Figure 2) were generated for each species to assess genetic dissimilarities among collection sites.

Table 4. Pairwise F_{ST} (Bottom Diagonal) and Nei's Genetic Distance (Top Diagonal) between collection sites in A) *N. serpentarius* and B) *N. trifoliolatus* with reference for color gradient.

A

	MM	NA	NAS	NR
MM	–	0.187	0.150	0.223
NA	0.062	–	0.148	0.190
NAS	0.048	0.053	–	0.198
NR	0.112	0.110	0.109	–

B

	MT	NW	SF	SS	WS
MT	–	0.035	0.042	0.038	0.080
NW	0.016	–	0.024	0.028	0.053
SF	0.014	0.012	–	0.038	0.052
SS	0.013	0.012	0.013	–	0.038
WS	0.027	0.020	0.018	0.012	–

0.000
0.010
0.020
0.030
0.040
0.050
0.060
0.070
0.080
0.090
0.100

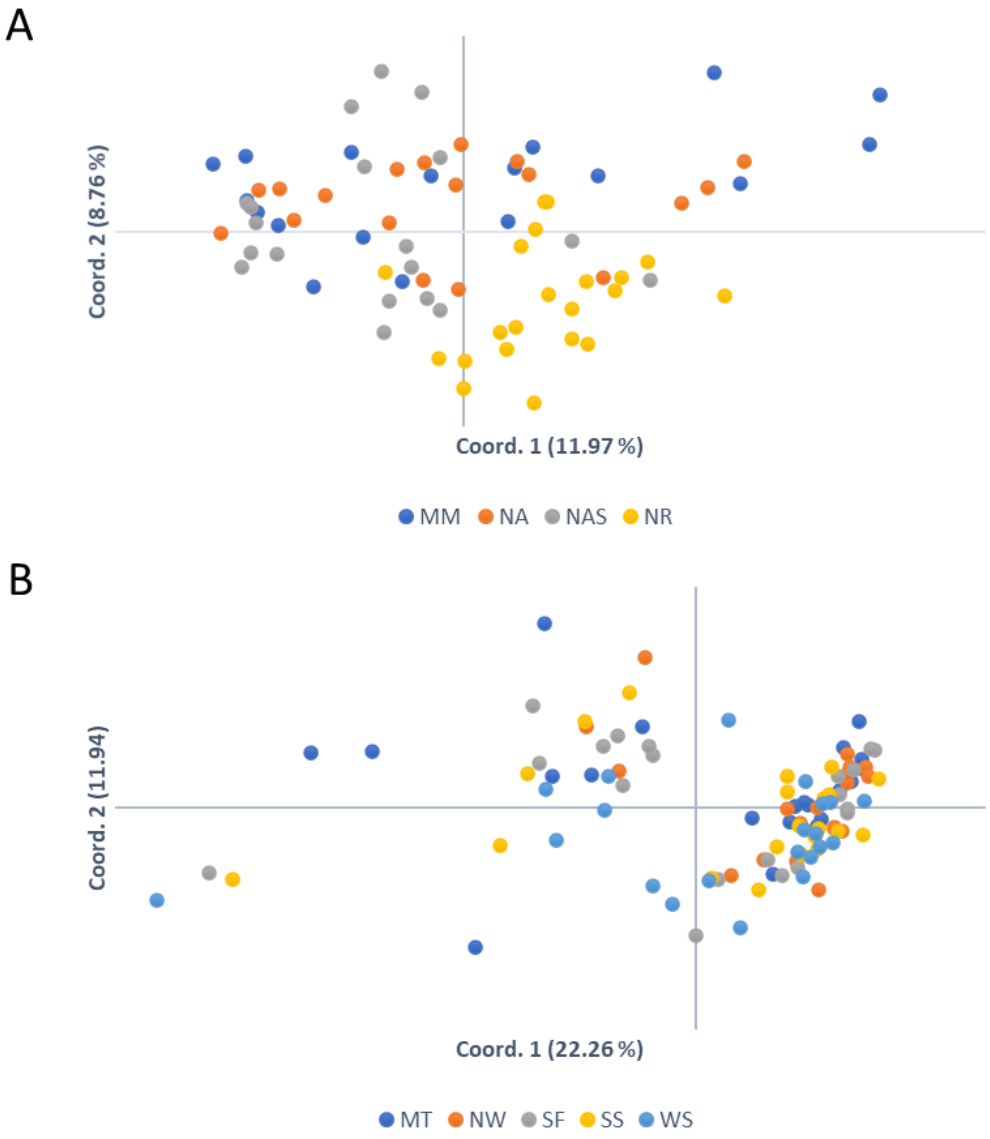

Figure 3. Principal coordinates analysis (PCoA) of the four collection sites of A) *N. serpentarius* (Airport (NA), Airport South (NAS), Middle Moors (MM), and Nabalus Raceway (NR)) and five collections sites of B) *N. trifoliolatus* (Squam Farm (SF), Norwood Farm (NW), Masquetuck (MT), Squam Swamp (SS), and Windswept (WS)).

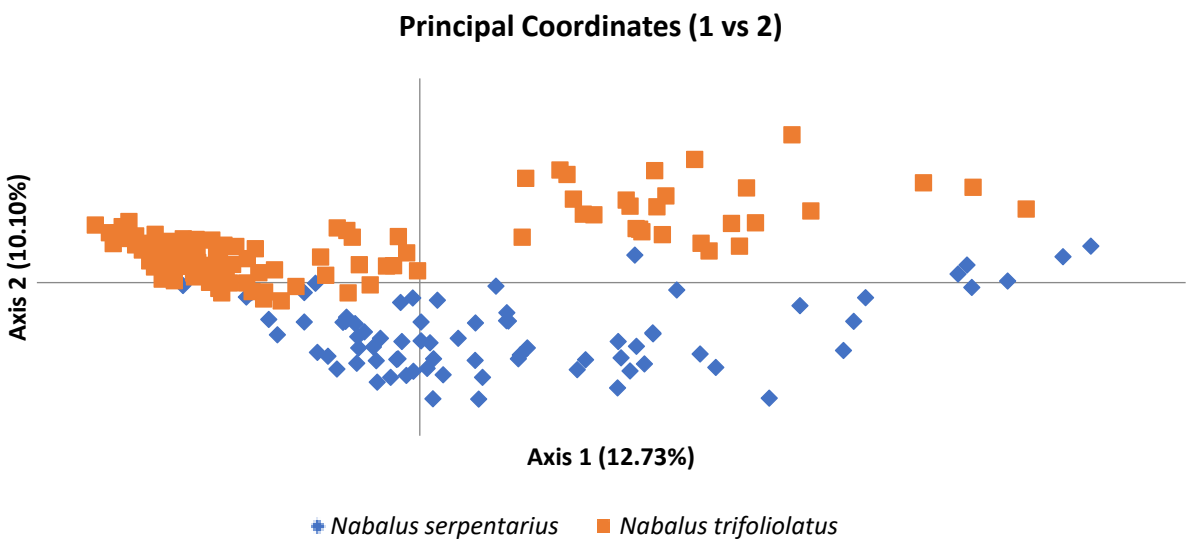

Figure 4. PCoA between all individuals of *N. trifoliolatus* and *N. serpentarius* among shared loci.

STRUCTURE was used to assign potential K values to each species. The output of STRUCTURE was uploaded to Structure Harvester (Earl & vonHoldt, 2012) where the most likely K value was assigned. Using the Evanno method (Evanno, et al., 2005) for *N. serpentarius* K= 4 ($\Delta K = 13.77$) and for *N. trifoliolatus* K = 2 ($\Delta K = 17.33$).

The software package CLUMPP was used to permute each independent run from STRUCTURE. The output from CLUMPP was run through distruct to generate bar plots (Figure 3) for each species to represent relative structure observed in each species on the island of Nantucket.

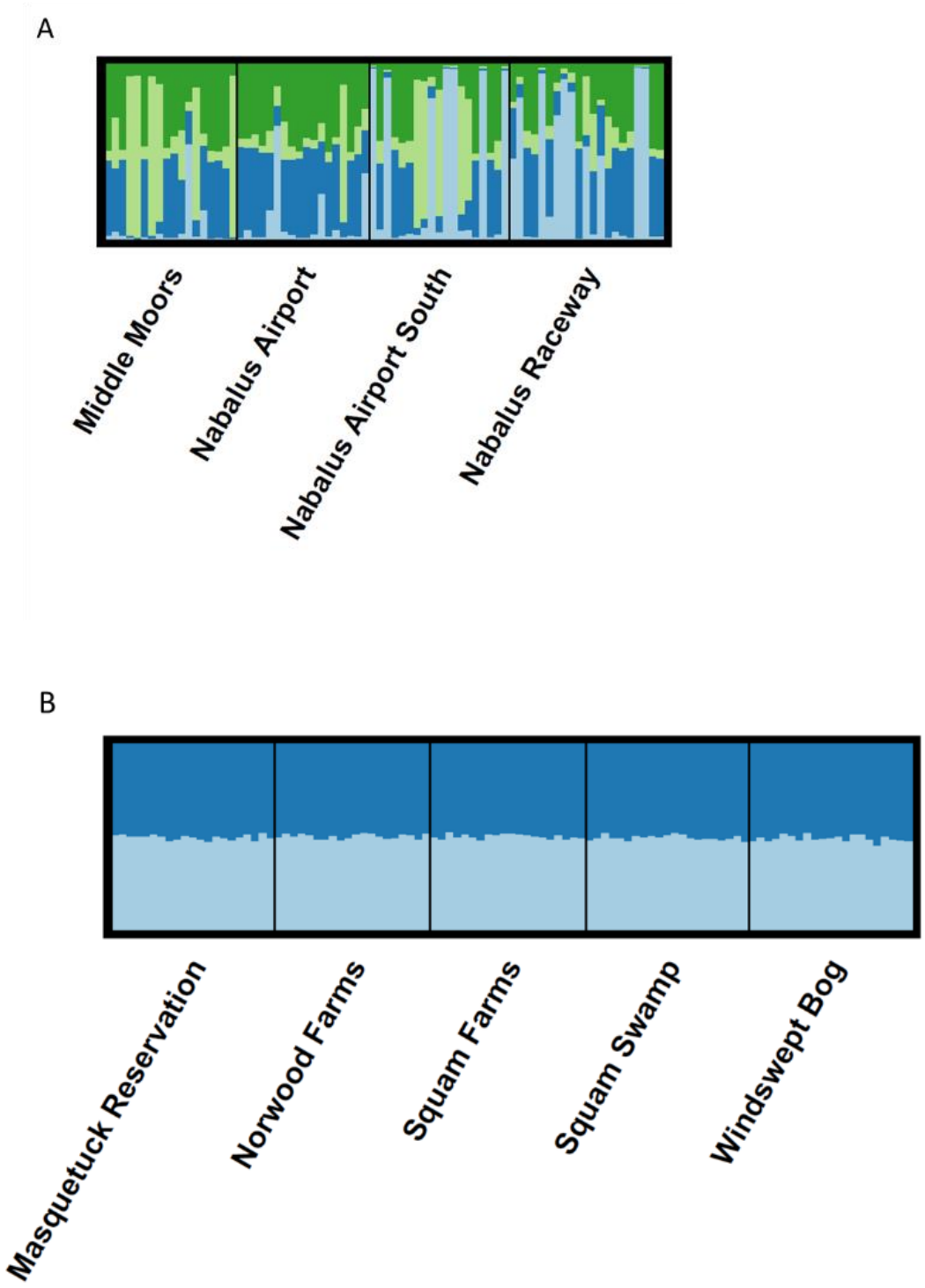

Figure 5. Patterns of population genetic structure observed in A) *N. serpentarius* (K = 4) and B) *N. trifoliolatus* (K = 2).

Discussion

Levels and distribution of genetic variation

In this study, both *N. serpentarius* and *N. trifoliolatus* on the island of Nantucket harbored moderate levels of population genetic variation based on twelve ($H_E = 0.537 \pm 0.032$) and eight ($H_E = 0.534 \pm 0.043$) loci respectively. A general trend is that widespread common species harbor higher levels of genetic diversity when compared to related species that are rare or have restricted distributions (Gitzedanner & Soltis, 2000). However, for the population genetic markers surveyed here, no significant differences ($p = 0.994$) were seen in levels of population genetic diversity between the two species. This differs also from Purdy & Bayer (1996) who found that the endemic species harbored greater variability than the widespread congener.

In terms of population genetic structure, nearly all the genetic variation observed for *N. serpentarius* is within populations and not among populations ($F_{ST} = 0.066$). While the common *N. trifoliolatus* exhibited a slightly but not significantly lower level of population genetic structure ($F_{ST} = 0.016$; $p = 0.061$), the two values are both relatively low especially when compared to other studies of short-lived perennial plant species (mean $F_{ST} = 0.31$; Nybom, 2004; mean $F_{ST} = 0.21$ (across multiple species); Gitzendanner & Soltis, 2000; mean $F_{ST} = 0.134$; Kyrkjeeide, et al., 2020). The FSTs for these *Nabalus* species are closer to the reported values of the endemic *Achellia millefolium* ssp *megacephala* (GST = 0.078; Purdy & Bayer, 1996). The low structure on the island is to expected considering the suspected wind dispersal (Everett & Lepley, 2002) and maximum distances between rare and common sites are 8 km and 3.2 km respectively. With little distance between populations, gene flow is expected. An interesting note is that the slightly higher pairwise F_{ST} value for the NR population of *N. serpentarius* may

indicate isolation of this population by distance. This site is somewhat distant from the other sites (average of about 6.5 km as opposed to 3.2km; see also PCoA in Figure 2). The analysis of the most likely K via the Evanno method (Evanno, et al., 2005) indicated that *N. serpentarius* comprises four genetic clusters, whereas *N. trifoliolatus* comprised two. However, given the very low levels of population genetic structure in both species, the STRUCTURE results should be interpreted with caution as low levels of structure may produce spurious, or conflicting results with different measures of population structure such as F_{ST} (Hubisz, et al., 2009; Babron, et al., 2012), and I suggest data from the F_{ST} measures and PCoA represent more reliable measures of genetic differentiation.

Hybridization is an important consideration when dealing with rare species. Hybridization has the potential to lead to extinction through the mechanisms of introgression and reduction of hybrid viability (Rhymer & Simberloff, 1996). Special attention was given here due to the record of hybridization in the common species (*Nabalus* ×*mainensis* (A. Gray) A. Heller; ICN portal, 2021). The level of structure between *N. serpentarius* and *N. trifoliolatus* (F_{ST} = 0.14) does not raise concern of threat of hybridization when compared to the other levels of structure found in this study.

The use of transferable SSR markers

Due to the lack of published markers for *Nabalus* before this study, the transferability of markers from *Taraxacum* were tested for viability. Of the 17 primers tested, nine produced amplicons between 100 and 500 base pairs reliably. Unbiased expected heterozygosity was used to assess levels of variation seen in these markers and the values were compared to those of the *Nabalus* primers designed by me in this project. The *Nabalus* primers exhibited significantly

greater mean levels of variation than the *Taraxacum* primers for both species (*N. serpentarius*: p = 0.046 and *N. trifoliolatus* p = 0.038). This is possibly because the primers that were transferrable were representative of loci from conserved regions of the *Taraxacum* and *Nabalus* genomes. Genes that are conserved may be under purifying selection, or evolutionary constraint, and likely to harbor lower levels of variation across species (Pashley, et al., 2006). The majority of the *Nabalus* specific primers developed here work across both species and should be useful for population genetic studies in other *Nabalus* study.

Conservation Implications

For more than half of a decade, conservation agencies have been protecting land on Nantucket. Population genetics is a key tool for conservation because it allows conservation agencies to better allocate potentially limited funding and personnel for the protection of populations of threatened species. *N. serpentarius* has a moderate level of population genetic variation similar to the level we see in the common congener, *N. trifoliolatus*. Some population genetic structure exists for the rare species on the island, but most populations are equally variable. For these reasons, the best course of action for local management may be to focus on maintaining numbers of individuals of the rare species. These efforts may be strengthened by creating or ensuring the existence of suitable habitat for the species. A study by Patterson and Clarke (2006) indicated that prescribed burning was beneficial to the growth of *N. serpentarius* and this species can be found within and around fire breaks (pers. obs.). Further, Patterson and Clarke (2006) state that mowing may reduce habitat suitability for rare plants in the absence of prescribed fire treatments and plowing may be detrimental to establishment of *Nabalus* species (Clarke, 2006). Given that *N. serpentarius* harbors moderate levels of variation and low levels of population structure, the loss of any one population may not be overly detrimental to the species

on the island. However, with individuals being more different within populations than among populations, it may be wise to maintain or increase population numbers to mitigate detriment of demographic or environmental stochasticity (Schaffer, 1981).

Future Directions

Much is left to learn about *Nablus serpentarius*, which alone may be sufficient reason to warrant protection. Improving our understanding about the threatened populations of this species may allow us to better control or mitigate harm done to currently secure populations. Some future studies would address mainland, *N. serpentarius* variation, seed dispersal distances, and clonality of the species. At the start of this study, the only known sites of *N. serpentarius* in Massachusetts were on Nantucket Island and Martha's Vineyard (Clarke, 2006). Recently, at least one individual was found on the mainland by a community scientist on the social network, iNaturalist. Assessment of genetic material from the mainland can offer another point of reference for understanding variation and answer the degree to which there is gene flow between the island and mainland. Clonality within *Nabalus* is another mystery to be solved. Currently, little evidence has been published to suggest clonality within the genus or any particular species. Bogler (2006) suggested clonality of some species of *Prenanthes* that are now recognized as *Nabalus*, however, this mode of reproductive has not yet been confirmed in *N. serpentarius*. Finally, little is known about the ability of *Nabalus* species on the island to hybridize which could explain the moderate levels of genetic variation and low population structure. Combined AMOVA analyses suggest restricted gene flow and reproductive isolation between the two species ($F_{ST} = 0.14$) though the potential for hybridization cannot be ruled out and is another fertile area for study in these species.

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Appendix

Table S 1. Four varying recipes were used for all (TKS and NS) primer trials and subsequent amplifications of experimental DNA.

TAQ/DNA Trial Recipe 1		
Ingredients	**Amount/ Reaction (µL)**	**Amount/ Mix (µL)**
Deionized H_20	9.4	84.6
10x PCR Buffer w/ $MgCl_2$	1.5	13.5
10 µM M13 w/o fluorophore	0.7	6.3
25 µM $MgCl_2$	0.35	3.15
20 µM R Primer	0.35	3.15
5 µM F Primer	0.25	2.25
20 µM dNTPs	0.2	1.8
TAQ	0.75	6.75
DNA	1.5	-
Total	15	-

TAQ/DNA Trial Recipe 2		
Ingredients	**Amount/ Reaction (µL)**	**Amount/ Mix (µL)**
Deionized H_20	9.65	86.85
10x PCR Buffer w/ $MgCl_2$	1.5	13.5
10 µM M13 w/o fluorophore	0.7	6.3
25 µM $MgCl_2$	0.35	3.15
20 µM R Primer	0.35	3.15
5 µM F Primer	0.25	2.25
20 µM dNTPs	0.2	1.8
TAQ	0.5	4.5
DNA	1.5	-
Total	15	-

TAQ/DNA Trial Recipe 3		
Ingredients	**Amount/ Reaction (µL)**	**Amount/ Mix (µL)**
Deionized H_20	10.15	91.35
10x PCR Buffer w/ $MgCl_2$	1.5	13.5
10 µM M13 w/o fluorophore	0.7	6.3
25 µM $MgCl_2$	0.35	3.15
20 µM R Primer	0.35	3.15
5 µM F Primer	0.25	2.25
20 µM dNTPs	0.2	1.8
TAQ	0.5	4.5
DNA	1	-
Total	15	-

TAQ/DNA Trial Recipe 4		
Ingredients	**Amount/ Reaction (µL)**	**Amount/ Mix (µL)**
Deionized H_20	8.75	78.75
10x PCR Buffer w/ $MgCl_2$	1.5	13.5
10 µM M13 w/o fluorophore	0.7	6.3
25 µM $MgCl_2$	0.25	2.25
20 µM R Primer	0.35	3.15
5 µM F Primer	0.25	2.25
20 µM dNTPs	0.2	1.8
TAQ	1.5	13.5
DNA	1.5	-
Total	15	-